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Biocontrol Agents: Mode of Action and Interaction with Other Means of Control

editors:

Yigal Elad, Stanley Freeman & Enrique Monte

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IOBC / WPRS

Working Group "Biological Control of Fungal and Bacterial Plant Pathogens"

Proceedings of the Sixth WG Meeting

Biocontrol Agents: Mode of Action and Interaction with Other Means of Control

at

Sevilla, Spain

November 30 - December 3, 2000

Edited by
Yigal Elad, Stanley Freeman and Enrique Monte

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PREFACE

This bulletin contains the proceedings of the Sixth meeting of the "Biological Control of Fungal and Bacterial Plant Pathogens" working group of the IOBC/WPRS. The meeting of "Phytopathogens" Working Group was held in Sevilla, Spain from 30.11-3.12.2000. It was organized by Enrique Monte from Salamanca, Spain and Yigal Elad from Bet Dagan, Israel and was entitled "Biocontrol Agents Mode of Action and Their Interaction with Other Means of Control". A record of 180 persons from 30 countries attended the meeting.

Ninety-eight studies were presented in the meeting. The works present a very interesting collage of research in aspects of biocontrol that varied between basic approaches to field implementation of biocontrol of plant diseases. These included reports on biological control of diseases on plant propagation material, soil and foliage plant parts, harvested products and wood. The development that was striking is the maturation of several biocontrol agents as preparations that can be used under commercial conditions. Biocontrol agents were used successfully, either alone or in combination, with various other means of disease control or with other biocontrol agents. Thus, optimization of control was achieved and the use of chemical agents was minimized. Thorough research of many of the microbial biocontrol agents revealed interesting and innovative modes of action that were further developed by studying enzymes involved in biocontrol. The corresponding genes were cloned and may eventually be developed as control agents alone.

There are many people and organizations that should be thanked for their contribution to the success of the meeting. These include E. Monte, persons of the scientific and local organizing committees and the collaborating and funding organizations. Thank you for the enthusiasm and hard work.

It is the first meeting of the WG that I am responsible for since I replaced Nyckle J. Fokkema. I wish to thank Nyckle for the management of the WG since its foundation about a decade ago and for his fruitful activity for the benefit of the working group. In the name of all WG members I wish Nyckle peaceful and enjoyable retirement. I hope to carry on leading the WG in new meetings and interesting discussions in the future.

Yigal Elad, Convenor
IOBC/WPRS WG on "Biological Control of Fungal and Bacterial Plant Pathogens"
Fabruary 2001

As the editors of the present volume, we wish to thank all the contributors for summarizing their scientific work. The contributions present valuable information for the science and implementation of biocontrol of plant diseases. The contributors were very cooperative and patient with the editors and thus facilitated rapid completion and publication of this volume.

The editors

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CONTENTS

IOBC/WPRS - EFPP WORKING GROUP:
BIOLOGICAL CONTROL OF FUNGAL AND BACTERIAL PLANT PATHOGENS
Sixth Meeting of the Working Group
30 November 2000 – 3 December 2000

BIOCONTROL-2000 Seville-Spain

BIOCONTROL AGENTS MODES OF ACTION AND THEIR INTERACTION WITH OTHER MEANS OF CONTROL

Convenor:
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El MONTE Conference Hall, Calle Laraña 4, Sevilla

MEETING PROGRAM

Thursday 30.11.2000 21:00 Get together
Friday 1.12.2000 10:00-11:00 Opening Session
Chair persons: Joy Chimombe, Enrique Monte, Yigal Elad, Candido Santiago

Enrique Monte: Welcome
Andalucia Minister of Agriculture, Research Vice-rector of Seville University and President of El Monte: Greetings
Claude Alabouvette: IOBC/WPRS
Yigal Elad: IOBC/WPRS Phytopathogens WG, Meeting Introduction

Friday 1.12.2000 11:30-12:30 Oral Session S1: Mode of action of BCAs I
Chairperson: Tahía Benítez
S1-1 Alison Stewart, Kim Eade, Nimal Rabeendran, Hayley Ridgway: Mechanisms of biological control of Sclerotinia lettuce drop by *Coniothyrium minitans* - more than just sclerotial parasitism.
S1-2 Ulla Meyer, Erwin Fisher, Olga Barbul, Yigal Elad: Biocontrol agents effect on specific antigens present in the extracellular matrix of *Botrytis cinerea*, which are important for pathogenesis.
S1-3 Roberta Roberti, Luciana De Vero, Annamaria Pisi, Augusto Cesari: Biological control of wheat foot rot by antagonistic fungi and their modes of action.
S1-4 John Larsen, Lars Bødker: Mode of interaction between the arbuscular mycorrhizal fungus *Glomus mosseae* and the root pathogen *Aphanomyces euteiches.*

Friday 1.12.2000 12:30-13:45 Oral Session S2: Mode of action II
Chairperson: Barrie Seddon
S2-1 Rosalind McHugh, Barrie Seddon: Mode of action of *Brevibacillus brevis* - biocontrol and biorational control.
S2-2 Zoria Lapsker, Yigal Elad: Involvement of active oxygen species and antioxidant enzymes in the disease caused by *B. cinerea* on bean leaves and in its biological control by means of *Trichoderma harzianum* T39.
S2-3 Annegret Schmitt, Michael Schuld, Barrie Seddon, Stavroula Konstantinidou- Doltsinis, Aleid Dik, Annegret Ernst, Nikolaos Malathrakis, Wittko Francke: Improved plant health by the combination of biological disease control methods.
S2-4 Nanjian Raman, Mariappan Gnanaguru: Biological control of *Fusarium* wilt of tomato by VA mycorrhizal fungus, *Glomus fasciculatum.*
S2-5 Kris Audenaert, Theresa Pattery, Pierre Cornelis, Nico Koedam, Monica Höfte: Induced resistance to *Botrytis cinerea* by *Pseudomonas aeruginosa*: role of siderophores and pyocyanin.

Friday 1.12.2000 15:00-16:30 Oral Session S3: BCAs in agriculture and forestry systems
Chairperson: Aleid J. Dik
S3-1 Alba Marina Cotes: Biocontrol of fungal pathogens - from discovery of potential biocontrol agents to implementation of formulated products.
S3-2 Aleid Dik, Jos Wubben: Biological control of *Botrytis cinerea* in greenhouse crops.
S3-3 David McNeil, Janaki Kandula, Christine Stark, Sandra Romero, Alison Stewart, Stuart Larsen: Bacteriophages as a potential biocontrol agent against walnut blight (*Xanthomonas campestris* pv *juglandis*).
S3-4 Jan Stenlid, Andrei Iakovlev: How do basidiomycetes interact in conifer wood?

S3-5 Nicola La Porta, Paolo Ambrosi, Renata Grillo, Kari Korhonen: Biological control of *Heterobasidion annosum* in Norway spruce forest by non-pathogenic wood decay fungi.
S3-6 Chris A. Gilligan: The use of models to understand variability in biological control.

Friday 1.12.2000 16:30-19:00 Posters group 1: Biocontrol in soil
Chair person: John Whipps
P1-1 Inmaculada Larena, Antonieta De Cal, Paloma Melgarejo: Involvement of population levels of *Penicillium oxalicum* in the biocontrol of tomato wilt.
P1-2 John Clarkson, Tina Payne, John Whipps: A screening system for identifying biological control agents of *Sclerotium cepivorum.*
P1-3 Victor Rubio, Vicente González, María de los Angeles Portal, María Julián, Oscar Salazar, Horacio López-Córcoles, Prudencio López-Fuster: Biological control of damping-off on pine (*Pinus* spp.) with a new fungal species, *Ceratobasidium albasitensis* isolated in Albacete (Spain).
P1-4 Francisco M. Cazorla-López, Guido V. Bloemberg, Ben J.J. Lugtenberg: Biocontrol of white root rot on avocado plants using rhizobacterial strains.
P1-5 Eirian Jones, John M. Whipps: Biological Control of *Sclerotinia sclerotiorum* in glasshouse lettuce.
P1-6 Ramón Penyalver, Begonya Vicedo, María M. López: Comparison of strains K84 and the GEM K1026 in biological control of crown gall caused by *Agrobacterium* spp.
P1-7 J. C. (Mike) Tu, Wei-Zheng Zhang, Barbara Harwood, Chun Ma: Biological Control of Pythium root rot of tomato.
P1-8 Roudolf Azizbekyan, Anatoly Kuzin, Tatyana Smirnova, Tatyana Shamshina: Biological control of plant fungal diseases.
P1-10 Trazilbo J. de Paula Jr., Claudia Rotter, Bernhard Hau: Effects of soil moisture and planting depth on *Rhizoctonia solani* and on the antagonist *Trichoderma harzianum.*
P1-11 Ximena Besoaín, Rodrigo García, Carla Raggi Eduardo Oyanedel, Jaime Montealegre, Luz-María Perez: Biological control of *Phytophthora parasitica* in greenhouse tomatoes using *Trichoderma harzianum.*
P1-12 Francisca Suárez-Estrella, María Antonia Elorrieta, María Carmen Vargas-García, María José López, Joaquín Moreno: Selective isolation of antagonist microorganisms of *Fusarium oxysporum* f.sp. *melonis.*
P1-15 Trini Campos, Josep Roselló, María Rosa Hermosa, Belén Rubio, Isabel Grondona, Enrique Monte: Antagonistic effect of a *Trichoderma* formulation against *Sclerotinia sclerotiorum* in lettuce.
P1-16 Kurze, Sauerbrunn, Bahl, Berg: Effects of antagonistic rhizobacteria on plant health, yield, and the bacteria rhizosphere community on strawberry.
P1-17 Gabriele Berg, Petra Marten, Arite Minkwitz, Stefan Brückner: Efficient biocontrol of fungal plant diseases by Rhizovit on the basis of *Streptomyces* sp. DSMZ12424.
P1-18 P Maria Johansson: Suppression of wheat seedling blight caused by *Fusarium culmorum* and *Microdochium nivale* using seed-applied bacteria.
P1-19 Chang Seuk Park , Jin Woo Kim, Okhee Choi: Biological control of tomato bacterial wilt caused *by Ralstonia solanacearum* in rockwool hydroponic culture employing *Pseudomonas fluorescens* B16.

Friday 1.12.2000 16:30-19:00 Posters group 2: Biocontrol of foliar and postharvest diseases
Chairperson: Stanley Freeman
P2-1 Bharti Odhav, Kugen Permaul, Shaun Ramsunder, Thiroshnee Padayachee, Viresh Mohanlal, Lalini Reddy: Incidence and biocontrol of some mycotoxins in South Africa.
P2-2 Eftihia Tsomlexoglou, Barrie Seddon, Eunice J. Allan: Biocontrol potential of *Bacillus* antagonists selected for their different modes of action against *Botrytis cinerea.*

P2-3 Diego Romero, Alejandro Pérez García, Daniel del Pino, Eugenia Rivera, J.A.Torés, Antonio de Vicente: Isolation and evaluation of potential agents for biological control of powdery mildew in cucurbits.

P2-4 Gilbert Bompeix, Danielle Cholodowski-Faivre: Biocontrol of some post-harvest diseases by the use of natural plant products, and their interaction with thermotherapy.

P2-6 Stanley Freeman, Olga Barbul, Dalia Rav David, Yehuda Nitzani, Aida Zveibil, Yigal Elad: *Trichoderma* spp. for biocontrol of *Colletotrichum acutatum* and *Botrytis cinerea* in strawberry.

P2-7 Capieau Kristof, Stenström Elna, Stenlid Jan: Biological control of *Botrytis cinerea* of pine seedlings in a forest nursery in Sweden.

P2-8 Lillian Holmer, Jan Stenlid, Caroline Woods, Olof Pettersson: Potential in wood-decaying fungi to control *Heterobasidion annosum.*

P2-9 Chrysoula M. Koumaki, Barrie Seddon, Nikolaos E. Malathrakis: Control of cucumber powdery mildew (*Sphaerotheca fuliginea*) with bacterial and fungal antagonists.

P2-10 Vanamala Anjaiah, VP Rao, RP Thakur, Kiran K. Sharma, Pierre Cornelis, Nico Koedam: A biological control approach making use of rhizobacteria and soil fungi for soilborne post harvest infection of *Aspergillus flavus* in groundnut.

P2-11 Michael Wisniewski, Charles Wilson, Ahmed El Ghaouth, Samir Droby: Increasing the ability of the biocontrol product, Aspire, to control postharvest diseases of apple and peach with the use of salt solutions and chelating agents.

P2-12 Ahmed El Ghaouth, Charles Wilson, Michael Wisniewski: Evaluation of two biocontrol products, Bio-coat and Biocure, for the control of postharvest decay of pome and citrus fruit.

P2-13 Pilar Plaza, Rosario Torres, Neus Teixidó, Josep Usall, Maribel Abadias, Immaculada Viñas: Control of green mold by the combination of *Pantoea agglomerans* (CPA-2) and sodium bicarbonate on oranges.

P2-14 Rosa Segura, Antonio Trapero: Screening of epiphytic fungi from olive leaves for the biological control of *Spilocaea oleagina.*

P2-15 Jürgen Köhl, Geert J.T. Kessel: Epidemiology of *Botrytis* spp. in different crops determines success of biocontrol by competitive substrate exclusion by *Ulocladium atrum.*

P2-16 Geert Kessel, Lia de Haas, Wopke van der Werf, Jürgen Köhl: Mechanistic aspects of competitive substrate colonisation by *Botrytis cinerea* and *Ulocladium atrum.*

P2-17 Paulo Germán García, Yanira Jiménez, Alejandro Neisa, Alba Marina Cotes: Selection of native yeasts for biological control of postharvest rots caused by *Botrytis allii* in onion and *Rhizopus stolonifer* in tomato.

Sunday 3.12.2000 09:00-10:00 Oral Session S4: Combination of control agents I
Chairperson: Rafael Jimenez Díaz

S4-1 Walid Fakhouri, N. Neemann, F. Walker, Heinrich Buchenauer: Combination of fluorescent pseudomonads with benzothiadiazole (BTH) induces synergistic disease resistance in tomato and cucumber against bacterial and fungal pathogens.

S4-2 Guetsky Ruth, Elad Yigal, Shtienberg Dani, Dinoor Amos: Combination of *Pichia guilermondii* and *Bacillus cereus* for the control of gray mold (*Botrytis cinerea*) in strawberries.

S4-4 Birgit Jensen , Finn Vestergaard Poulsen, Inge M.B. Knudsen, Dan Funck Jensen: Combining microbial seed treatment with priming of carrot seeds for control of seed borne *Alternaria* spp.

Sunday3.12.2000 10:00-11:00 Oral Session S5: Combination of control agents II
Chairperson:Claude Alabouvette

S5-1 Dimitrios G. Georgakopoulos, Phil Fiddaman, Carlo Leifert, Nikolaos E. Malathrakis: Evaluation of antagonistic bacteria and fungi for biological control of sugarbeet and cucumber damping-off caused by *Pythium ultimum.*

S5-2 Stanley Freeman, Aida Zveibil, Haim Weintal, Marcel Maimon: Isolation of nonpathogenic mutants of *Fusarium oxysporum* for biocontrol of *Fusarium* wilt in cucurbits.

S5-3 Isabel Grondona, M. Rosa Hermosa, Juan A. Vizcaíno, Pablo García Benavides, José Redondo, Carlos Rico, Enrique Monte, Isabel García Acha: Integrated control of rhizomania disease by *Trichoderma* and cultural management

S5-4 John M Whipps, Simon P. Budge: Integrated control of *Sclerotinia sclerotiorum* in glasshouse lettuce using the mycoparasite *Coniothyrium minitans.*

Sunday3.12.2000 11:30-12:30 Oral Session S6: Mode of action III - SAR

Chairperson: Nico Koedam

S6-1 Bart P.J. Geraats, Peter A.H.M. Bakker, L.C. van Loon: Can enhanced susceptibility to pathogens of ethylene-insensitive plants be overcome by biocontrol agents that induce systemic resistance?

S6-2 Gaëtan Le Floch, Patrice Rey, Anne Sophie Renault, Nicole Benhamou, Yves Tirilly: *Pythium oligandrum*-induced resistance against grey mould of tomato associated with pathogenesis-related proteins.

S6-3 Fabio Mascher, Yvan Moënne-Loccoz, Geneviève Défago: Soil conditions and regulatory genes modulate persistence and cell culturability of biocontrol agent *Pseudomonas fluorescens* CHA0.

S6-4 Samir Droby, Ron Porat, Victor Vinokur, Lea Cohen, Batia Weiss, Avinoam Daus: Induction of resistance to postharvest decay by the yeast biocontrol agent *Candida oleophila.*

Sunday3.12.2000 12:30-13:30 *Oral Session: Post harvest and induced resistance*

Chairperson: Samir Droby

S7-1 Mohamed Haïssam Jijakli, Cathy Grevesse, Philippe Lepoivre: Modes of action of biocontrol agents of postharvest diseases: Challenges and difficulties.

S7-2 Raffaello Castoria, Leonardo Caputo, Vincenzo De Cicco: Resistance to oxidative stress and antagonism of biocontrol yeasts against postharvest. Pathogens.

S7-3 Ahmed El Ghaouth, Charles Wilson, Michael Wisniewski: Induction of Systemic Resistance in Apple by the Yeast Antagonist, *Candida saitoana.*

S7-4 Inge M.B. Knudsen, Kirsten A. Thomsen: Thermotherapy and microbiological control of storage fungi on acorns (*Quercus robur*).

Sunday 3.12.2000 15:00-16:45 Oral Session S8: Enzymes and Genes involved in biocontrol

Chairperson: Dan Funck Jensen

S8-1 Jens Frankowski, Gabriele Berg, Hubert Bahl: Purification and properties of two chitinolytic enzymes of the biocontrol agent *Serratia plymuthica* C48.

S8-2a Jesús Delgado-Jarana, Tahía Benítez: Isolation of genes from *Trichoderma harzianum* CECT 2413 expressed at different pHs.

S8-2a Manuel Montero, Manue Rey, Fran González, Luis Sanz, Enrique Monte, Antonio Llobell: β -1, 6-glucanase isozyme system in *Trichoderma harzianum.* Isolation of two new genes coding for proteins with β -1,6- endoglucanase activity.

S8-3 Laura Vila, Mar Rufat, Maria Angels Planell, María Coca, Isabel Murillo, Ana Beatriz Moreno, Valle Lacadena, Alvaro Martinez del Pozo, Blanca San Segundo: Engineering disease resistance in crop plants through the expression of fungal and bacterial genes.

S8-4 Vladimir V. Tikhonov, Luis Vicente Lopez-Llorca, Hans -Bortje Jansson, Elena Monfort, Jesús Salinas: Purification and characterisation of chitinases from the biocontrol agents *Verticillium chlamydosporium* and *V. suchlasporium.*

S8-5 C. Peter Romaine, Xi Chen, Manuel D. Ospina-Giraldo, Daniel J. Royse: Molecular Genetics and Pathogenicity of biocontrol and mushroom *Trichoderma.*

S8-6 Sandra A. I. Wright, Maria Johansson, Berndt Gerhardson: Biocontrol of fungal pathogens in wheat and barley with bacterial seed dressings – possible mechanisms.

Sunday3.12.2000 16:45-19:00 Posters group 3: Integrated control and other means of control
Chairperson: Jürgen Köhl

P3-1 Annegret Schmitt, Stavroula Konstantinidou-Doltsinis, Kalliopi Tzebelikou, Niki Petsikos-Panayotarou, Emilia Markellou, Aleid Dik, Annegret Ernst: Efficacy of a new liquid formulation from *Reynoutria sachalinensis* as inducer of resistance against powdery mildew in cucumber and grape vine.

P3-3 Annierose Tamayo Albertsen, Helge Green, Dan Funck Jensen, John Larsen: Influence of organic matter on the interaction between the biocontrol agents *Glomus intraradices* and *Burkholderia cepacia.*

P3-4 Duncan White, Annegret Schmitt, Annegret Ernst, Barrie Seddon: Interaction of the biocontrol agent *Brevibacillus brevis* with other disease control methods.

P3-5 Andrés Nico, Cecilia Mónaco , María Cristina Rollán, Gustavo Dal Bello: Organic amendments effect on survival and pathogenic ability of *sclerotinia minor* slerotia.

P3-6 MC Rollán, J Stancovich, C Sgarbi, C Mónaco, B L Ronco, A Mittidieri: Steam soil disinfecting: effect on *Sclerotinia sclerotiorum, S. minor* and *Trichoderma* sp.

P3-7 László Kredics, Zsuzsanna Antal, László Manczinger: Isolation and characterisation of heavy metal resistant mutants from mycoparasitic *Trichoderma* strains.

P3-8 Silvia Raquel Zapata, Norma Beatriz Vecchietti: Fusarium wilt in tobacco burley: cultural and biological management in Tucumán (República Argentina).

P3-9 Richard Walker, Stephen Rossall, Michael J.C. Asher: Compatibility with seed treatment chemicals of rhizobacteria antagonistic to the sugar-beet damping-off pathogen *Aphanomyces cochlioides.*

P3-10 Leszek B. Orlikowski: Control of *Phytophthora* spp. with grapefruit extract.

P3-12 Walid Fakhouri, Zhensheng Kang, Heinrich Buchenauer: Microscopic studies of fluorescent pseudomonads alone or in combination with benzothiadiazole derivative effective against *Fusarium oxysporum* f.sp. *lycopersici* in tomato plants.

P3-13 Pasi Haansuu, Paivi Soderholm, Pia Vuorela, Kielo Haahtela: Biological activity of an antibiotic produced by Frankia Aips1.

P3-14 Bedini Stefano, Filippi Carlo, Bagnoli Giovanna1, Russo Anna, Nuti Marco Paolo: Soil colonization by *Bacillus subtilis* M51 pre-conditioned in organic matrix and its survival in soil bulk.

P3-15 Alba Marina Cotes, Adriana Cárdenas, Hernán Pinzón: Effect of seed priming in the presence of *Trichoderma koningii* on seed and seedling disease induced in tomato by *Rhizoctonia solani* and *Fusarium oxysporum* f.sp. *lycopersici.*

P3-16 Giora Kritzman, Avraham Gamliel: Organic soil amendments as a biological control of soilborne pathogens.

P3-17 Luciano Avio, Cristiana Sbrana, Carlo Filippi, Giovanna Bagnoli, Manuela Giovannetti: Antagonistic bacteria tightly associated to spores of three arbuscular mycorrhizal fungal species.

P3-18 Ahmed Sid, Consuelo Pérez, Mohammed Ezziyyani, M. Emilia Candela: Effect of *Trichoderma harzianum* treatments on systemic resistance in pepper plants (*Capsicum annuum*) to *Phytophthora capsici* and its relation with capsidiol accumulation.

Sunday 3.12.2000 16:45-19:00 Posters group 4: Plant, biocontrol agent, enzymes, genes and substrate interactions

Chairperson: Monica Höfte

P4-1 Zsuzsanna Antal, László Kredics, László Manczinger, Lajos Ferenczy: Extracellular enzyme profiles of mycoparasitic *Trichoderma* strains.

P4-2 Daniel J. Royse, Manuel D. Ospina-Giraldo, Xi Chen, C Peter Romaine: Phylogenetic analyses of *Trichoderma* spp. associated with mushroom culture or used for biological control of plant pathogens.

P4-3 G. Innocenti, M. Montanari, M.A. Sabatini: Do *Trichoderma* influence Collembola, pathogenic fungi, plant interactions?

P4-4 Martine Maes: Bacterial inoculation for protection of critical plant growth stages.

P4-5 Ana Rincón, José Antonio Pintor-Toro, Tahía Benítez: Heterologous expression of a fungal β-1,3-glucanase in plants.

P4-7 Luz-María Pérez, Mauricio Reyes, Mónica Lespinasse, Jaime Montealegre, Ximena Besoain: The expression of enzymes involved in biological control of tomato phytopathogens by *Trichoderma* depends on the phytopathogen to be controlled and on the biocontrolle isolate.

P4-8 Mainul Hassan, Gabriel Corkidi, Enrique Galindo, Celia Flores, Leobardo Serrano-Carreón: Accurate and rapid viability assessment of biocontrol agent *Trichoderma harzianum* using fluorescence-based digital image analysis.

P4-9 Mercedes Dana, Tahía Benítez, Christian P. Kubicek, José A. Pintor-Toro: Chitinase 33 gene expression during mycoparasitism in *Trichoderma harzianum.*

P4-10 Cristina Cepeda, María de las Mercedes Dana, Irene García, Beatriz Cubero, José A Pintor-Toro: Increased hypersensitive response of transgenic tobacco plants expressing chitinases from *Trichoderma harzianum.*

P4-12 Peter Laux, Wolfgang Zeller: Studies on the mode of action of *Rahnella aquatilis* Ra39 against *Erwinia amylovora.*

P4-15 Patricia Santorum, Mª Angeles Castillejo, Luis Sanz, Isabel Grondona, Manuel García Roig, Francisco Javier González, Enrique Monte: Enzyme production by a biocontrol strain of *Trichoderma atroviride.*

P4-16 Sonia Sousa, Manuel Rey, Antonio Llobell: Homologous and heterologous overexpression of a beta-1,6-glucanase (BGN16.3) from *Trichoderma harzianum.*

P4-17 Belén Suárez, Manuel Rey, Enrique Monte, Antonio Llobell: Purification and characterization of a protease, Pra1, from *Trichoderma harzianum* with affinity for fungal cell walls.

Sunday 3.12.2000 19:30-20:15 Closing session

Chairperson: Antonio Llobell

Speakers: Enrique Monte, Alberto Alonso, Claude Alabouvette, Yigal Elad

Sunday 3.12.2000 21:30 Meeting Dinner at Benazuza Palace

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Mechanisms of biological control of Sclerotinia lettuce drop by *Coniothyrium minitans* - more than just sclerotial parasitism

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Abstract: *Coniothyrium minitans* A69 gave effective control (70%) of *Sclerotinia minor* disease of lettuce when applied as a maize perlite soil amendment at planting time. Laboratory and field studies indicated that disease control was achieved by a dual mechanism of action. *C. minitans* A69 protected lettuce plants from infection during the first 4 weeks after transplanting by suppressing mycelial growth of the pathogen through localised antibiosis and hyphal parasitism. *C. minitans* also made contact with and parasitised sclerotia in the vicinity of the developing plant and, thereby, reduced the inoculum which would contribute to mid-late season infection.

Key words: biological control, *Coniothyrium minitans*, lettuce drop, *Sclerotinia minor*

Introduction

Coniothyrium minitans Campbell is a sclerotial parasite of *Sclerotinia* species (Campbell, 1947, Turner & Tribe 1997) with numerous reports of successful biocontrol of *Sclerotinia* diseases in the glasshouse and field (Budge *et al.*, 1995; Luth, 1995). A New Zealand isolate of *C. minitans*, designated A69 (Jones & Stewart 2000), was shown to have good activity against *Sclerotinia* diseases in glasshouse and field crops. In particular, excellent disease control (70-80%) was achieved against *S. minor* Jagger on lettuce (Rabeendran, 2000). In previous field trials, the fungus was generally applied as a soil amendment 4-6 weeks prior to planting, the rationale being to allow time for the fungus to contact and parasitise sclerotia, reducing the amount of inoculum available for crop infection. However, in a recent field trial, improved control was achieved by applying *C. minitans* A69 to the soil at planting. This control cannot be explained solely on the basis of sclerotial parasitism given the short time period from transplanting to first signs of disease.

We describe the results of a lettuce field trial and several laboratory experiments that investigated the ecology and mechanism/s of action of *C. minitans* A69 in the control of Sclerotinia lettuce drop.

Materials and methods

Lettuce field trial

A lettuce field trial, set up in a *S. minor* infested site at Lincoln University, evaluated three treatments; *C. minitans* A69 incorporated into the soil as a solid substrate 6 weeks before planting, the same treatment applied at planting and an untreated control. *C. minitans* was prepared as a maize perlite inoculum (4 week old infested substrate; 10^6 spores/g) and applied to the surface of the plots at a rate of 0.8 L/m^2 and worked into the top 5 cm with a fork (Rabeendran, 2000). Plots were sown with 6 week old lettuce transplants (*Lactuca sativa* cv

Casino). There were 50 transplants per plot (2 x 1 m plot size) and 10 replicate plots per treatment arranged in a randomized block design.

Soil samples were taken from each plot 6 weeks prior to planting and at 0, 6 and 12 weeks after planting and a soil dilution series performed (Jones & Stewart, 2000) to determine *C. minitans* A69 population levels. Initially, colonies of A69 were assessed visually on plates using key colony characteristics and then a representative group analysed using an isolate specific PCR probe to confirm the identity of *C. minitans* A69 (Goldstein *et al.*, 2000). In addition, samples of field sclerotia contained in soil in mesh bags (10 sclerotia/per bag) were buried adjacent (1-2 cm) to individual lettuce plants. These bags were recovered at weekly intervals and sclerotial viability and presence of *C. minitans* A69 recorded. Disease counts were made at weekly intervals. Data was analysed by ANOVA and treatment means compared using Fishers LSD.

Parasitism assay

Mycoparasitism was assessed using the method of Laing & Deacon (1991). Water agar coated cover slips (35 x 65mm) were dual inoculated, 30 mm apart, with small pieces of agar cut from the growing edge of 7-10 day old cultures of *C. minitans* and *S. minor*. The cover slips were incubated in Petri dishes in the dark at 20°C until the hyphae of the two fungi met. The cover slips were then removed and inverted onto the surface of a viewing chamber made up of glass tubing glued onto the surface of a microscope slide. Interactions between the two fungi were examined using the compound microscope.

Germination and growth assay

Spore germination and germ tube growth of *C. minitans* A69 was examined on soil extract agar and non sterile soil in the presence/absence of sclerotial leachates and extracts, and germinating sclerotia using the method of Grendene & Marciano (1999). Percentage spore germination (from 100 spores chosen at random) and mean germ tube growth (from 20 observations) were determined after 24 and 48h. Data was analysed by ANOVA and treatment means compared using LSD.

Results

Lettuce field trial

Sclerotinia disease was first observed in the control plots 2 weeks after transplanting (20%) and increased to a final disease count of 80% at harvest (Figure 1). Disease was reduced in both *C. minitans* treatments but application at planting time was significantly better than application prior to planting (20% disease compared to 60%).

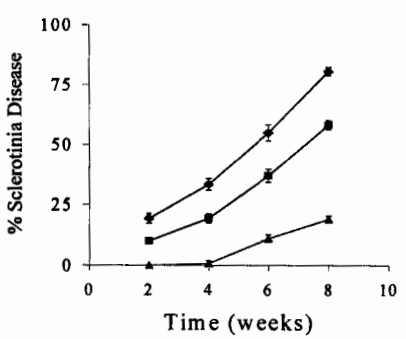

Figure 1: Percentage of Sclerotinia disease observed in the control (◆), *C. minitans* pre-planting (■) and planting (▲) treatments.

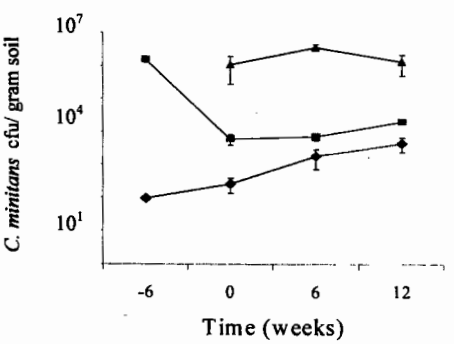

Figure 2: Colony forming units of *C. minitans* isolated from the control (◆), *C. minitans* pre-planting (■) and planting (▲) treatments.

When applied at planting, *C. minitans* cfu counts were maintained at 10^6 /g soil over the course of the trial. In contrast, when applied 6 weeks prior to planting, *C. minitans* counts declined dramatically after application and were only 10^3/g soil at the time of transplanting (Figure 2). This level increased slightly during the growing season to 10^4/g soil. The background level of resident *C. minitans* in the control plots was 10^2/g soil increasing to 10^3g/soil during the cropping period.

C. minitans A69 was recovered from 32% of the sclerotial baits 2 weeks after transplanting. This level increased to 60 and 80% at 4 and 6 weeks, respectively. However, loss of sclerotial viability was not evident until 3 to 4 weeks after transplanting with 25% sclerotial death recorded at 4 weeks increasing to 60% at harvest.

Parasitism assay

Parasitism of hyphae of *S. minor* by *C. minitans* A69 was frequently observed. Infection occurred from main hyphae of *C. minitans* A69 or following the production of side branches. Prior to contact between *C. minitans* A69 and young actively growing hyphae of *S. minor*, the hyphal compartment of the pathogen appeared granular and vacuolated. Similarly, in dual culture, no inhibition zones were observed but localised antibiosis was seen at the interaction sites with pathogen hyphae appearing granular and distorted and some hyphal tip lysis evident.

Germination and growth assay

C. minitans A69 spore germination was stimulated in the presence of *S. minor* sclerotial leachates and exudates. A greater proportion of these spores produced more than one germ tube and germ tube length was greater than the control at 48h (Table 1). The same response was evident when *C. minitans* was incubated in non-sterile soil.

Table 1. *C. minitans* A69 spore germination and germ tube growth on soil extract agar.

Treatment	% Germ. (24h)		% Germ. (48h)		% Spores with >1 germ tube		Mean germ tube length (μ m)	
A69 control	2	a	52.6	a	0	a	45.3	a
A69+sclerotial leachate	5.3	a	86.7	b	6.7	a	49.8	a
A69+germin. sclerotia	14.0	a	89.3	b	31.9	b	63.2	a
A69+sclerotial extract	64.3	b	100	b	70.9	c	116.9	b
LSD (P<0.05)	14.7		16.4		10.4		39.7	

Discussion

The lettuce field trial showed that best disease control was achieved when population levels of *C. minitans* A69 were maintained at 10^5-10^6 cfu/g soil during the cropping period as occurred with *C. minitans* A69 treatment at planting but not the pre-planting treatment. In the presence of the host plant, both the applied *C. minitans* and the indigenous *C. minitans* populations in the soil were stimulated, presumably in response to increased nutrient availability. These results, together with the laboratory assay showing stimulated spore germination and germ tube growth in the presence of sclerotial leachates and exudates, suggests that *C. minitans* proliferates in the presence of the host plant and pathogen. Thus, it would appear that application of *C. minitans* as a pre-planting treatment is not the best strategy to control *S.*

minor on lettuce as it does not take advantage of the stimulatory environment provided by the host plant/pathogen interaction.

The disease control observed in the *C. minitans* treated plots in the first two weeks after transplanting cannot be attributed to sclerotial parasitism since the sclerotial baits revealed no loss of sclerotial viability until 3-4 weeks. The laboratory parasitism assay provided evidence that *C. minitans* could parasitise *S. minor* mycelium and there was some evidence of localised antibiosis in dual culture. Antimicrobial compounds produced by *C. minitans* have recently been identified and characterized by other workers (McQuilken *et al.*, 2000) supporting our observations.

It is postulated that *C. minitans* provides early season protection through localised antibiosis and mycoparasitism which suppresses mycelial growth of the pathogen in the vicinity of the roots. Sclerotial parasitism reduces the number of germinating sclerotia which contribute to mid-late season infection.

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Effect of biocontrol agents on antigens present in the extracellular matrix of *Botrytis cinerea*, which are important for pathogenesis

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Abstract: The effect of five microorganisms on the extracellular matrix of two isolates of *Botrytis cinerea*, BC I4 and BC I16, was studied on *Phaseolus vulgaris* and *Lycopersicum esculentum* using immunological techniques with the monoclonal antibodies BC-8.DH2 and BC-12.CA4, enzymatic measurements and scanning electronic microscopy. All biocontrol agents reduced the disease. Greatest reduction in disease severity was obtained with the *Trichoderma* sp. T115. In the presence of this isolate significant reductions in antigen levels in the inoculation site was measured with the monoclonal antibody BC-12.CA4. At the same time the immunofluorescence pattern of the antigen recognized by BC-12.CA4 was weaker and more dispersed than the control. Scanning electronic microscopy showed attachments of the *Trichoderma* conidia to the extracellular matrix of *B. cinerea*. The antigen recognized by BC-8 DH2 and associated with penetration sites, was absent in the presence of T115. At the same time the number of penetrated hyphae was reduced by up to 50%. Enzymatic measurements showed an increase in the production of glucanases in the inoculation sites.

Key words: chitinases, glucanases, monoclonal antibodies, *Trichoderma harzianum*, T39, TRICHODEX

Introduction

Botrytis cinerea Pers.;Fr. is a polyphagous fungus – which causes damage and losses in a wide range of crops. Antigens present in the extracellular matrix of *B. cinerea*, which are glycoproteins with polysaccharides as the active antigenic binding sites were previously investigated for their importance in the fungal pathogenesis. Two antigens were found to be important, in that reductions in pathogenicity was observed when the antigenic binding sites were blocked during initial growth by adding specifically raised monoclonal antibodies (MAb) to the inoculation sites (Meyer & Elad, unpublished). These findings mean that targeting the antigens could be a new way to control the pathogen. The monoclonal antibodies used in this study were all previously raised against *B. cinerea* isolate P-6g, using cell free surface washing of fungal cultures or juice from infected grapes as immunogen (Meyer & Dewey, 2000). The antigen recognized by the monoclonal antibody BC-12.CA4 had a distinct binding site on L-Rhamnose and the antigen seems to have importance as an oligosaccarin (Darvill *et al.*, 1992; Meyer & Elad, unpublished). By immunofluorescence it was observed that the antigen recognized by MAb BC-8.DH2 was expressed mainly near points of attachment of the pathogen to the plant surfaces and penetration sites. The antigen recognized by MAb BC-12.CA4 was expressed extensively along the entire length of the hyphae. The two antigens may function as oligosaccharins, with polysaccharide side chains as the active sites. In the current study we examined how various biocontrol agents (Elad, 2000; Elad *et al.*, 2000; Guetsky *et al.*, 1998, 2001; Kapat *et al.*, 1998) affect the expression of these antigens and investigated whether these microorganisms are able to target the sites with cell wall

degrading enzymes like glucanases and chitinases.

Materials and methods

Cultures of antagonistic microorganism and pathogen

Five biocontrol agents from the collection of The Volcani Center, Bet Dagan were used. Three isolates of *Trichoderma harzianum,* T39 (of TRICHODEX), T115 and Tncim (Elad, 2000; Elad *et al.*, 2000; Kapat *et al.*, 1998), one yeast, S2, all were grown on PDA and the one bacterium, 6Ba (Guetsky *et al.*, 1998, 2001) was grown on nutrient agar. Bacterial or yeast cells were used in a 8 X 10^6 cells ml^{-1}, while *T. harzianum* suspensions were adjusted to 6 X 10^6 conidia ml^{-1} in water with 0.05% glucose and KH_2PO_4. *B. cinerea* isolates from the collection at The Volcani Center, BC I4 and BC I16, were grown on PDA for 10 days at 20°C. Conidia were suspended in water containing 0.05% of respectively glucose and KH_2PO_4 to a final concentration of 2 X 10^5 conidia ml^{-1}. Experiments were conducted in an incubator at 20°C.

Host plants and their treatment

Leaves of 3-4 week old bean plants (*Phaseolus vulgaris* L.) and 5-6 week old tomato (*Lycopersicon esculentum* Mill.) were detached and placed with the adaxial surface up on a plastic mesh in a sealed plastic box with water soaked filter paper in the bottom. Boxes were kept in an illuminated walk-in growth chamber at 20°C for 4-5 days. The leaves were inoculated with 5-10 40 1 droplets of spore suspensions after the method of Elad *et al.* (1994). The spore suspensions of the pathogens were mixed 1:1 with the suspension of the antagonist. As a positive control the pathogen suspension was mixed with buffer (0.05% glucose and KH_2PO_4), and as a negative control the buffer was used alone. Leaf disks of the inoculation site were made with a cork borer, size 10 mm, transferred to glass slides and stained with cotton blue for 20 min before evaluation of germination and estimating germ tube length under light microscopy. For scanning electron microscopy the leaf samples were fixed in formalin:ethanol:acetic acid (FAA), dried in critical point drier (CPD), gold sputter coated and observed using a Jeol T330 SEM. Penetrations were observed on leaf disks immunofluorescence- labeled with the monoclonal antibody BC-12.CA4 as described below.

Antigen expression observed by immuno-fluorescence

Two antigens present in the extracellular matrix of *B. cinerea* were immuno-labeled with the monoclonal antibodies BC-8.DH2 and BC-12.CA4 raised by Meyer & Dewey (2000). Leaf disks of the inoculation site were places in wells in 48-well tissue culture plates and processed after the method described by Meyer and Dewey (2000), however using the goat-anti-mouse FITC (F 9006 Sigma) in a 1:200 dilution. Disks were arranged on glass slides and mounted in a glycerol/PBS solution (Citifluor Ltd., London, UK) and covered with a cover slip. Disks were examined under blue/violet light using a Zeiss HBO-50 microscope with epifluorescence optics with filter set for FITC Ex. 480 visualization. All steps were performed at room temperature.

Antigen expression observed by Enzyme Linked Immunosorbent Assay (ELISA)

For each treatment, five inoculation sites were collected into one Eppendorf tube by suction and transfer of the remaining of the droplets on the leaf disks. Five leaf disks were taken with a cork borer size 5 mm or the area of the infection site and lesion expansion zone into the same Eppendorf tube. Each Eppendorf tube were added 1 ml buffer (PBS containing 0.5M Urea). Samples were vortexed and boiled for 5 min to remove any natural peroxidases. The supernatant was used to coat microtitre plates (Nunc), and processed by ELISA after the method of Meyer & Dewey (2000).

Enzymatic and protein measurement

Cell wall-degrading enzymes were evaluated in the liquid collected from the drops were *B. cinerea* interacted with the different biocontrol agents. Substrates were *p*-nitrophenyl-N-acetyl- β -D-glucoseaminidine (to test N-acetyl-β -D-glucoseaminidase activity), *p*-nitrophenyl-diacetyl chitobiose (to test chitobiosidase activity), *p*-nitrophenyl-diacetyl chitotriose (to test endochitinase activity) and *p*-nitrophenyl-β -D- glucopyranosside (to test β 1-3 glucanase) in citrate buffer. All samples of fractions were tested for protein by Bio-Rad Protein microassay and specific activities (μg *p*-nitrophenyl/μg proteine) were calculated.

Results and discussion

Disease, germination, germ tube length and penetration of B. cinerea

Disease was visible after 20 hours post-infection (hpi) when it was decreased by T39, T115, Tncim, S2 and 6Ba whereas at 48 hpi significant control was achieved by T115 and S2. Germination after 4 hpi was reduced by 40-70 % by all biocontrol agents. After 20 hpi the presence of Tncim, T39 and S2 reduced germination by 20-40 %, T115 reduced germination by up to 70 %. The germ tube length in the first 20 hpi was significantly reduced by up to 30 %, in the presence of *Trichoderma* isolates (results shown only as germtube biomass). Penetrations were reduced by all biocontrol agents, at 4 hpi T39, T115, S2 and 6Ba reduced it by 84, 100, 75 and 100% and at 8 hpi T115 and 6Ba reduced it by 50-51%, respectively (Table 1).

Antigen expression observed by immunofluorescence

Immunofluorescence patterns were compared to the controls of *B. cinerea* isolate alone. The pattern of the antigen recognized by BC-12.CA4 was continuously expressed along the entire length of the hyphae- however in the presence of Tncim, the matrix was intensely visible, while in the presence of T115 a more diffuse and weaker pattern was observed. The pattern of the antigen recognized by BC-8.DH2 in the control was expressed intensively at the penetration sites. The presence of Tncim or S2 resulted in a weakened pattern, while in the presence of T115 the signal was completely missing.

Antigen expression measured by ELISA

The direct measurements of absorbance values from ELISA tests using BC-12.CA4 showed significant reduced levels of this antigen in the interaction sites. Calculations of the absorbance levels per biomass shows that this antigen is significantly expressed in lower amounts or degraded in the presence of T115 and S2, while the expression of the antigen is increased in the presence of Tncim.

Polysaccharide degrading enzymes

When BC I4 was inoculated onto bean in the presence of T115 an increase in glucanase activity was observed. The levels of endochitinases were increased when BC I16 was inoculated onto bean in the presence of all *Trichoderma* isolates. Protein levels were significantly reduced in the presence of S2, while the *Trichoderma* isolates T39 and T115 resulted in increments.

Scanning electronic microscopy

The microscopy revealed an intense adhesion of non-germinated *Trichoderma* conidia along the length of the germinated *B. cinerea* hyphae. These conidia adhered to the extracellular matrix produced by *B. cinerea.*

All biocontrol agents had significant effect on disease reduction. Some biocontrol agents have the ability to affect antigens present in the extracellular matrix of *B. cinerea.* Conidia of *Trichoderma* sp. adhere to the extracellular matrix and the presence of certain isolates e.g. T115 results in visible reduction of the antigen productions. This can also be confirmed by

measurements by ELISA, where antigen production was significantly reduced, not only due to reductions in germination and germ tube length, but also antigen production per biomass. The immunofluorescence pattern of the antigen recognized by BC-8.DH2 which often is seen in association with penetration sites, was visibly absent in the presence of T115. The number of penetrating hyphae was also reduced. Increase in glucanase activity was measured in inoculation sites were T115 was present. Increased enzymatic activity could affect the antigens since their active binding sites are found on polysaccharides. Isolate T115 that had the best effect on disease control, also affected the antigens investigated. Initial screenings for microorganisms that target these antigenic binding sites could be performed by immunological techniques and thus increase efficiency of screening large collections of microorganisms for their ability to act as biocontrol agents.

Table 1. Effect of 5 biocontrol agents on *B. cinerea* BC I4 in drop-inoculation sites on bean leaves.

Treat-ment	Hpi	Disease severity (%)	Germtube biomass (germination x germ tube length)	Penetration (%)	Absorbance BC-12.CA4 (OD U x 1000)	Glucanase (µg *p*NP /µg protein)	Endo chitinase (µg *p*NP /µg protein)	Protein (µg/µl)
BC I4	4	0 ± 0	2059.0	17.3 ± 2.2	8 ± 2.6	-	-	-
	8	-	3900.6	89.7 ± 3.4	14 ± 1	0	0	0.30
	20	2.7± 0.9	6509.0	78.7 ± 9.0	57 ± 3.9	2.74	1.74	1.42
	48	9 ± 3.1	-	-	147 ± 1.8	5.74	1.42	5.50
BC I4	4	0 ± 0	2296.0	2.7 ± 0.9	9 ± 0.3	-	-	-
& T39	8	-	4475.3	82.7 ± 2.0	13 ± 0.6	2.71	10.6	4.13
	20	0.8 ± 0.3	7136.0	89.7 ± 2.7	67 ± 1.7	1.63	4.13	5.74
	48	7.2 ± 1.2	-	-	64 ± 1.5	4.02	1.42	8.06
BC I4	4	0 ± 0	434.8	0.0 ± 0	4 ± 0.6	-	-	-
&	8	-	1190.0	45.0 ± 3.5	10 ± 1.2	1.95	6.9	2.60
T115	20	0.04 ± 0.04	2532.7	71.7 ± 2.6	25 ± 0.3	3.07	1.97	4.94
	48	0.04 ± 0.04	-	-	42 ± 0	34.66	4.55	9.56
BC I4	4	0 ± 0	1293.3	15.3 ± 1.8	10 ± 0.3	-	-	-
&	8	-	1849.3	74.7 ± 2.3	22 ± 1.5	1.81	7.83	3.40
Tncim	20	0.42 ± 0.06	7252.0	87.3 ±4.1	111 ± 3.5	2.6	2.41	4.47
	48	8 ± 0.95	-	-	147 ± 3.7	10.11	1.76	2.25
BC I4	4	0 ± 0	1277.7	4.3 ± 0.3	7 ± 0.9	-	-	-
& S2	8	-	2320.0	69.0 ± 4.7	11 ± 0.6	4.09	4.09	0.66
	20	0.24 ± 0.11	5566.0	62.7 ± 5.2	25 ± 0.6	4.3	1.36	0.73
	48	4.8 ± 0.66	-	-	113 ± 1.2	3.53	0.63	3.53
BC I4	4	0 ± 0	159.0	0.0 ± 0	5 ± 0.7	-	-	-
& 6Ba	8	-	3294.2	44.4 ± 13.2	9 ± 1.2	1.73	1.47	4.13
	20	0.12 ± 0.08	8046.0	93.0 ± 1.5	49 ± 1.3	1.26	1.04	5.82
	48	7.4 ± 1.33	-	-	109 ± 0.7	3.43	0.71	7.30

Hpi=Hours post infection

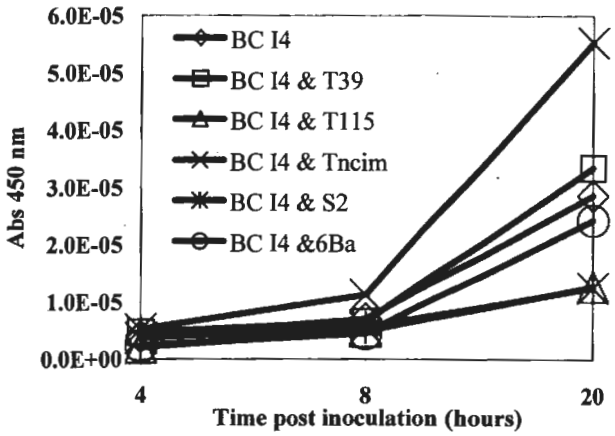

Figure 1. The antigen (BC-12.CA4) production per biomass (germination x germ tube length) of *B. cinerea* I4 when inoculated onto bean leaves in the presence of other microorganisms.

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Mode of interaction between the arbuscular mycorrhizal fungus *Glomus mosseae* and the root pathogen *Aphanomyces euteiches*

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Arbuscular mycorrhiza (AM) has been shown to have a positive influence on plant health, especially by suppressing a broad range of root pathogens. In order to be able to integrate such natural control mechanisms in agricultural practices it is important to understand how AM fungi interact with pathogens. Pathogen suppression has been ascribed to both general physiological changes in mycorrhizal plants and direct effects of the fungal symbiont on the pathogen through antibiosis. However, the actual role of AM in disease control, is still poorly understood. The objective of the present work was to study the mode of interaction between the AM fungus *Glomus mosseae* (Gm) and the root pathogen *Aphanomyces euteiches* (Ae) in pea roots. Signature fatty acids were used to estimate biomass and amount of energy reserves of both these root-inhabiting fungi. The phospholipid fatty acids (PLFA) 14:0 and 16:1 ω5 were used to estimate biomass of Ae and Gm, respectively. The neutral lipid fatty acids (NLFA) 14:1ω9 and 16:1ω5 were used to estimate the amount of energy reserves of Ae and Gm, respectively.

Pisum sativum L. was grown in a semi-sterile soil:sand mix with and without Gm. Pots were placed in a temperature-regulated container providing a constant soil temperature of 21 °C. Two weeks after seedling emergence 10^5 zoospores of Ae in dilute salts were delivered to the stem base of plants in one half of the pots and plants, and the other half of the pots received dilute salts only. Plants in four replicate pots of each treatment (± Gm * ± Ae) were harvested 5, 10 and 20 days after zoospore application.

Inoculation of pea with Ae resulted in a decrease in shoot and root dry weight. The symbiosis between pea and Gm had no effect on the disease severity (discolouration of roots) caused by Ae. However, root length with oospores of Ae, as revealed after clearing and staining roots with trypan blue in lactoglycerol, was lower in mycorrhizal roots than in non-mycorrhizal roots. Similarly, the presence of Gm in pea roots reduced both the biomass and level of energy reserves of Ae as indicated by a reduction in the amount of both PLFA 14:0 and the NLFAs 14:0 and 14:1ω9. Biomass and level of energy reserves of Gm was on the other hand also depressed in pea roots infected by Ae as indicated by a reduction in both PLFA and NLFA 16:1ω5.

In conclusion, the results from the present work demonstrate that signature fatty acids can be used to quantify biomass and amount of energy reserves of an AM fungus and *A. euteiches* in pea roots, simultaneously. Furthermore, the mutual inhibition between Gm and Ae in terms of biomass and amount of energy reserves might indicate that these root-inhabiting fungi are competing for nutrients, which add further to the clarification of their mode of interaction.

Biological Control of Fungal and Bacterial Plant Pathogens
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pp.13- 16

Biological control of wheat foot rot by antagonistic fungi and their modes of action

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Abstract: The effectiveness of five antagonists*, *Trichoderma atroviride* 312, *T. longibrachiatum* 9, *T. harzianum* 144, *Gliocladium roseum* 11 and *Penicillium frequentans*, applied to wheat seeds naturally infected with *Fusarium culmorum*, was tested in two experiments in a two year field study. All biological treatments, in the first experiment, gave a significant increase in emergence, winter survival, number of heads and yield, and a low disease incidence and severity when compared to the infected control. In the second experiment, a yeast culture was added to seeds treated with *G. roseum* and *T. harzianum*; this addition did not seem to induce an improvement in the antagonistic activity. All antagonists colonized wheat rhizosphere and showed different *N*-acetyl-ß-D-glucosaminidase, chitin 1,4-ß-chitobiosidase and glucan-1,3-ß-glucosidase enzymatic activities in culture filtrates added with cell walls of *F .culmorum*, *F. nivale* and *F. graminearum*. A consistent protease activity was detected by *P. frequentans* in the presence of each pathogen. All antagonists produced non volatile and volatile metabolites active in reducing *F. culmorum* colony growth. Scanning electron microscopy (SEM) observations of *Trichoderma* spp., *G. roseum* and *P. frequentans* hyphae revealed different types of interaction with *Fusarium* species.

Key words: biological control, antagonistic fungi, wheat foot rot, *Fusarium* spp.

Introduction

Wheat foot rot caused by *Fusarium* species is a very common disease, economically important in Italy (Innocenti, 1996) as well as in many other wheat producing areas (Wiese, 1987). The use of microorganisms for biological control is of interest due to the undesirable side effects of pesticides. The objectives of our study were: a) to verify the antagonistic activities of *Trichoderma atroviride* 312, *T. longibrachiatum* 9, *T. harzianum* 144, *Gliocladium roseum* 11 and *Penicillium frequentans** against *F. culmorum* (*F.c.*) in the open field; b) to check their rhizosphere competence; c) to investigate mechanisms of action against *F.c.*, *F. nivale* (*F.n.*) and *F. graminearum* (*F.g.*).

Material and methods

Biological control

A 2-year field study was conducted, with two experiments, to evaluate the effectiveness of the antagonists applied to durum wheat seeds naturally infected by *F. c.* (20%). Seeds for the first

*T. *longibrachiatum* 9, *T. atroviride* 312, *T. harzianum* 144 and *G. roseum* 11 were isolated from vegetable root, vegetable seed, loamy soil and wheat crown respectively and identified by CBS (Baarn, Netherlands); numbers refer to our collection. *P. frequentans* was provided by Dr. P. Melgarejo (INIA, Madrid, Spain).

experiment were coated with a conidial suspension ($1x10^8$ conidia/ml) of all five antagonists and talcum powder, while, for the second experiment, seeds were coated respectively with *G. roseum* and *T. harzianum* conidial suspensions combined with talcum powder and a yeast culture. Treatments were assessed from emergence (December) to crop harvest (May to June).

Rhizosphere competence and mechanisms of action

The rhizosphere competence of the antagonists was detected in a complete sterile system following the method described by Ahmad & Baker (1987) partially modified. The hyperparasitism of these microorganisms was studied using a scanning electron microscope (SEM) according to Pisi & Filippini (1994). Ability of the antagonists to produce lytic cell-wall-degrading-enzymes (*N*-acetyl-ß-D-glucosaminidase, chitin-1,4-ß-chitobiosidase, glucan-1,3-ß-glucosidase and protease) was determined in culture filtrates added with cell wall constituents of *F. c.*, *F. n.* and *F. g.* as sole carbon sources (Elad *et al.*, 1982; Tronsmo & Harman, 1993). Non volatile and volatile metabolites were tested *in vitro* according to Dennis & Webster (1981).

Results and discussion

Biological control

First experiment - It was observed that the biological treatments compared to the infected control, caused a significant increase in emergence, winter survival, number of heads and yield, and a low disease incidence and severity in both years. Table 1 shows the emergence, disease severity and yield inferred from the first year.

Table 1. Effect of biological seed treatments on foot and root rot of durum wheat caused by *F. culmorum* in the first experiment, the year one.

Treatments	Emergence (No seedlings/m)	Disease severity[1] (%)			Yield (kg/ha)
	December	January	April	June	June
Infected control	48.0 a[2]	35.3 e	55.2 f	60.5 d	3,421 a
G. roseum	61.6 b	15.6 bc	17.9 bc	19.9 b	5,622 cd
P. frequentans	60.0 b	20.9 d	24.5 e	26.4 c	5,715 d
T. atroviride	64.6 b	17.0 c	20.2 cd	22.8 bc	5,150 b
T. longibrachiatum	61.4 b	19.3 d	22.6 de	23.2 bc	5,350 bc
T. harzianum	63.0 b	14.7 b	17.3 b	20.8 bc	5,460 bcd
Chemical[3]	65.4 b	2.6 a	7.6 a	9.2 a	6,120 e
Non infected control	65.0 b	2.5 a	5.3 a	6.7 a	5,680 cd

[1] Disease index was estimated using a scale of 0 to 4, where 0 = no signs of infection, 1 = very slight infection , 2 = slight infection, 3 = moderate infection and 4 = aboundant infection. Disease severity was calculated as: Σ (disease index × number of diseased plants in each index) × 100/4 × (total number of examined plants). [2] Means in column not followed by the same letter are significantly different ($P \leq 0.05$) according to Duncan's multiple range test.
[3] 0.23 g prochloraz + 0.80 g mancozeb / kg seed.

Second experiment – A yeast culture added to seeds did not seem to induce any improvement in the antagonistic activity of *G. roseum* and *T. harzianum*. A decrease in disease incidence was observed for both years but only in the last assessment when compared to the treatments without yeast. Furthermore, *G. roseum* showed a low disease severity in the last assessment, but only for the first year.

Rhizosphere competence and mechanisms of action

All antagonists were recovered from the rhizosphere adhering to the first 6 cm of seedling roots; *T. longibrachiatum* had the highest colonization ability (Figure 1).

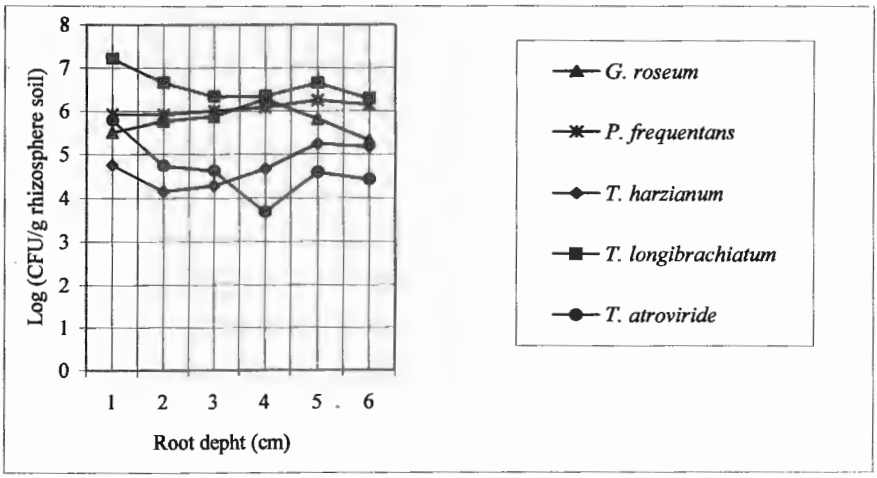

Figure 1. Population density in the wheat rhizosphere inoculated with the antagonists.

SEM observations revealed that *Trichoderma* spp., *G. roseum* and *P. frequentans* hyphae interacted with *Fusarium* spp. Hyphal coiling, hooks, pincer-shaped structures, short contact branches and hyphal depressions were mainly observed when *T. longibrachiatum* interacted with *Fusarium* species. Figure 2 shows *T. longibrachiatum* parasitising *F.c.* hyphae.

Figure 2. Mycoparasitic action of *T. longibrachiatum* on *F. culmorum* hyphae (bar = 0.1 mm).

The glucosaminidase activity for all the antagonists was always higher than that of chitobiosidase; *G. roseum* had the highest values of both enzymatic activities. *T. atroviride*

produced the highest level of glucosidase especially when grown with *F.g.* cell walls. A consistent protease activity was detected when *P. frequentans* was with all *Fusarium* spp.

Volatile and non-volatile metabolites produced by all the five biocontrol agents tested our experiments were able to reduce the growth of *F. c.* colonies. Furthermore, *T. longibrachiatum* and *T. atroviride* had an inhibitory effect on *F. c.* colony growth in the presence of their non-volatile metabolites.

Our study supports the hypothesis that the antagonists tested, when applied to wheat seeds, may serve as biological agents in controlling *F. c.* The *in vitro* studies on the mechanisms of action of the five antagonists against *F.n.* and *F.g.*, also involved in wheat foot rot disease, demonstrated a parasitic action accompanied by a production of lytic enzymes and toxic metabolites. Furthermore we should not exclude competition or induced resistance that are other modes of action of the antagonists.

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Biological Control of Fungal and Bacterial Plant Pathogens
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Mode of action of *Brevibacillus brevis* - biocontrol and biorational control

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Abstract: A biorational approach to biocontrol of *Botrytis cinerea* was adopted through fractionation of *Brevibacillus brevis*. Such fractions possessed either one or none of the known active components of *B. brevis* (gramicidin S or biosurfactant). Whole cultures and fractions were used in field trials with tomato and lettuce crops. In tomato crops, treatments containing only the biosurfactant were as effective as *B. brevis* WT in reducing grey mould leaf symptoms, suggesting that the biosurfactant was responsible for disease control. However, *B. brevis* WT gave far superior control of grey mould stem lesions with less than half as many lesions as treatments containing only one active component. In spring lettuce, treatments containing either active component performed equally well whereas in a more rapidly growing preliminary trial spores of WT (containing only gramicidin S) had given superior control. It is suggested that the biosurfactant is mainly responsible for disease reduction in regions of free air movement such as open leaf canopies. However, when the environment is such that high humidity renders the biosurfactant inactive, gramicidin S is responsible for disease control.

Key words: biorational, *Brevibacillus brevis*, *Botrytis cinerea*, lettuce, tomato

Introduction

Brevibacillus brevis wild type (WT) has been shown to control grey mould caused by *Botrytis cinerea* in protected Chinese cabbage in the UK (Edwards *et al.*, 1994) and tomatoes in Greece (Markellou *et al.*, 1998). It has two modes of antagonism: production of gramicidin S which inhibits conidial germination and production of a biosurfactant which reduces periods of leaf wetness that encourage infection. The biosurfactant is released into the culture medium while Gramicidin S remains largely bound to the bacterial spore. A gramicidin S-negative mutant (E1) aids elucidation of the operative mode of action. A biorational approach can thus be adopted, whereby, if the biosurfactant is operative in biocontrol, supernatant preparations should perform as well as whole cultures. If, however, gramicidin S is the sole mode of action, washed spores of *B. brevis* WT should be as effective as WT whole cultures. When both components play a role in disease control whole cultures of *B. brevis* WT should perform best, whereas *B. brevis* E1 spores should give no protection. The validity of this interpretation was tested in this study.

Materials and methods

B. brevis WT and E1 whole cultures were incubated in tryptic soy broth (TSB) for 14 d in a rotary incubator (150 rpm, 37°C). To prepare supernatant and washed spore spray treatments, WT and E1 whole cultures were centrifuged (3000*g* for 10 minutes). The supernatant was reserved for use as spray treatments and the pellets resuspended in sterile distilled water (SDW) before shaking vigorously to resuspend and wash the spores. The spore suspension was centrifuged (3000 g for 10 minutes) twice more, the supernatant being discarded each

time and the pellet resuspended in SDW to produce the final washed spore spray treatment (10^7spores/ml). Whole cultures (WT and E1, 10^7spores/ml), supernatants (SWT and SE1) and washed spores (WTSp and E1Sp) of *B. brevis* WT and E1 were used in randomised block polytunnel trials with lettuce and tomato. Control treatments (SDW) and Rovral (iprodione) fungicide at recommended rates (1 g/l tomatoes; 0.5 g/l lettuce) were also used. Three blocks, each with two plots of 20 plants per treatment, were used in lettuce trials and five blocks, each with two plots of three plants per treatments were used in tomato trials. Treatments were applied every 14 d using hand-held sprayers and disease severity was recorded weekly as the percentage of leaf area affected by grey mould or by number and severity of stem lesions. Spring lettuce crops were scored at harvest in order that basal grey mould disease symptoms obscured beneath plants could be observed.

Results and discussion

Whole cultures of both WT and E1 were equally effective in reducing leaf disease in tomato (disease control of 40-50%) and SWT and SE1 were generally as effective as whole cultures (Fig 1). This suggests that the biosurfactant was responsible for disease control since use of *B. brevis* WT containing gramicidin S did not provide better disease control.

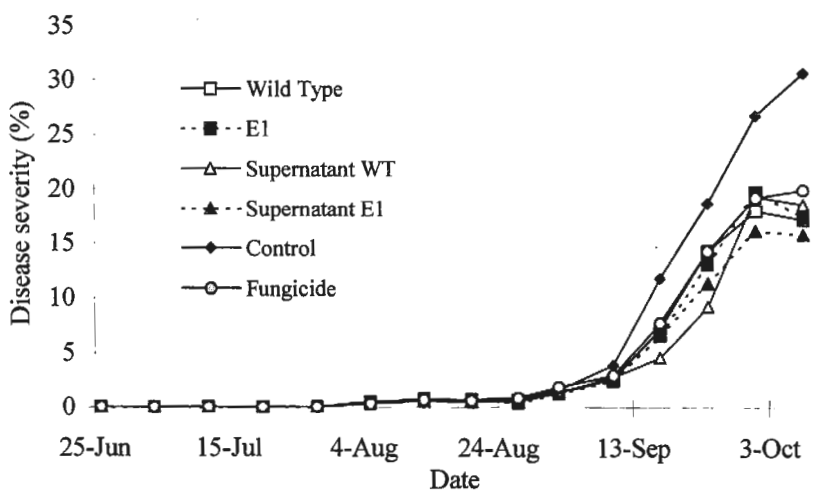

Figure 1. Grey mould disease progress on tomato leaves.

When grey mould disease was assessed in tomato stems, however, few (30%) WT treated tomato plants had any stem lesions as opposed to the majority (80%) of control plants. Intermediate levels (60 to 70%) of tomato plants treated with WTSp, E1 and SE1 were affected by stem lesions. The total number of lesions was lowest in WT treated plants and, of the lesions found in WT, E1 and WTSp treated plants, 50-65% were less than 10cm as opposed to 35-40% of those on E1Sp treated and control plants (Fig 2). E1Sp containing neither active component, did not reduce disease.

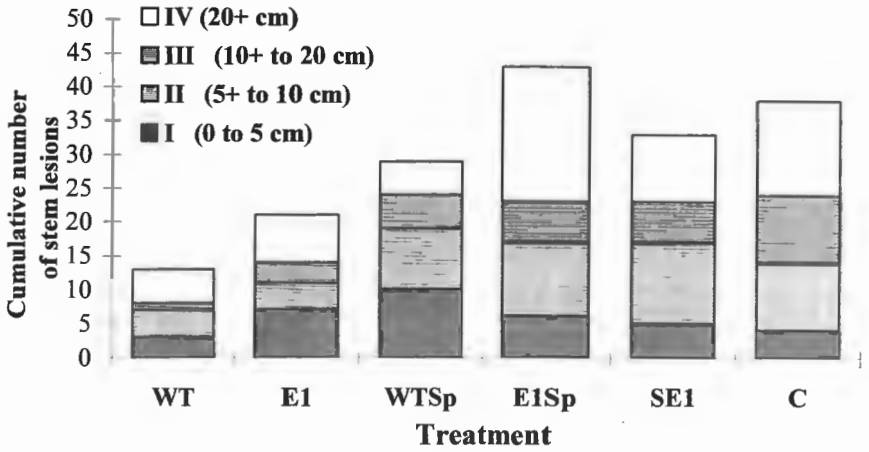

Figure 2. Number and severity of grey mould stem lesions in tomato. Each column represents the total number of lesions on thirty plants.

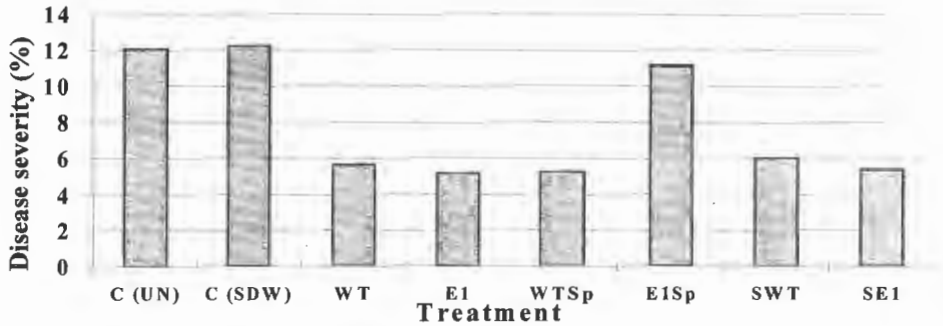

Figure 3. Grey mould disease severity at harvest in spring lettuce. C (UN), unsprayed control; C (SDW), sterile distilled water sprayed control; WT, *B. brevis* WT whole culture; E1, *B. brevis* E1 whole culture; WTSp, washed spores of WT; E1Sp, washed spores of E1; SWT, supernatant of WT; E1, supernatant of E1.

In spring lettuce (Fig. 3), *B. brevis* treatments (WT, E1, WTSp, SWT and SE1) reduced disease to the same extent (50-60%) and E1Sp had no effect suggesting that both the biosurfactant and/or gramicidin S were effective in controlling grey mould, although there was no additive effect since *B. brevis* WT containing both gramicidin S and biosurfactant was only as effective as treatments containing either active component.

In a previous preliminary trial, where a region of high humidity had quickly developed at the base of more rapidly growing lettuce plants, WTSp had given superior control (65%) to all other treatments (up to 35%) indicating that under these conditions the biosurfactant is less effective.

It appears that disease reduction in tomato leaves was mostly attributable to the biosurfactant since *B. brevis* WT (containing gramicidin S and biosurfactant) gave similar control to biocontrol treatments containing only biosurfactant. However, both gramicidin S and the biosurfactant appear to have contributed to disease reduction in tomato stems since *B. brevis* WT gave superior biocontrol but disease was reduced in all treatments containing either the biosurfactant or gramicidin S. E1Sp, containing neither biosurfactant nor gramicidin S, was not effective in reducing disease indicating that it is the gramicidin S and biosurfactant that contribute to disease control. In spring lettuce, both the biosurfactant and gramicidin S appear to play a role in disease control since treatments containing either one or both were equally successful. There is some evidence that in rapidly growing crops, where a high humidity environment quickly develops beneath plants, the biosurfactant is rendered less effective and gramicidin S is more responsible for disease control.

It is clear that both modes of *B. brevis* antagonism are important in disease control in both tomato and lettuce. In less humid environments and on plant parts with a large surface/volume ratio (open leaf canopies), biocontrol is achieved through the action of the biosurfactant. In more humid regions or with plant material with a low surface/volume ratio (beneath lettuce plants and on tomato stems), gramicidin S may play a more active role in disease reduction. The biorational approach adopted here could be developed to formulate a range of biofungicide preparations of *B. brevis* for disease control purposes.

Acknowledgements

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Involvement of reactive oxygen species and antioxidative processes in the disease caused by *B. cinerea* on bean leaves and in its biological control by means of *Trichoderma harzianum* T39

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Abstract: *Botrytis cinerea* causes necrotic lesions on bean (*Phaseolus vulgaris*). The biocontrol agent *Trichoderma harzianum* T39 controls the disease either when applied simultaneously with the pathogen or when it is spatially separated from the pathogen infection site. T39 was either applied to the roots or to the primary or secondary leaves. The formation of reactive oxygen species (ROS) in the bean leaves in the rot or in the symptomless tissue around it was tested as a result of infection and/or as a result of T39 treatment. Further, the formation of the antioxidative enzymes catalase, peroxidase, superoxide dismutase (SOD) and the process of lipid peroxidation in the rotten tissue and around it was also tested. *B. cinerea* infection on the primary leaves resulted in elevated ROS in the same leaves either in the rot or around it and to a lower extent also in the secondary leaves. This was not the case when the secondary leaves were infected. All the antioxidant enzymes were elevated in the primary infected leaves whereas only peroxidase was increased on the secondary infected leaves. Infection of the secondary leaves also resulted in antioxidant enzyme changes in the primary leaves. In the absence of *B. cinerea*, T39 treatment to the soil resulted in decreased ROS in the primary and secondary leaves, in comparison with the untreated leaves. The respective treatment of T39 to the primary and to the secondary leaves decreased the ROS in the secondary and in both leaf types. T39 application to the roots resulted in an increase of the antioxidant enzymes in the primary leaves and/or in the secondary leaves. T39 treatment to the primary leaves resulted in increased peroxidase in the same leaves and in increased lipid peroxidation in the secondary leaves, whereas T39 treatment to the secondary leaves increased SOD in the same leaves and increased peroxidase and catalase in the primary leaves. T39 leaf treatment decreased in the area around the rot of the *B. cinerea*- infection- induced ROS whereas root treatment decreased all ROS levels. The biocontrol agent decreased the peroxidase, catalase and SOD levels that were induced by *B. cinerea* in the symptomless tissue around the rot and the SOD that was induced by the pathogen in the rot itself.

Key words: *Botrytis cinerea*, *Trichoderma harzianum*, peroxidase, catalase, lipid peroxidation, superoxide dismutase, reactive oxygen species, induced resistance

Introduction

Reactive oxygen species (ROS) and free radicals are involved in many different facets of stress physiology including pathogen attack of their tissues. Among the reactive species are singlet oxygen ($^1O_2$), superoxide (O_2^-) and perhydroxy (HO_2^-) radicals, hydrogen peroxide (H_2O_2), hydroxyl radical (OH^-) nitric oxide, free radicals of lipids and other carbohydrates and more. The cells have evolved an efficient radical scavenging system that employs antioxidant enzymes and various compounds such as βcarotene, vitamins, organic acids etc. However, during periods of stress the ROS overwhelm the antioxidative capacity of the cells and a collapse of the cell may occur (Baker & Orlandi, 1999).

One of the most studied commercial biocontrol agents is isolate T39 of *Trichoderma harzianum*, the active ingredient of TRICHODEX. T39 controls the foliar pathogens, *Botrytis cinerea*, *Pseudoperonospora cubensis*, *Sclerotinia sclerotiorum* and *Sphaerotheca fusca* (syn. *S. fuliginea*) in cucumber, tomato, grapes and other crops under commercial cultivation.

cinerea, *Pseudoperonospora cubensis*, *Sclerotinia sclerotiorum* and *Sphaerotheca fusca* (syn. *S. fuliginea*) in cucumber, tomato, grapes and other crops under commercial cultivation. Involvement of locally and systemically induced resistance has been demonstrated. Cells of the biocontrol agent (BCA) applied to the roots, and dead cells applied to the leaves of cucumber plants induced control of powdery mildews and grey mould. T39 also suppressed enzymes of *B. cinerea,* such as pectinases, cutinase, glucanase and chitinase, through the action of protease secreted on plant surfaces. A combination of several modes of action is responsible for the biocontrol. However, biocontrol is not achieved by means of antibiotics or by mycoparasitism, in spite of the fact that the BCA has the potential to degrade cell-wall polymers, such as chitin (Elad, 1994, 1996, 2000ab; Elad & Kapat, 1999; Elad *et al*, 1998ab).

Botrytis cinerea causes necrotic lesions on bean (*Phaseolus vulgaris*). In the present study primary or secondary leaves of bean were separately infected by *B. cinerea*. T39 affects the disease either when applied simultaneously with the pathogen or when it is spatially separated from the pathogen infection site. T39 was either applied to the roots or to the primary or secondary leaves. The formation of reactive oxygen species (ROS) in the bean leaves in the rot or in the symptomless tissue around it was tested as a result of infection and/or as a result of T39 treatment. The effect of T39 on *B. cinerea* induced ROS and plant antioxidant activity is also reported here.

Materials and methods

Plants and microorganisms

Bean plants (*P. vulgaris* L.) were grown in 1l pots containing a common growing mixture. Four-five week old plants were used. A suspension of *T. harzianum* T39 (TRICHODEX, Makhteshim Chemical Works, Beer Sheva, Israel) at a concentration of 10^6 conidia/ml (0.4% preparation) was sprayed on lower, primary leaves or on upper, secondary leaves or was injected to the root zone of the plants. Twenty µl drops of *B. cinerea* suspension (10^5 conidia/ml) supplemented with 0.05% glucose and KH_2PO_4 were inoculated on primary or secondary leaves.

Assays of enzymes and reactive oxygen species

Antioxidant enzyme activity and products of lipid peroxidation

Presence of natural antioxidants in the leaves was evaluated in the infected tissue, in tissue surrounding the infection and in non-infected leaves. The samples were taken 10-72 h after treatment. Samples were immediately frozen in liquid N_2, lyophilized and stored at -80 C. Enzymes were extracted from homogenized dry tissue in phosphate buffer 0.01M, pH 6, 0.2% EDTA, 0.4% PVP at 4 C. All samples of fractions were tested for protein by Bio-Rad Protein microassay and assayed for Peroxidase, Catalase and Superoxide Dismutase (SOD) activities.

Peroxidase was assayed using guaiacol as the hydrogen donor (0.25% v/v guaiacol in 0.01 M sodium phosphate buffer, pH 6.0 and 0.1M H_2O_2). Enzyme extract (0.1 ml) was added to initiate the reaction that was followed colorimetrically at 470 nm. Activity was expressed as the increase in absorbance at 470 nm min^{-1} mg^{-1} protein. **Catalase** was assayed by following the disappearance of peroxide spectrophotometrically at 240 nm. One ml of substrate solution 0.059 M H_2O_2 in 0.05 M phosphate buffer, pH 7.0 was added to 2 ml of diluted enzyme solution in 0.05 M phosphate buffer pH 7.0. Readings were taken at 20-second intervals for 2 minutes and determined delta OD mg $protein^{-1}$ min^{-1}. **SOD** was assayed by measuring its ability to inhibit the photochemical reduction of nitro blue tetrazolium (NBT). The 3-ml reaction mixture contained 50 mM phosphate buffer, pH 7.8, 13 mM methionine, 75 µM NBT, 2 µM riboflavin (added last), 0.1 mM EDTA and 0-50 µl enzyme extract. The tubes were placed 30 cm below a light bank of two 15 W fluorescent lamps to

allow the reaction to take place. Absorbance was read at 560 nm. A non-irradiated reaction mixture did not develop colour and served as control. The reaction mixture-lacking enzyme developed the maximum colour and this decreased with increasing volume of enzyme extract added. Log A560 was plotted as function of the volume of enzyme extract used in the reaction mixture. From the resultant graph the volume of enzyme extract corresponding to 50% inhibition of the reaction was read and was considered as one enzyme unit. The process of **Lipid peroxidation** in the leaf tissue was measured in term of malondialdehyde (MDA, a product of lipid peroxidation) content determined by the thiobarbituric acid (TBA) reaction. All steps of the assay were carried out at 4 C. Each 0.01g dry leaf sample was homogenized in 1 ml 0.1% TCA. The homogenate was centrifuged at 14 000 g for 15 min. To 1 ml aliquot of the supernatant 4 ml 20% TCA containing 0.5% TBA were added. The mixture was heated at 95 C for 30 min and quickly cooled in ice-bath. After centrifuging at 10 000 g for 10 min the absorbance of the supernatant at 532nm was read and the value for the non-specific absorption at 600 nm was subtracted. The concentration of MDA was calculated using its extinction coefficient of 155 $mM^{-1}cm^{-1}$. Activity was expressed as μM MDA/g dry weight tissue. The fluorogenic compound 2,7-dichlorodihydrofluorescein diacetate (DCF-H = D-399) is used for detection of hydrogen peroxide and nitric oxide and was found to be very sensitive to peroxynitrite oxidation. Leaf samples in shapes of 1cm-diameter disks were used for H_2O_2 determination in a fluorimeter (FL600 Microplate fluorescence reader, Bio Tek). The leaf disks were incubated for 15 min in water and in darkness and then incubated for 45 min in a solution of 20 μg/ml DCF-H in a 10 mM MES buffer pH 7.0 that replaced the water. The readings were carried out at wavelength of 485 nm excitation and 530 nm emission. Several readings were taken during the time of incubation and the results are presented as change in fluorescence units per minute.

Results and discussion

B. cinerea causes necrotic lesions on bean (*P. vulgaris*). In the present study primary or secondary leaves of bean were separately infected by *B. cinerea*. *T. harzianum* T39 is a biocontrol agent of *B. cinerea*. T39 affected the disease either when applied simultaneously with the pathogen or when it is spatially separated from the pathogen infection site. T39 was either applied to the roots or to the primary or secondary leaves. The formation of reactive oxygen species (ROS) in the bean leaves in the rot or in the symptomless tissue around it was tested as a result of infection and/or as a result of T39 treatment. Further, the formation of the antioxidative enzymes catalase, peroxidase, superoxide dismutase (SOD) and lipid peroxidation in the rot tissue and around it was also tested.

B. cinerea infection on the primary leaves resulted in elevated ROS in the same leaves either in the rot or around it and to a lower extent also in the secondary leaves. This was not the case when the secondary leaves were infected. All the antioxidant enzymes were elevated in the primary infected leaves whereas only peroxidase was increased on the secondary infected leaves. Infection of the secondary leaves also resulted in antioxidant enzymes change in the primary leaves.

In the absence of *B. cinerea*, T39 treatment to the soil resulted in decreased ROS in the primary and secondary leaves, in comparison with the untreated leaves. The respective treatment of T39 to the primary and to the secondary leaves decreased the ROS in the secondary and in both leaf types, respectively. T39 application to the roots resulted in an increase of the antioxidant enzymes in the primary leaves and/or in the secondary leaves. T39 treatment to the primary leaves resulted in increased peroxidase in the same leaves and in increased lipid peroxidation in the secondary leaves, whereas T39 treatment to the secondary

leaves increased SOD in the same leaves and increased peroxidase and catalase in the primary leaves.

The disease was evaluated 1, 2, 3 and 4 days after infection. At these dates *B. cinerea* incited 1.8, 4.6, 9.3 and 20.0% disease severity. Disease severity on the T39 leaf treated plants was 0, 0, 0 and 0.5%, respectively and on leaves of the T39 root treated plants it was 0, 3.11, 4.5 and 9.0% respectively.

T39 leaf treatment decreased the rot in the area around the *B. cinerea*- infection- induced ROS whereas root treatment decreased all ROS levels. The biocontrol agent decreased the peroxidase, catalase and SOD levels that were induced by *B. cinerea* in the symptomless tissue around the rot and the SOD that was induced by the pathogen in the rot itself (Table 1).

In conclusion, infection is associated with increased ROS and therefore antioxidants are significantly elevated to combat it. The biocontrol agent *T. harzianum* T39 decreased the ROS levels in the uninfected tissue and negated the *B. cinerea* induced ROS. T39 treatment also was associated with lower levels of antioxidant enzymes in the *B. cinerea* infected tissue probably because of the reduced disease ROS levels. The effect of *T. harzianum* T39 was obtained in the same treated leaves and also systemically transferred from roots to leaves and from leaves to spatially separated leaves, confirming the systemic induction of resistance by this biocontrol agent.

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Table 1. Levels of reactive oxygen species (ROS) and antioxidant enzymes in bean leaves infected by *Botrytis cinerea*, treated by *Trichoderma harzianum* T39 or treated by T39 and infected by *B. cinerea*.

Treatment[1]		Leaf Tested[2]	ROS[3]	Antioxidant enzymes																
				Peroxidase[4]					Catalase[5]				SOD[6]				Lipid peroxidation[7]			
B.cinerea	*T.harzianum*		Time after treatment with *B. cinerea* and/or *T. harzianum* T39																	
			18	10	24	48	72	10	24	48	72	10	24	48	72	10	24	48	72
-	-	1st[6]	172	76	6.8	69	76	20	1130	1770	2980	13	5	9	9	4.3	6.6	3.9	4.7
-	-	2nd	134	53	25	11	13	20	4870	410	830	2	5	3	4	2.8	3.7	2.9	3.3
-	Soil	1st	89	118	38	89	89	10	2960	3200	4900	7	8	11	5	5.4	6.3	6.2	3.3
-	Soil	2nd	71	161	151	143	29	10	1120	1290	540	11	3	7	6	2.4	5.1	4	3.1
-	1st leaf	1st	154	56	42	102	80	10	2420	8400	5000	4	10	6	5	5.1	6.9	4.4	4
-	1st leaf	2nd	95	17	2.6	7.8	5	0	780	250	250	3	1	2	2	2.4	7.7	3.9	3
-	2nd leaf	1st	92	65	53	125	369	10	332	3330	1970	4	11	17	30	4.3	4.8	6.2	3.9
-	2nd leaf	2nd	75	21	7.7	35	41	0	410	760	1070	2	3	7	7	3.2	3.6	3.1	2.5
-	2nd leaf	2nd Untreated	56	10	4.2	40	39	10	1100	790	1900	3	2	8	7	3	4	3	2.7
1st leaf	-	1st Around	1179	94	69	125	151	20	4900	4820	2430	4	13	12	19	3.8	4.6	2.6	7
1st leaf	-	1st Rot	1090	65	41	58	138	20	2610	2400	2370	7	33	18	26	4.5	6.7	3	3.8
1st leaf	-	2nd	334	13	20	33	30	10	1640	910	1290	3	2	8	10	3.6	2.6	5.6	4.4
2nd leaf	-	1st	89	64	24	11	138	10	690	1570	2940	7	4	4	13	5.4	5.6	5.8	4
2nd leaf	-	2nd Around	95	26	6.5	55	26	10	650	600	560	6	1	6	5	4.6	3.7	3.4	0.3
2nd leaf	-	2nd Drop	89	1.6	27	10	0.9	0	1710	900	500	19	5	9	5	2.7	2.9	3	2.9
1st leaf	Soil	1st Around	102	60	45	119	111	8	4432	3322	2001	4	8	5	12	3.1	4.6	4.0	3.0
1st leaf	Soil	1st Rot	227	55	39	101	90	9	2000	1400	1012	15	5	4	23	2.3	3.8	2.9	2.0
1st leaf	Soil	2nd Untreated	98	25	21	47	21	3	850	620	1550	3	6	4	9	3.0	2.5	2.2	2.2
1st leaf	1st leaf	1st Around	810	65	51	122	119	10	5600	4170	2380	5	11	5	14	4.1	5.2	4.8	4
1st leaf	1st leaf	1st Rot	1179	20	25	50	73	10	1870	2400	2100	12	7	10	28	3.5	4.7	2	1.2
1st leaf	1st leaf	2nd Untreated	430	9.6	12	30	42	0	970	730	1740	4	7	3	11	3.4	2.8	2.6	2.9

[1] Infection by *Botrytis cinerea* was carried out on primary or secondary leaves and *Trichoderma harzianum* T39 was applied to the soil or to primary leaves or to secondary leaves (2nd); [2] Leaves tested were primary (1st) or secondary (2nd) either not infected or in the rot area or in the symptomless tissue around the rot; [3] Reactive oxygen species (Fluorescence units/min); [4] Peroxidase (OD/mg/min); [5] Catalase (–OD/ µg/min); [6] Superoxide dismutase (U/mg/min); [7] Lipid peroxidation that gave rise to MDA (U/mg fresh weight).

The use of models to understand variability in biological control

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Successful biological control depends on the skilful interference with the population dynamics of three or more interacting species, including the biological control agent (BCA), the target parasite, the host crop and sometimes vectors and an indigenous microflora. The problems centre on the dynamics of invasion and persistence: *Will the parasite invade? Will the BCA invade? Will the parasite and the BCA both persist or will one or other be eliminated? How long is the time to extinction? How do these outcomes affect the chance of successful control? How much BCA should be applied, when and where?* These questions are complicated by the intrinsic variability of biological control caused by spatial heterogeneity within and between sites and stochastic variation in the behaviour of the parasite and the BCA as well as changing environmental conditions. *How is the variability between replicate epidemics affected by the presence of a BCA? How can we predict population behaviour from the effects measured on individual plants?* It is not surprising that it is difficult to translate biological control from the laboratory to the glass-house, field and forest. While empirical experimentation is essential, progress in understanding the underlying dynamics in order to predict the chance of success requires a theoretical framework that just captures the essential features of the dynamics: too complicated and it can't be tested and thoroughly analysed; too simple and it will fail to reproduce the complicated biological interactions. Using examples from the biological control of plant parasitic fungi, I propose to show how we have begun to establish a theoretical framework that identifies how to devise criteria for invasion and persistence in biological control; how to scale-up from small-scale experiments on individuals to population behaviour and how to predict variability between epidemics as a prelude to risk assessment.

The presentation will draw from amongst our recent work involving control of *Rhizoctonia solani* on radish by *Trichoderma viride*, control of *Sclerotinia minor* on lettuce over five successive seasons by *Sporidesmium sclerotivorum* using data by Dr. D.R. Fravel, USDA, Beltsville, USA and the potential for control of Dutch elm disease over large regions by transmissible hypovirulence. Particular attention will be given to the underlying epidemiological ideas rather than to the mathematical detail.

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Biological Control of Fungal and Bacterial Plant Pathogens
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pp.29 - 32

Improved plant health by the combination of biological disease control methods

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Abstract: The antagonists *Brevibacillus brevis* and *Pseudozyma flocculosa* and the plant extract from *Fallopia sachalinensis* (formerly *Reynoutria sachalinensis*) [Milsana®(VP99)] are under investigation in tomato, cucumber and grape against powdery mildew and grey mould. Before combining the different control methods with their different modes of action, single methods were tested. *B. brevis* (wild type) reduced infection with *Botrytis cinerea* on stored grapes to 58 %. Efficacy of 65 % was found for application of Milsana® on tomato against *L. taurica*. In cucumber, number of dead plants due to *B. cinerea* infection was reduced by the antagonists to approx. 50 %, but not by Milsana®. Results reported here, together with results from other studies will serve as a basis for studies on combinations of the three control agents against powdery mildew and grey mould of tomato, cucumber and grape.

Key words: biological control, *Botrytis cinerea, Brevibacillus brevis,* cucumber, grape, induced resistance, *Leveillula taurica,* plant extract, *Pseudozyma flocculosa, Sphaerotheca fuliginea*, tomato, *Uncinula necator*

Introduction

In the frame of an EU-project (FAIR CT98-4413; Biocombi) the effect of combinations of biological disease control methods with different modes of action is being studied against grey mould (*Botrytis cinerea*) and powdery mildew of cucurbits (*Sphaerotheca fuliginea*), tomato (*Oidium lycopersicum* and *Leveillula taurica*) and grapes (*Uncinula necator*). The aim is to enhance plant health by induction of resistance and by integration of bacterial and fungal antagonists that inhibit fungal spore germination and mycelial development. The biorational / biological agents used are a formulation of a plant extract from *Fallopia sachalinensis* (syn. *Reynoutria sachalinensis)* [Milsana® (VP99)], and the antagonistic microorganisms *Brevibacillus brevis* and *Pseudozyma flocculosa* (syn. *Sporothrix flocculosa).* All three give moderate to good protection in one or more of the listed host-pathogen systems, when applied as stand-alone

treatments. When used in combination, these control strategies target different stages in the infection process of the pathogens, which is expected to result in higher reliability and efficacy. This is especially important in situations where a single biological method does not give sufficient protection or requires a complicated manipulation (Malathrakis, 1997). Furthermore, combined non-chemical control of both diseases prevents the use of fungicides against one of the pathogens which may have an adverse side effect on the biological control method used against the other pathogen.

As a first step, the single biocontrol agents are tested in the different host-pathogen-systems. Thereafter, compatibility of the plant extract and the antagonists with each other, with chemical control methods and with beneficial insects is evaluated. Trials will then be carried out in which different combinations and different timing of applications will be investigated. At a later stage, effective combinations will be implemented in commercial cultivation systems. Studies on the mode of action, chemical nature of resistance-inducing compounds in *F. sachalinensis,* and the biosurfactant from *B. brevis*, will supplement this study.

Material and methods

Trial against grey mould* (Botrytis cinerea) *on stored grape berries

Grape bunches of the table grape Soultanina were harvested. Before storing in plastic trade boxes (11x11x7cm^3), bunches were treated with 1. water, 2. *B. brevis* wild type (WT), 3. *B. brevis* gramicidin S-deficient mutant (E1). Applications took place a) one day before b) together with and c) one day after artificial inoculation. There were nine combinations in total with six replicates each. *B. brevis* was applied at a concentration of 10^9 spores/ml in liquid culture (tryptone soy broth). *B. cinerea* was applied at a concentration of 10^6 spores/ml. Treated grapes were incubated at 0.5°C for 10 weeks. The percentage grey mould infected berries per bunch was recorded weekly.

***Greenhouse trial in tomato against powdery mildew* (Leveillula taurica)**

In Greece, tomato plants of the cv. Manthos were used. Plots were arranged according to complete randomised block design with 4 treatments in 5 replicates. The treatments were: 1. water, 2. Milsana® (VP99) 2 ml/l and 3. 0.5 ml/l, 4. fungicides myclobutanil (Systhane 12E EC; 0.4 ml/l), penconazol (Topas 10EC; 0.15 ml/l), triforine (Saprol 16EC; 1.5 ml/l) (alternated). Treatments were applied weekly for 26 weeks except for fungicides which were applied on as-needed base. Percentage area affected with powdery mildew was recorded on all individual leaves of 3 plants / plot weekly, 14 times in total. Tomato fruits were harvested and number and weight of fruits / plot was recorded.

***Greenhouse trial in cucumber against grey mould* (B. cinerea)**

In The Netherlands, cucumber plants cv. Jessica were transplanted to two identical greenhouses. Plots were arranged according to complete randomised block design with 6 treatments in 4 replicates, two blocks per greenhouse. The treatments were: 1. control with water, 2. *B. brevis* (WT), 3. *B. brevis* gramicidin S-deficient mutant (E1), 4. formulated *P. flocculosa* (5 g/l), 5. Milsana® (VP99) 5 ml/l, 6. fenarimol (Rubigan; 0.2 ml/l) and tolylfluanid (Eupareen M; 1.5 g/l). Treatments 1 to 5 were applied eight times in weekly intervals, starting before disease appearance. Treatment 6 was applied nine times on as-needed basis. Percentage leaf area covered with powdery mildew was assessed on all leaves of 5 plants per plot. *B. cinerea* severity was assessed by counting and removing the infected fruits, assessing the number of stem lesions per plant and recording dead plants per plot. Number and weight of harvested fruits were recorded.

Results

Trial with* B. brevis *against grey mould* (B. cinerea) *on stored grape berries

B. brevis (WT) showed significant reduction of infection with *B. cinerea* compared to the control, while *B. brevis* (E1) proved to be ineffective in controlling grey mould in stored grapes at 0.5°C. Nine weeks after the start of the experiment, levels of Botrytis infection in the control reached 25 %. Reduction amounted to 58 and 28 % for *B. brevis* (WT) and (E1), respectively. By the end of the experiment (10th week), infection rose to 33 % in the control, 32 % in E1- and 23 % in WT-treated grapes. Time of application (before, together with or after inoculation) had no significant influence on efficacy.

***Large scale greenhouse trial with Milsana® in tomato against powdery mildew* (L. taurica)**

In comparison to the control, weekly treatments of tomato plants with Milsana® at a concentration of 0.5 % and 0.2 % reduced the Area Under the Disease Progress Curve (AUDPC) for *L. taurica* by 65 and 40 %, respectively. Treatment with chemical fungicides led to a reduction of 92 %. All treatments were significantly different from each other. Despite large differences in disease severity and development, yield, in terms of number and weight of harvested fruit was not influenced significantly by any of the treatments.

***Large scale greenhouse trial with* B. brevis, P. flocculosa *and Milsana® in cucumber against grey mould* (B. cinerea)**

Stem infection with *B. cinerea* was significantly decreased by *P. flocculosa* (Table 1), while Milsana® and *B. brevis* had no significant effect. The number of dead plants due to grey mould was significantly lower in the treatment with *P. flocculosa* and *B. brevis* WT. *B. cinerea* infection on fruits was low in all experimental plots (no significant differences).

Table 1. Area Under the Disease Progress Curve (AUDPC) for *Botrytis cinerea* stem lesions, dead plants due to *B. cinerea* infection and total number of fruits infected by *B. cinerea* per plant in a large greenhouse trial on cucumber.

Treatment	AUDPC (lesion days) for *B. cinerea* stem infection	AUDPC (number days) for dead plants due to *B. cinerea*	Total number of fruits infected by *B. cinerea* per plant
Water	11.4 b	36.6 a	1.15 a
Brevibacillus brevis (WT)	9.3 bc	15.8 b	0.95 a
Brevibacillus brevis (E1)	7.2 bc	29.3 ab	1.00 a
Pseudozyma flocculosa	3.3 c	18.6 b	0.65 a
Milsana® (VP99) 0.5%	12.6 b	30.8 ab	1.45 a
Fungicide	19.4 a	42.6 a	0.90 a

*Numbers within a column followed by different letters are significantly different at $P \leq 0.05$.

Discussion

Protection against grey mould during storage of grape berries by *B. brevis* (WT) was effective and confirmed earlier observations (Seddon *et al.*, 2000). Since storage temperature is 0.5 °C and *B. brevis* grows at 37°C and only the gramicidin S-producing WT-strain gave good

control, it is likely that this metabolite plays a major role in protection. This was also reported for other systems (Seddon et al., 2000).

Milsana® (0.5 %), reduced disease severity of L. taurica in greenhouse-grown tomato significantly. However, yield in the trial was not different between treatments and control. In tomato/O. lycopersicum, Dik (1999) found that yield was not significantly influenced by disease levels up to 2000 percentage-days. According to Malathrakis (pers. communication), high infection by *L. taurica* does reduce tomato yield. Hence, it will be important to elucidate the factors that are determining influence of powdery mildew on yield of tomato.

Significant reduction of *B. cinerea* by *P. flocculosa* in commercial trials is reported here for the first time. *In vitro* studies have demonstrated that *P. flocculosa* can antagonize *B. cinerea* (Bélanger & Deacon, 1996) but similar effect *in vivo* was not shown before. The effect may be explained by a well synchronised presence of both organisms at potential sites of infection in the course of this experiment. It should be interesting to validate the reproducibility of these results. *B. brevis* WT significantly reduced plant mortality caused by *B. cinerea* but not *Botrytis* stem infections. This finding will be investigated in future trials. Significant reduction of *B. cinerea* in cucumber stem infection was not found for Milsana®. Trials with combinations of Milsana®, *B. brevis* and *P. flocculosa* are currently being conducted.

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Biological Control of Fungal and Bacterial Plant Pathogens
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Biological control of *Fusarium* wilt of tomato by VA mycorrhizal fungus, *Glomus fasciculatum*

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Abstract: Biochemical and enzymatic changes in VA mycorrhizal inoculated *Lycopersicon esculentum* infected by *Fusarium oxysporum* was carried out. The enzymes responsible for defense response were enhanced in VAM inoculated plants than other treatments and the control.

Key words: Biocontrol, *Fusarium* wilt, tomato, *Glomus fasciculatum*

Introduction

Fungal wilt diseases continue to devastate plants in many areas of the world. *Fusarium* wilt of tomato (*Lycopersicon esculentum* Mill.) caused by *Fusarium oxysporum* f. sp. *lycopersici* (Sacc.) Snyd.& Hans. is a worldwide important pathogen. Among the promising strategies for minimizing damage from plant pathogens, biological control by microorganisms such as *Trichoderma* spp. (Chet, 1987) and induced systemic resistance in plants by inoculation of VAM fungi (Schwab *et al.*, 1991) are two of the most talked about issues in agriculture. The present study was undertaken to gain better insight into the influence exerted by *Glomus fasciculatum* in stimulating defense mechanisms in tomato plants infected by *F. o.* f. sp. *lycopersici*.

Materials and methods

VAM fungal inoculum was produced with *Allium cepa* L. (onion) as the host plant. Tomato (*Lycopersicum esculentum* Mill. cultivar Co.1, a susceptible variety to *F. o.* f. sp. *lycopersici*) seedlings, 40-day-old were used for the experiments. The experimental design was: a) Con - Control - heat killed inoculum of VAM fungus and pathogen; b) MI - VAM inoculated fungus (*Glomus fasciculatum*); c) PI - Pathogen (*F. o.* f. sp. *lycopersici*) inoculated; d) DI - Dual inoculation - *F. o.* f. sp. *lycopersici* and *G. fasciculatum* inoculated on the same day; e) PrI - Pre inoculation - *F. o.* f. sp. *lycopersici* inoculated 10-days prior to *G. fasciculatum* inoculation; f) PoI - Post inoculation - *F. o.* f. sp. *lycopersici* inoculated 10-days after *G. fasciculatum* inoculation.

G. fasciculatum inoculum, of 10 g (soil having 50 spores/g) and pathogen inoculum, 5 mL conidial suspension containing 10^6 conidia/mL were used. Control seedlings were inoculated with heat killed conidia. Leaf and root samples were collected randomly for physiological and biochemical studies for 60 days at 10-day intervals. Reducing sugar content, total soluble sugar, free amino acids, o-dihydric phenol, phenol contents, phosphorus content, cytokinin and total soluble protein were estimated. Antifusarial substances (tomatine) was extracted and measured. Enzymes such as catalase, peroxidase, phenol oxidase, phenylalanine ammonia-lyase, acid phosphatase and alkaline phosphatase were estimated.

Results and discussion

In some VA mycorrhizal associations, enhanced P nutrition apparently accounts for the altered host water status (Koide, 1985). Interestingly, the P content of MI plants was high. In *Solanum tuberosum* inoculated with *G. fasciculatum*, P level increased (McArthur and Knowles, 1993). The PI plants had reduced level of P than the control plants. This may be due to the destruction of root cells by the pathogen, since *Fusarium* is a necrotroph which destroys the cells from which it obtains its nourishment. The development of *Fusarium* mycelia blocks the xylem vessels affecting conductivity. The effect of growth regulators as mediators of mycorrhizal sink strength may be considered, because their balance changes the symbiotic or parasitic associations (Lewis, 1975). Auxin, gibberellin and cytokinins increase in VAM infected plants (Raman *et al.*, 1994) and these could affect distribution of photosynthates. P not only influences plant growth but also reduces pathogenic infection (Reuveni *et al.*, 1993). The PoI plants had higher concentration of P in both roots and leaves followed by DI plants. Therefore, the mycorrhizal fungus supplements P nutrition of plants through its active external hyphae extending beyond the depletion zone surrounding the absorbing roots and root hairs (Cooper, 1984). Furthermore, the external hyphae of VAM fungi may have more absorbing sites for P than the surface of roots (Cress *et al.*, 1979). Mycorrhizal infection increased P levels in the shoots (Waidyanath *et al.*, 1979). Arbuscules were present in DI and PoI roots. The arbuscules help in the uptake and accumulation of P in the roots. The MI tomato plants showed an increase in reducing and total sugars both in leaves and roots, but more in leaves. Leaves of VAM fungus-infected plants generally contain more sucrose, reducing sugars and starch than non-mycorrhizal plants (Nemec and Vu, 1990). In leaves and roots of PI plants, initially both total sugars and reducing sugars increased and then decreased. *Fusarium* produces a number of enzymes such as exo and endo B-1,4 galactonases and exo B-1,3 and B-1,5 arabinases, that degrade sugar polymers (Mahadevan and Sridhar, 1986).

In PoI and DI tomato roots, during the first stage of fungus development, quantity of sugars was not significantly affected. But when *G. fasciculatum* was well established, sugars increased in infected roots. The degree of colonization influenced the carbohydrate concentration of the roots of *Trifolium subterraneum* (Pearson and Schweiger, 1993). In DI and PoI plants, level of reducing sugars was high. The increased level of reducing sugars in mycorrhizal roots lowers disease incidence (Schenck, 1981). The amount of total sugar in the roots of PoI and DI plants was lower than in leaves. The carbon losses from the root are sufficient to sustain the activities of *G. fasciculatum*. As hyphal uptake of P occurs, root P content increases, membrane permeability is reduced and more of the carbon is allocated to the mycorrhizal fungus in the root, resulting in less exudation out of the root (Graham *et al.*, 1981). An increased level of amino acids was found in MI, DI and PoI tomato plants. Krishna and Bagyaraj (1983) found higher levels of amino acids in *Arachis hypogea* inoculated with *G. fasciculatum*. The amino acid content decreased in leaves and roots of PI plants. This indicates that *F. o.* f. sp. *lycopersici* by producing protease breaks down the proteins and successfully utilized the amino acids as carbon and nitrogen source.

The increase in total phenols in MI plants could be attributed to general triggering of pathways of aromatic biosynthesis (Mahadevan, 1991). Krishna and Bagyaraj (1984) reported an increase in phenols of roots of *Arachis hypogea* colonized by *G. fasciculatum*. In DI and PoI plants, both phenols and O-dihydricphenols increased. The increased level of phenols was correlated with disease resistance to pathogens (Mahadevan, 1991). The activities of two key enzymes in phenolic metabolism- phenylalanine ammonia lyase and peroxidase may participate in the restriction of hyphae of *F. o.* f. sp. *lycopersici*. Increased levels of peroxidase, phenol oxidase and phenylalanine-ammonia lyase were recorded in DI and PoI

plants. The end products of these reactions of these enzymes are closely associated with the defense mechanism of plants (Mahadevan, 1991). In both roots and leaves, proteins increased in MI plants. Such increased levels of proteins have been observed in *Glycine max* with *G. fasciculatum* (Pacovsky, 1989). This is in agreement with Grzelinska (1969) who found that *F. oxysporum* f. sp. *lycopersici* reduced the protein content in tomato plant.

Enhanced cytokinin levels in mycorrhizal plants suggest significant impacts on plant growth and development. Allen *et al.*, (1980) reported increase of cytokinin content in plant tissues associated with VAM fungi. Mycorrhiza enhances cytokinin synthesis (Raman *et al.*, 1994). Leaves and roots of PoI plants had higher amount of cytokinins followed by MI and DI plants. It may be assumed that at least a part of the increased amount of cytokinins in DI and PoI plants resulted due to infection by *G. fasciculatum*. Tomatine plays a major role in resistance by inhibiting spore germination and production (Smith and MacHardy, 1982). The concentration of tomatine increased in the leaves and roots of MI plants. The increased level of tomatine is an important factor in the resistance of PoI and DI plants to the pathogen. Roots and leaves of DI and PoI plants had increased levels of tomatine. Catalase increased in all test plants at the early stages of infection and then decreased. However, higher activity was found in DI and PoI plants. This increased activity is correlated with susceptibility of plants to VAM fungal infection. Resistance also depends upon the level of catalase in plant tissues (Rubin and Artsikhovskaya, 1963). In PI, DI and PoI plants, phenol oxidase activity initially increased and then decreased. This initial increase of phenol oxidase may be due to contribution of the pathogen (Maraite, 1973) to detoxification of phenols (Mahadevan, 1974). Both acid and alkaline phosphatase activities were high in MI plants. But the activity of alkaline phosphatase was lower than acid phosphatase in these plants. Krishna *et al.* (1983) reported no increase in acid phosphatase but increased alkaline phosphatase activity in mycorrhizal roots of *Arachis hypogea*.

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Biological Control of Fungal and Bacterial Plant Pathogens
IOBC wprs Bulletin Vol. 24 (3) 2001
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Induced resistance to *Botrytis cinerea* by *Pseudomonas aeruginosa*: role of siderophores and pyocyanin

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Abstract: *Pseudomonas aeruginosa* 7NSK2 can induce resistance to *Botrytis cinerea* leaf infections on bean and tomato. *P. aeruginosa* 7NSK2 produces three siderophores: pyoverdine, pyochelin and salicylic acid (SA), a precursor of pyochelin. SA-negative mutants of 7NSK2 are unable to induce resistance in tomato and bean against *B. cinerea*. KMPCH, a pyoverdine and pyochelin-negative mutant of 7NSK2 that still produces SA, produced nanogram amounts of SA on bean and tomato roots in non-sterile soil. It was proven that these amounts of SA are sufficient to induce PAL expression in the roots and to increase free SA levels in leaves. In contrast to KMPCH, however, the wild type strain 7NSK2 does not produce SA on roots and does not induce PAL expression in the roots. In this paper evidence is given that the resistance induced by the wild type 7NSK2 is not caused by SA production but by a combination of pyochelin and pyocyanin production. A pyochelin-negative mutant of 7NSK2 (7NSK2-562) and a pyocyanin mutant of 7NSK2 (Phz1) were unable to induce resistance to *B. cinerea* when inoculated alone on tomato roots. However, when both mutants were inoculated together on tomato roots, the ability to induce resistance was restored. In animal cells it has been shown that ferripyochelin and pyocyanin act in concert to produce the very reactive hydroxyl radical that can cause cell damage. Probably, a similar mechanism leads to the production of active oxygen species on plant roots resulting in induced resistance.

Key words : pyochelin, phenazines, induced systemic resistance

Introduction

P. aeruginosa 7NSK2, a plant-growth promoting rhizobacterium, induces resistance to *B. cinerea* leaf infections on bean (De Meyer & Höfte, 1997) and tomato. Under iron-limiting conditions, *P. aeruginosa* 7NSK2 produces three siderophores: pyoverdine, pyochelin and salicylic acid (SA) (Buysens *et al.*, 1996). The latter is also a precursor for pyochelin biosynthesis (Serino *et al.*, 1995). In addition, *P. aeruginosa* 7NSK2 produces the phenazine compound pyocyanin. It has been shown that SA-negative mutants of 7NSK2 are unable to induce resistance in tomato and bean against *B. cinerea*. Moreover, *P. aeruginosa* 7NSK2 no longer induced resistance in NahG tomato plants. These results suggest that 7NSK2 induces resistance via a SA-dependent signal transduction pathway. For the pyoverdine- and pyochelin-negative, but still SA-producing mutant KMPCH it was proven that this strain produces nanogram amounts of SA on bean roots and that this amount of SA is sufficient to induce PAL expression in the roots and to increase free SA levels in leaves of bean (De Meyer *et al.*, 1999).

In this paper we show that in contrast to mutant KMPCH, the wild type strain 7NSK2 does not produce SA on tomato roots and does not induce PAL expression in the roots. We also provide evidence that 7NSK2 induces resistance to *B. cinerea* by a combination of pyochelin and pyocyanin production.

Material and methods

Bacterial strains

P. aeruginosa 7NSK2 is a wild type strain isolated from the roots of barley. KMPCH is a pyoverdine- and pyochelin-negative mutant of 7NSK2 that still produces SA. KMPCH-567 is a SA-negative mutant of KMPCH (De Meyer *et al.*, 1999). 7NSK2-562 is a SA-negative mutant of 7NSK2 (De Meyer & Höfte, 1997). The pyocyanin mutant Phz1 was constructed by mini Tn5-PhoA transposon mutagenesis.

Quantification of SA and determination of PAL activity

For rhizosphere extraction of SA, five samples of 2.5 g of root pooled from five plants were analyzed per treatment. Free and bound SA in plant tissue was quantified as described by Meuwly & Métraux (1993). PAL activity was determined as described by Edwards & Kessmann (1992). Six plants were analyzed per treatment. Treatments were compared by Student's t test at $P \leq 0.05$ on pooled data from two experiments.

***Assay for ISR to* B. cinerea**

P. aeruginosa root colonization was achieved by a combined seed and soil treatment (De Meyer & Höfte, 1997) and plants were grown in a greenhouse for 5 weeks. *Botrytis* infections were carried out on detached leaves by infecting each leaf with 10 droplets of 4 µl solution containing 10^6 spores per ml, 0.01M glucose and 0.0067 M KH_2PO_4. Four days after inoculation, infection was evaluated by looking at spreading of lesions and statistically analyzed as a dichotomous variable by logistic regression.

Results and discussion

7NSK2 does not produce SA on tomato roots and does not induce PAL expression

Figure 1 shows that the wild type strain 7NSK2 does not produce SA on tomato roots in contrast to mutant KMPCH. KMP567 is a SA-negative mutant of KMPCH and this strain is also unable to produce SA on tomato roots. Figure 2 indicates that 7NSK2 inoculation of tomato roots does not result in an enhanced PAL expression in contrast to results obtained with mutant KMPCH.

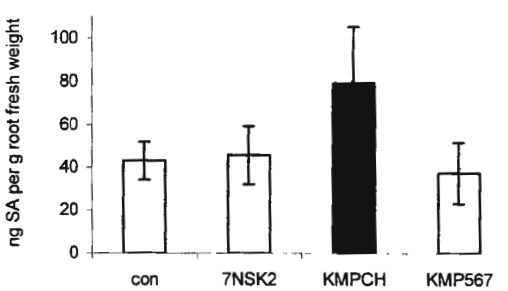

Figure 1. Levels of free salicylic acid on tomato roots bacterized with *P. aeruginosa* 7NSK2 and mutants. Only KMPCH causes a significant increase in free SA levels on tomato roots (black bar).

These results came as a surprise since 7NSK2 does produce SA *in vitro*. Our hypothesis was that on roots SA is very efficiently converted to pyochelin and that the resistance induced by

7NSK2 is not caused by SA, but by a combination of pyochelin and pyocyanin production. This hypothesis was supported by the fact that *P. aeruginosa* PNA1, an effective antagonist of *Pythium* and *Fusarium* (Anjaiah *et al.*, 1998), is unable to induce resistance. PNA1 produces SA and pyochelin, but does not produce pyocyanin. Instead, it produces the phenazine antibiotics phenazine-1-carboxylate and oxychlororaphin that are involved in direct antagonism. Moreover, in animal cells it was shown that ferripyochelin and pyocyanin act in concert to produce the very reactive hydroxyl radical that can cause cell damage (Britigan *et al.*, 1992).

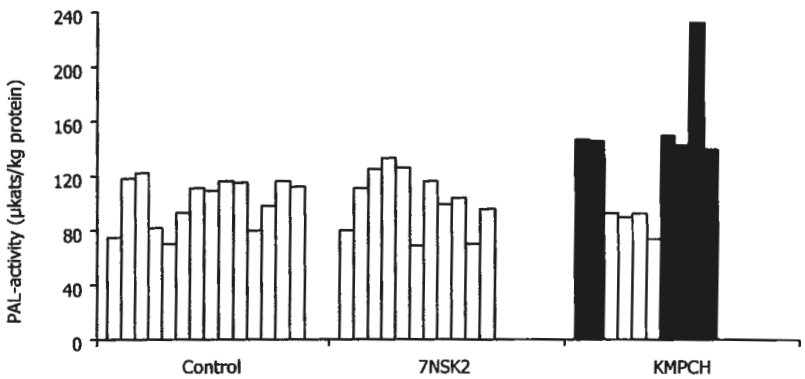

Figure 2. PAL activity on tomato roots inoculated with *P. aeruginosa* 7NSK2 (wild type) or P. aerugosa KMPCH (SA-producing mutant). Only the PAL activity on some tomato roots inoculated with KMPCH (black bars) is significantly different from the activity on non-inoculated roots.

7NSK2 induces resistance via pyochelin and pyocyanin production

To test our hypothesis, we constructed a pyocyanin-negative mutant of 7NSK2 by mini Tn5-PhoA transposon mutagenesis. Mutant Phz1 appeared to be mutated in a gene coding for o-methyltransferase (99% identity with gene PA4209 on the PAO1 genome) and was completely unable to produce pyocyanin. This mutant was subsequently used in a tomato-*B. cinerea* bioassay, together with mutant 7NSK2-562 that still produces pyocyanin, but is unable to synthesize pyochelin due to a mutation in the SA biosynthetic gene *pchA* (Serino *et al.*, 1995). Figure 3 shows that both mutants are unable to induce resistance to *B. cinerea* when inoculated alone on tomato roots. However, when the mutants were co-inoculated at the same density, the ability to induce resistance was restored. These results strongly suggest that unlike KMPCH, 7NSK2 does not induce resistance by SA production but by a combination of pyochelin and pyocyanin production. It is known that in mammalian cells pyocyanin can undergo redox cycling resulting in the continuous generation of hydrogen peroxide (H_2O_2) and superoxide (O_2^-). Ferripyochelin catalyzes the generating of the very reactive hydroxyl radical from hydrogen peroxide and superoxide via the Haber-Weiss reaction (Brigitan *et al.*, 1992). Probably, these reactive oxygen species are also produced on the tomato roots resulting in the induction of resistance.

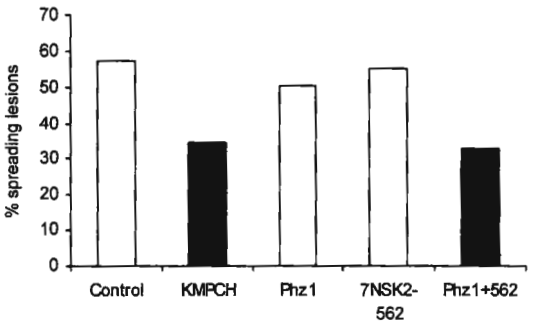

Figure 3. ISR induced by *P. aeruginosa* mutants in tomato to *B. cinerea*. Only mutant KMPCH and a combination of mutant Phz1 (pyocyanin mutant) and mutant 7NSK2-562 (pyochelin mutant) can induce resistance (black bars).

Acknowledgements

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Biological Control of Fungal and Bacterial Plant Pathogens
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Biocontrol of fungal plant pathogens - from the discovery of potential biocontrol agents to the implementation of formulated products

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Abstract: In order to develop efficient biopesticides that could be used as components for integrated pest management programs, a multidisciplinary approach was adopted. Control of more than 85% of the postharvest pathogens *Botrytis allii* and *Rhizopus stolonifer* in onion and tomato was obtained at 22°C and 6°C by using a strain of the yeast *Pichia onychis*. This isolate was produced through liquid fermentation and formulated as an emulsion and a gel that will be used under commercial conditions. For soilborne plant pathogens, attention was focused in protection of spermosphere or rhizosphere by combining seed priming with a strain of *Trichoderma koningii*, and by adding a suspension of the antagonist twice into the soil. This strategy resulted in 82% and 62% protection against *Rhizoctonia solani* in bean and tomato, respectively, and 70% protection against *Fusarium oxysporum* f. sp *lycopersici* in tomato. Conidia of *T. koningii* produced by solid state fermentation were formulated as granules for soil application under field conditions, which can also be dispersed as a suspension. For the control of *Sclerotium cepivorum* in onion more than 60% protection was obtained by using native isolates of *Trichoderma* sp., *Chlonostachys* sp. and *Beauveria* sp.

Introduction

In recent years interest in Colombia has focused on biological control alternatives for pest management. Nevertheless, practical application of this strategy is still limited, in part due to the small number of commercial BCAs available for use against plant pathogens and technical difficulties in obtaining high quality biomass and adequate formulation with reliable efficacy under field conditions. Taking into account that the development of stable, cost effective and easily applied biocontrol formulations is critical for the advancement of biological control (Lisansky, 1985), the purpose of the present work was to develop biopesticides for the control of the main pathogens affecting vegetables.

Materials and methods

We focused on the isolation of fungi from soil, rhizoplane and rhizosphere for soilborne pathogen control of *R. solani*, *F. oxysporum* and *S. cepivorum*, and yeasts from fruits and flowers for postharvest disease control. Samples originated from the same agroecological areas in which the crop and the disease are prevalent, as well as from exotic areas. Microorganisms were isolated by techniques such as plate count agar and soil granule analysis (Hennebert, 1974). Once cultivated in axenic conditions and identified, potential BCAs were deposited in the Colombian Collection of Microorganisms (CoCM), by using at least two different conservation methods (oil, soil, cryopreservation). All information regarding the origin and characteristics of each strain was included in the documentation of the CoCM.

In vivo selection of BCAs was performed taking disease etiology into account, the crops and cultivation methods. For *R. solani* and *F. oxysporum* control in tomato and bean,

experiments were conducted under greenhouse conditions (Mezui *et al.*, 1994). For protection under seedbed, seeds primed in the presence of potential antagonists were sown in soil artificially infested with plant pathogens. For protection after transplant against the above pathogens and *S. cepivorum*, roots of seedlings were dipped in a suspension of potential antagonists before sowing in soil artificially infested with plant pathogens. Suspensions of the biocontrol agent were applied twice during the experiments. In all cases, concentration of the suspension was 10^7 conidia/ml of the BCA. Selection of the BCA for the postharvest plant pathogens *B. allii* in onion and *R. stolonifer* in tomato was performed (Janisiewicks, 1997; Jijakli *et al.*, 1993), at two different temperatures (6^0C and 22^0C) by applying 25 µl of yeast suspension containing 10^7 cells/ml into wounds.

For mass production of the selected BCAs, fungi were grown in a solid medium consisting in ground wheat bran with a small particle size, supplemented with a starch solution and mineral salts. Results were expressed as conidia produced per gram of substrate. Yeasts were produced by using standardized liquid fermentation. The determination of optimal conditions was achieved by determining response surfaces and by the elaboration of a second order polinomial model, produced by an equation including all the variables of the process.

In order to develop formulated products, microorganisms were submitted to preformulation procedures consisting of the determination of physical characteristics of propagules (size, shape, surface), determination of environmental conditions affecting viability or activity under storage and under field conditions. Product delivery systems and excipients to confer stability to the microorganism or to give desirable characteristics to the product were selected, and the effect of these excipients on fungal germination time and viability was determined. For granulated products excipients such as diluents, adherents, nutrients and hygroscopic agents were evaluated. For liquids to emulsify and gels, vegetable oils, surfactants, adherents, dispersants and stabilizing agents were evaluated.

Results

This research strategy involved the conformation of a multidisciplinary professional group which relies on the participation of disciplines such as phytopathology, agronomy, biology, microbiology, pharmaceutical chemistry and biochemical engineering, working on different topics related to biopesticides development. This collection includes nowadays over 3500 accessions of potentially BCAs.

A strain of *T. koningii* (TH11) with high biocontrol activity against the main soilborne plant pathogens in tomato and bean was selected. Results obtained were reliable and efficient. The antagonist application method consisted of seed priming in the presence of *T. koningii* and the addition of this antagonist into soil. This treatment resulted in 82% and 62% control of diseases caused by *R. solani* in beans and tomato, respectively, 85% and 75% protection respectively, against *P. splendens* in bean and cucumber, and 70% protection against *F. oxysporum* in tomato. This assay allowed the establishment of enhanced biocontrol activity of this strain with its wide activity spectrum, taking into account that it was able to control different pathogens in different crops (Fig. 1).

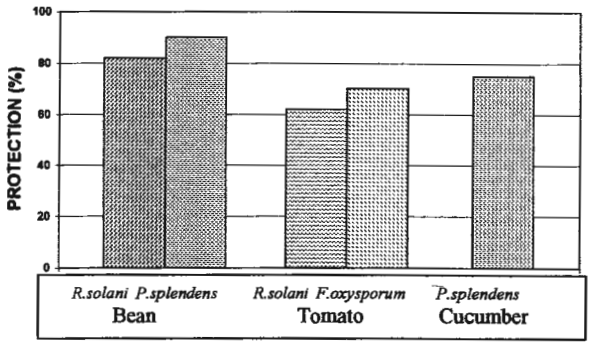

Figure 1. Biocontrol effect of seed priming in the presence of *T. koningii* on control of *R. solani* in tomato, cucumber and bean; *F. oxysporum* in tomato, and *P. splendens* in cucumber. The treatment evaluated was seed priming in the presence of *T. koningii*. Protection was determined taking into account the incidence and disease severity.

Additionally, for control of *S. cepivorum* in onion, different fungi exhibiting high biocontrol activity were selected, 60% protection was obtained with strains of *C. rosea* (Cn 001), *Beauveria* sp. (Bv 043) and *Trichoderma* spp. (TH 034 and TH 035) (Fig. 2). On the other hand, for biological control of postharvest diseases, two native yeasts identified as Lv 027 and Lv 031 were selected because of their ability to protect onion efficiently against *B. allii* and tomato against *R. stolonifer*. In both onion and tomato, protection of over 85% was obtained at 22°C and 6°C. These results demonstrated the adaptation of these yeasts to different temperatures and their capacity to control different pathogens in different vegetables, suggesting a wide action spectre for biological control purposes.

Once selected, isolates of *C. rosea*, *Beauveria* sp. and *Trichoderma* spp. were produced in mass on solid medium. In all cases, after 8 days of incubation more than 10^9 conidia per gram of substrate were obtained. Mass production of the selected yeasts produced 5.2 x 10^9 cells/ml.

Nowadays, different biopesticide prototypes of selected microorganisms for biological control of soilborne plant pathogens have been developed as granules for soil application. The *B. bassiana* product has a particle size of 2 mm and a concentration of 10^{10} UFC per gram. The formulation of *T. koningii* has a particle size of 1 mm at a concentration of 10^8 conidia per gram. For seed treatment or soil application as a drench, granules can be dispersed in water, where particles are easily disintegrated forming a stable suspension. These biopesticides include excipients as a nutritional base for enabling the microorganism to establish in soil. The nutritional substrate of the granules was fully occupied by the antagonist, to avoid competition by pathogens, as reported by Harman *et al.*, 1981. The biopesticides also contain diluents that provide weight to the product to avoid wind drift, and a substance for water absorption in order to enhance available water to the fungus and to improve its germination and colonization.

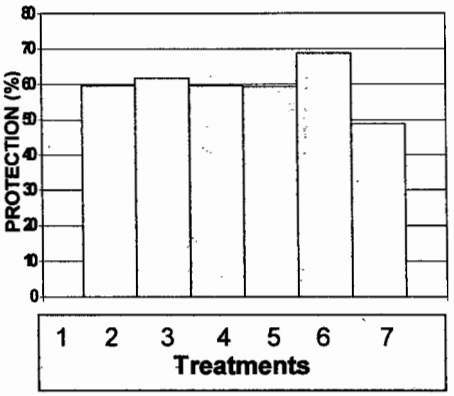

Figure 2. Biocontrol effect of different native fungi against *S. cepivorum* in onion. Plants were sown in soil artificially infested with the pathogen. Treatments evaluated were:
1:*Trichoderma* sp. Th 034
2: *Trichoderma* sp. Th 035
3: *Clonostachys rosea* Cn 001
4: *Beauveria* sp. Bv 043
5: TRICHODEX
6. Iprodione

Biopesticide prototypes based on yeasts for postharvest diseases control were formulated as two delivery systems. One consists of a liquid to be emulsified, which is formed by the yeast suspended in vegetable oils with a surfactant mixture to be reconstituted in water, at a concentration of 10^9 yeasts per ml. Reconstituted emulsion is stabile during four hours, enough time for application. The other yeast formulation consists of a stable gel having 10^7 cells per millilitre developed for direct application on the surface of fruits. This biopesticide includes excipients such as plastifiers and polymers. The film coating provides a better adhesion and reduces the number of excipients required for formulation and maximizes production efficiency. The final product does not affect the organoleptic characteristics of the fruits but adds natural brightness.

Because storage stability is an important criterion for an effective biocontrol formulation (Lewis, 1987), studies with optimised formulation are being conducted over two years to determine the shelf life of the biopesticides. Furthermore, to ensure quality of the biopesticides, a master formula of production is also being established, after standardization and validation of all unitary operations involved in the production process.

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Biological Control of Fungal and Bacterial Plant Pathogens
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Biological control of *Botrytis cinerea* in greenhouse crops

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Abstract: *Botrytis cinerea*, the causal agent of grey mould, is an important pathogen in many greenhouse crops, including tomato and cucumber. In cut flowers and in potted plants, *B. cinerea* almost only occurs at the post-harvest stage, when conditions are suitable for spore germination. *B. cinerea* is difficult to control with chemical fungicides and biological and integrated control are needed to reduce damage caused by this pathogen. In collaboration with Dr. Elad from the Volcani Center in Bet-Dagan, Israel and Dr Köhl from Plant Research International in Wageningen, The Netherlands, several biocontrol agents have been tested against *B. cinerea*. In tomato and cucumber, the experiments were carried out under different climatic regimes. The yeast strain PBGY1 and *Trichoderma harzianum* T39 (TRICHODEX) were effective under a range of conditions in tomato and cucumber. Integration of the biocontrol agent with a suitable climatic regime can increase the overall control of *B. cinerea*. Isolate PBGY1 was also effective against post-harvest symptoms in roses even at low temperatures and low relative humidity conditions. In potted plants, *B. cinerea* in the post-harvest stage was also controlled. The fact that the yeast is effective over a range of crops under different climatic conditions makes it an excellent candidate for commercialisation.

Key words: cucumber, rose, Saintpaulia, tomato, *Trichoderma harzianum*, yeast

Introduction

Botrytis cinerea, the causal agent of grey mould, is an important pathogen in many greenhouse crops. In tomato and cucumber, it can infect flowers, fruits and, most importantly, cause stem cankers that may result in plant death. In roses, gerbera and other cut flowers as well as in potted plants, *B. cinerea* almost only occurs in the post-harvest stage, when conditions are suitable for spore germination. *B. cinerea* is difficult to control with chemical fungicides and biological and integrated control are needed to reduce damage caused by this pathogen.

In collaboration with Dr. Elad from the Volcani Center in Bet-Dagan, Israel and Dr Köhl from Plant Research International in Wageningen, The Netherlands, several biocontrol agents have been tested against *B. cinerea*. In tomato and cucumber, the experiments were carried out under different climatic regimes. In cut flowers and potted plants, experiments were carried out at the post-harvest stage.

Material and Methods

Experiments with several biocontrol agents in cucumber and tomato
Large greenhouse trials were conducted in cucumber and tomato in The Netherlands. Most experiments had the following treatments: 1. control with water or Tween 80 (0.01%); 2. Isolate PBG yeast 1 (PBGY1, 10^6 cells ml^{-1}); 3. Isolate PBG yeast 2 (PBGY2, 10^6 cells ml^{-1}); 4. *Trichoderma harzianum* T39 (Trichodex 25 WP, 4 kg ha^{-1}); 5. control with fungicide, *i.e.* tolylfluanid (Eupareen M, 1.5 g l-1) or iprodione (Rovral 50 WP, 0.5 g l^{-1}). Treatments were

applied weekly at a rate of 1000 l ha^{-1} in tomato and 1500 l ha^{-1} in cucumber. Infected fruits, stem lesions per plant and plant mortality were recorded weekly. Yield was measured at each harvesting date.

In cucumber, four experiments were conducted under two or three different climatic regimes in the greenhouses. In tomato, two experiments were done either with or without closing of horizontal screens in the glasshouses during the night. The screens are used as energy saving method but also increase humidity in the glasshouse.

In one experiment, PBGY1 was applied at concentrations of 10^5, 10^6 and 10^7 cell ml^{-1}.

Experiments with several biocontrol agents in roses

A number of experiments was carried out to investigate the efficacy of biological control agents under a range of climatic conditions. Freshly cut roses cv. First Red were inoculated with *B. cinerea* (10^5 spores ml^{-1}) and placed in a growth chamber at 20 °C and 80% relative humidity (RH). After 24 h, different treatments, including PBGY1 and PBGY2 suspended in Tween 80 (0.01%) at concentrations of 10^6 – 10^8 cells ml^{-1}, were applied to ten flowers per treatment. The control was sprayed with Tween 80 only. In experiments examining the effect of climatic conditions, the flowers were subsequently incubated for 24 h at different temperatures and RH's. Subsequently, RH was increased to 100% but the temperature remained unchanged. After one to four days, lesions were counted on one cm^2 per flower.

Microscopic studies were carried out to assess germination rate and germ tube elongation of *B. cinerea* on rose petals with or without isolate PBGY1. Germ tubes were measured with a semi-automatic image analyser (Zeiss). Germ tube length per germinated spore was calculated.

Experiments with several biocontrol agents in Saintpaulia

Saintpaulia plants (different cultivars, eight plants per treatment) were sprayed shortly after harvesting with PBGY1 and PBGY2 suspended in Tween 80 (0.01%) at concentrations of 10^6 – 10^8 cells ml^{-1}. Each experiment included a control treated with Tween 80 and a control with tolylfluanid. The plants were incubated at 11 °C for 7 days to mimic transport conditions and subsequently moved to 21 °C and ambient RH. After transport simulation and one and two weeks after moving to ambient conditions, the number of infected flowers per plant were counted.

Results

Experiments in tomato and cucumber

In tomato and cucumber, the yeast strain PBGY1 and *T. harzianum* T39 effectively controlled *Botrytis* on stems and fruits under a range of climatic conditions. Yeast strain PBGY2 was not as effective as the other two biocontrol agents. In tomato, the biocontrol agents were more effective when horizontal screens were closed during the night. In cucumber, climatic conditions did not strongly influence efficacy, but plant death was enhanced under dry conditions. In most experiments, the efficacy of the biocontrol agents was as good as or better than the efficacy of tolylfluanid and iprodione (Dik & Elad, 1999).

Experiments in roses

In roses, the two yeast strains differed markedly in their efficacy under different climatic conditions (Fig. 1). Strain PBGY1 was much more effective at low temperatures and low RH.

The percentage germinated spores of *B. cinerea* in the control treatment with Tween 80 and in the treatment with PBGY1 at different temperatures and RH's is shown in Table 1. At all conditions, germination was reduced by PBGY1. The average germ tube length per germinated spore was not affected (data not shown).

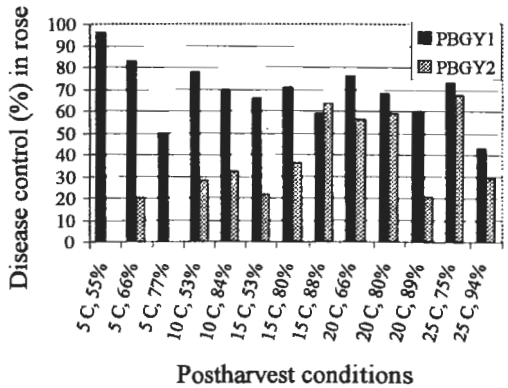

Figure 1. Efficacy (% reduction in lesions per cm^2) compared to control by strains PBGY1 and PBGY2 under different climatic post-harvest conditions in rose.

Table 1. Germination of *B. cinerea* spores on rose petals after incubation for 24 h at different temperatures (T) and vapour pressure deficits (VPD) with or without PBGY1.

T (°C)	RH (%)	VPD (Pa)	Germination (%) of *B. cinerea* spores	
			Control (Tween)	PBGY1
5	65	306	18.5	2.5
5	70	262	9.0	0.0
15	54	786	8.5	6.5
15	88	205	32.5	18.5
20	66	796	31.5	19.0

Experiments in Saintpaulia

The efficacy of the yeasts against *B. cinerea* in Saintpaulia varied between experiments. PBGY1 was more effective than PBGY2. The correlation between the concentration of the yeast suspension and disease was not very clear. In most experiments, the yeasts did not give complete control of *Botrytis*, but in general, efficacy of the yeasts was at least as good as that of a broad-spectrum fungicide.

Dose-response curves for PBGY1

In Fig. 2, dose-response curves are shown for PBGY1 in cucumber and rose. In cucumber, the effect on Area Under the Curve (AUC) for dead plants was clearly affected by the yeast concentration. The same can be said for number of lesions in rose. The efficacy was comparable for both host-pathogen combinations. The dose-response effect was less clear in Saintpaulia.

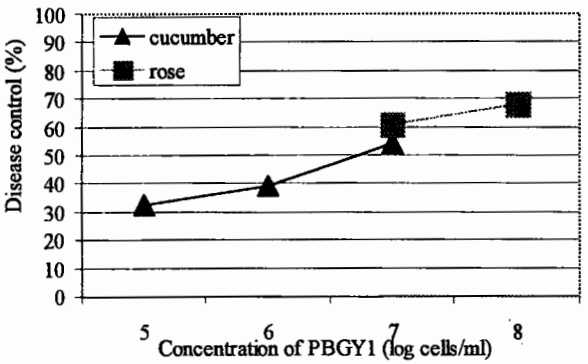

Figure 2. Efficacy of PBGY1 compared to control treatment for AUV for dead plants in cucumber and for lesions in rose.

Discussion

The biocontrol agents PBGY1 and *T. harzianum* T39 effectively controlled *B. cinerea* in cucumber and tomato during the cropping period under different climatic conditions. Integration of the biocontrol agents with climate control can increase the overall control of *B. cinerea*. PBGY1 was also effective against post-harvest symptoms in roses even at low temperatures and low relative humidity conditions, situations which are unfavourable for *T. harzianum* T39.

The mode of action of the yeast is still a subject of research. Spore germination is clearly affected by the yeast and this might well be the result of nutrient competition. Dead cells did not reduce infection by *Botrytis*, and dual plate culturing did not show an inhibition zone, which indicates that antibiotics are not important in the interaction. It is possible that the exopolysaccharides produced by the yeast have a direct inhibitory effect on *Botrytis*.

The yeast PBGY1 is compatible with several fungicides and pesticides registered in greenhouse crops. Therefore, it can very well be used in an integrated control schedule. Several cultural practices have been shown to reduce *B. cinerea* and integration of these practices with the biological control agent is expected to give very good control of grey mould. The fact that the yeast is effective over a range of crops under different climatic conditions makes it an excellent candidate for commercialisation. Current research focuses on 1. mass-production and formulation of the yeast; 2. integration of the yeast with cultural practices; 3. efficacy trials in other crops and against other pathogens.

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Biological Control of Fungal and Bacterial Plant Pathogens
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Bacteriophages as a potential biocontrol agent against walnut blight (*Xanthomonas campestris* pv *juglandis*)

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Abstract: During autumn 2000, 197 isolates of bacteria were collected from blighted nuts, leaves, buds and petioles of walnut trees from all over New Zealand. Among these isolates *Xanthomonas campestris* pv *juglandis* lines were identified by a range of cultural & morphological tests. Concurrently, 26 bacteriophages which attack *X. campestris* pv *juglandis* were isolated from the soils under the same trees. Phages were readily isolated from a depth of 2.5 cm. Phage typing was carried out to produce a set of phages capable of destroying all bacterial isolates. A subset of the isolated phages were also characterized for identity and ability to withstand environmental stresses such as long term storage, wetting agents, UV light and desiccation. Several phages were identified as being from the λ phage group with long unsheathed tails and hexagonal heads under the electron microscope. Desiccation and UV light had major effects whereas storage and wetting agents generally had little effect on phage viability. Differences were found among the phages and a search continues to locate effective, hardy phages.

Keywords: biocontrol, bacteriophage, desiccation, adjuvants, wetting agents, storage

Introduction

Walnut blight is both a devastating disease of walnuts (Bull, *et al.,* 1985; Ramos, 1998) and an excellent model system for a wide range of destructive plant / bacterial diseases. At present copper sprays are used to control blight (caused by the bacterium *Xanthomonas campestris* pv *juglandis*) in walnut orchards (Ramos, 1998). However, the bacteria are rapidly becoming resistant to copper sprays (Lee *et al.*, 1994) and copper residues are poisoning our environment (Radix & Seigle-Murandi, 1993). Alternative control chemicals are few and even more toxic while no effective plant resistance has been identified. A reliable and manageable biological control technology is needed. Bacteriophages provide highly specific control opportunities for bacterial diseases by specifically infecting and destroying the disease causing bacteria. With no proven alternatives for walnut blight control, investigating the potential for bacteriophage based biocontrol is warranted.

Lindow *et al.,* (2000) indicated spring applications of bactericides with a strong wetting agent effectively reduced bacterial numbers and disease levels in walnut. Replacing the bactericide by a phage mixture was selected as a window for disease control. Therefore, the objectives of the research were; 1) to isolate and characterize phages active against *X. campestris* pv *juglandis;* 2) to isolate and characterize a series of strains of *X. campestris* pv *juglandis* representing the New Zealand biotypes against which the phages could be tested; 3) to characterize the phages for survival and pathogenic ability in the presence of a range of adjuvants; and potential stresses; 5) to develop methods for counting levels of *X. campestris*

pv *juglandis* and bacteriophages within walnut buds with and without phage applications. The goal of these initial trials is to lay a foundation for later field testing of bacteriophage strains.

Material and methods

Bacterial isolations

Bacteria (197 isolates) were isolated on nutrient agar from a range of infected walnut tissues collected throughout New Zealand. Individual colonies were purified by streaking under sterile conditions through several passages. Confirmation of 89 of these isolates as *X. campestris* pv *juglandis* was made by: 1) comparing with authenticated lines from Landcare Research, Auckland, 2) using the MicroLog computer identification program at the Geisenheim Research Institute, Germany, 3) conducting a series of differentiating tests including; Gram staining, microscopic examination, colony characteristics on GYCA agar, sensitivity to Triphenyltetrazolium chloride (Lovrekovich & Klement, 1960), sensitivity to phages and characteristics on brilliant cresyl blue starch medium (Mulrean & Schroth, 1981).

Bacteriophage isolations and survival

Bacteriophages were isolated from the soil under walnut trees in the autumn of 2000. Methods of Crosse & Hingorani (1958) and Benson (1990) were used. An authenticated *X. campestris* pv *juglandis* isolate (134) was used for all phage isolations. Purified isolates were obtained by passage through at least 3 single plaque isolations. The phage solutions were filtered through 0.22μm filters and stored at 4^0C in sterile nutrient broth solution. Phage titres were measured by counting plaques formed in authenticated *X. campestris* pv *juglandis* lawns using standard dilution series methods in sterile water and the "double agar layer" method as described by Civerolo (1990). Phage typing was carried out as described in Benson (1990).

A set of bacteriophages purified and stored as indicated above had the number of plaque forming units counted (pfu/ml) at the start of storage and after 3 months storage. Drops of the phage solutions were then placed and spread out on sterile cover slips and dried at room temperature in a laminar flow cabinet until all the water had evaporated (approximately 30 minutes). Replicated samples were then counted immediately in trial 1 as well as after 1,2,7 and 10 days of desiccation and storage in the dark at room temperature in trial 2. To assess the effects of wetting agents on phages, counts were taken prior to and after having 1 of 10 wetting agents added at 0.1% to the initial stock for 0 to 48 hours.

Glasshouse trials

Branches of walnut line G026 from the Lincoln University walnut trial were removed from the trees and placed under a UV absorbent plastic in the greenhouse. The buds were sprayed either with water, phage solution in water (approximately 10^7pfu/ml of 6 mixed phages) or phage solution in water with the most successful wetting agent. After 16 and 28 hours three replicates each of 4 buds per treatment were ground in sterile water and bacteria counted on selective media (Lindow *et al.*, 2000). The solution was centrifuged at a low speed and filtered through a 0.22 μm filter and phages were counted.

Electron microscopy

Samples of 3 of the phage solutions were centrifuged at 2,500*g* for 10 minutes and the supernatant was then centrifuged at 49,000*g* for 90 minutes and redissolved in 0.1 ml of sterile water. The samples, which contained in excess of 10^8pfu/ml, were then examined using the transmission electron microscope at Landcare Research Lincoln.

Results and discussion

Bacteriophages of *X. campestris* pv *juglandis* were found to be widespread and very easily isolated from walnut orchards in New Zealand. The three isolates examined so far were not significantly different in size, all had long (181±2 nm) unsheathed tails and hexagonal heads (76nm long by 70nm wide ±2 nm). However, the soil containing large numbers of phages was restricted to a relatively narrow zone from 0 to 2.5 cm below the soil surface. No phages were isolated from greater depths in the soil (Table 1). Thus, while widespread, the phages appear to intercept the bacteria that are washed from trees or end up on the soil after leaf and nut fall.

Table 1. Proportion of soil samples taken from under walnut trees that produced phages active against *X. campestris* pv *juglandis* strain 134.

Soil Sampling Depth Cm	Lincoln University site % of total samples (11)	Other New Zealand sites % of total samples (12)
2.5	73	100
5.0	0	-
7.5	0	-

Table 2. Effect of storage and desiccation on phage activity.

Phage designation	After 3 months storage	After desiccation (trial 1)	After desiccation (trial 2)
	% survival		
P2V1	66	5.0	3.8
P3V1	65	10.1	6.8
P3V2	72	2.1	2.6
P4V2	55	5.9	-
P3V3	49	6.8	5.8
P7	38	2.8	-
P8	51	8.6	-
P9	78	15.8	-
P10	90	8.1	-
Significance	$P \leq 0.001$	$P \leq 0.001$	NS

Table 3. Effect of wetting agent addition on phage survivability over time.

Phage designation	P slope	Slope Log pfu/min	R^2adjusted
P2v1	0.003	-0.000126	0.12
P3v1	0.010	-0.000101	0.07
P3v2	0.000	-0.000215	0.22
P4v2	0.005	-0.000074	0.09

Table 2 shows that the bacteriophages survived well in the stored state and that there were differences in viability among the phages. P10 still had 90% of its activity after 3 months storage. There were also differences in viability after desiccation but there seemed to be little relationship between the two viabilities. Phage P3V1 showed some ability to survive desiccation in both trials suggesting that there may be a consistent difference in this ability. However, with generally more than 90% of the phages being inactivated by desiccation there is a great need to locate either sturdier phages or more benign desiccation media. There was a significant ($P \leq 0.001$) but relatively slow (0.07 log units per day) decline in phage viability during long term desiccated storage that was not significantly affected by phage type.

Table 3 indicates that the effects of wetting agents on phage survivability, though significant, were relatively minor particularly for P4V2. Indeed some of the wetting agents actually enhanced phage numbers though not significantly. Some wetting agents also seemed to assist bacteriophages to survive desiccation (data not shown). Thus addition of appropriate wetting agents to improve phage penetration into the walnut buds would seem to be a viable option without negative effects on the phages. It proved possible to count *X. campestris* pv *juglandis* bacteria within the buds with counts of around 10^4 bacteria per bud in late spring. However, at present bacteriophages have not been recovered from the buds and there has been no significant effect of the preliminary treatments with phages on bacterial numbers.

Acknowledgements

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Biological Control of Fungal and Bacterial Plant Pathogens
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Biological control of *Heterobasidion annosum* in Norway spruce forest by nonpathogenic wood decay fungi

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Abstract: A field experiment was carried out in 1998 with indigenous isolates of *Phlebia gigantea* (Ph-1, Ph-2) and with the Finnish *P. gigantea* (R), commercial preparation Rotstop®, together with a *Trichoderma viride* (T) strain and Urea treatment (U), in a 35-year-old Norway spruce forest. The experimental plot was a 1st generation plantation. The forest was located in Trentino (Eastern Italian Alps) at 900 m a.s.l. and had a very high intensity of *H. annosum* infection, up to 80%. A total of ninety freshly cut stumps of about 15 cm diameter were treated by spraying with *P. gigantea*, *T. viride*, Urea (conc. 30%) and control (sterile water) with equivalent concentrations for each treatment. Spore viability was measured before and after spraying. After two growing seasons (18 months) the stumps were evaluated by incubation of wood discs. Percentage of infected stumps and the area percentage on the upper surface of the discs colonized by *H. annosum* were 53 and 18 for control; 55 and 16 for Tr; 58 and 10 for Ph-2; 71 and 39 for U; 77 and 54 for Ph-1; and 14 and 2% for R. Rotstop was effective in controlling *H. annosum* with a very high significance when compared to all other treatments despite the fact that the *P. gigantea* (R) strain was selected in Finland under different environmental conditions. For some reason Ph-1 and U were more heavily infected than the control. Two possible explanations of these results are given based on the selective pressure on *H. annosum* spores stimulated by the weak antagonist P1 and by the dilution of Urea after the treatment due to rain and by nitrogen enrichment in the wood that is considered a limiting factor for the development of *H. annosum*.

Key words: biological control, *Heterobasidion annosum*, *Phlebia gigantea*, Rotstop, Norway spruce

Introduction

Heterobasidion annosum is considered, in economic terms, the most dangerous pathogen of conifers in the forests of northern temperate regions and thus it is also one of the most studied forest pathogens in the world (Woodward *et al.*, 1998). *H. annosum* is a pathogenic white-rot fungus that mainly colonizes freshly cut stumps via airborne spores during primary infection (Rishbeth, 1950). So far, biological control of *H. annosum* has focused mainly on preventing primary infection on the surface of fresh stumps since spore germination and early establishment are clearly the most critical stages targeted by antagonists. The efficacy of *Phlebia gigantea* in controlling *H. annosum* infections on pine has been reported (Risbeth, 1963). *P. gigantea* occurs throughout the natural lifestyle of *H. annosum* and plays an important role in the decomposition of conifer debris. Preliminary stump treatment experiments in Finland have indicated that *P. gigantea* can also be effective in preventing aerial infection of *H annosum* in both Scotch pine and Norway spruce (Korhonen *et al.*, 1994). In Trentino, as well as in Finland, *P. gigantea* is a common inhabitant of spruce stumps and during warm rainy fall it fruits quite abundantly on them. A rapid increase in the

number of sites infected by *H. annosum* in Italy, coupled with new requirements for "environmentally-safe" pesticides has stimulated research into potential biological control agents to prevent the spread of this pathogen.

The aim of this experimental work was to verify the efficiency of Rotstop in Southern Europe and to compare it with other indigenous *P. gigantea* as well as other saprotrophic fungi (*Trichoderma*) and chemical treatment.

Material and methods

Forest site and isolates

An experiment on treated stumps was performed in May 1998 in a pure even-age plantation of Norway spruce stand, approximately 35 years old, situated at 900 m a.s.l. in Trentino (Eastern Italian Alps) with a very high inoculum density of *H. annosum*, up to 80% infection. Two selected autochthonous strains of *P. gigantea* from Trentino, "Ph-1" and "Ph-2", and one selected strain of *T. viride*, from Finland, "Tr" were used to compare with Urea 30% treatments (U) and Rotstop® (R), a newly patented (Kemira Oy) biological pesticide based on a selected North European strain of *P. gigantea* (Korhonen *et al.*, 1994; Korhonen & Lipponen, 1995).

Treatments

The field trial was carried out during two growing seasons on healthy stumps chosen in Trentino. Very high disease incidence and sporulation of *H. annosum* was observed at the site. Viability tests and number of spore dilutions were conducted before the treatments to standardize all the biological tests.

Before spraying, spore concentrations were adjusted with sterile water to ca. $5X10^3$ to 10^4 spores/ml for *P. gigantea* and ca. $2X10^4$ spores/ml for *Trichoderma*. The treatments were applied as a spray on fresh stumps cut to 35 cm height. To ensure comparable shade conditions, plants were randomly cut in the forest plot, and treatments were also spatially randomized. Care was taken to spray the same amount of solution on each stump, approximately 10 ml/dm^2.

At the end of October 1999 the first 3 cm layer of each stump was cut and discarded. Then, a 5 cm thick disc was cut, de-barked and washed by brushing under running tap water. The discs were than incubated in clean plastic bags at room temperature for a week.

Using a dissecting microscope, the number and size of the treated surface of stumps infected by *H. annosum* and by *P. gigantea*, and proportion of infected wood in stumps were determined on both sides of wood discs.

Results and discussion

Infection on both sides of the discs was highly correlated besides the urea treatment where infections at the bottoms of the discs were significant (12% more than the upper side) (Fig. 1). All other differences were insignificant. Among all strains, Rotstop was the best treatment both in terms of number of colonized stumps and in proportion to colonized surface, 100% and 57%, respectively. The *P. gigantea* strains from Trentino varied in their biocontrol ability, ranging from a medium level to that similar to the control (Fig. 2). In all cases, *P. gigantea* colonized most of the sapwood in treated spruce stumps. All *P. gigantea* growth occurred only in the sapwood, while *H. annosum* was present mainly in the heartwood. *Trichoderma* treatments were poor in terms of *H. annosum* control. Percentage of infected stumps and the area percentage on the upper surface of the discs colonized by *H. annosum* were: 53 and 18

for control; 55 and 16 for *T. viride*; 58 and 10 for Ph-2; 71 and 39 for U; 77 and 54 for Ph-1; and 14 and 2% for R.

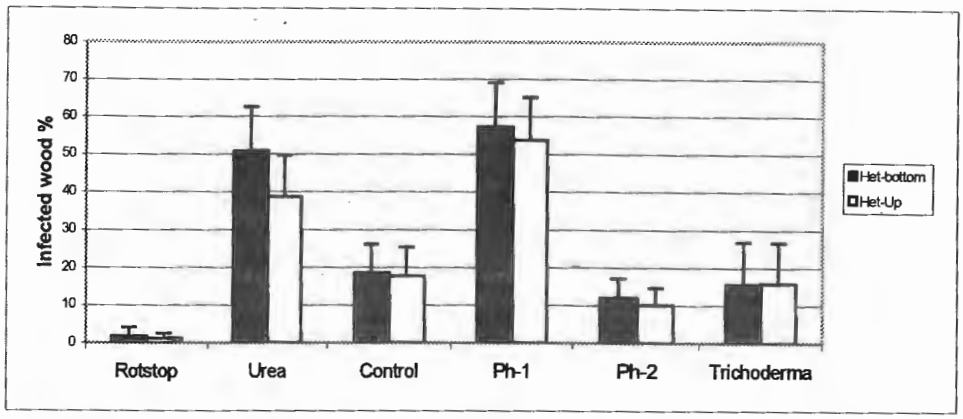

Figure 1. *H. annosum* infections among the six treatments on both sides of the discs. Vertical bars represent ± the standard error of the mean.

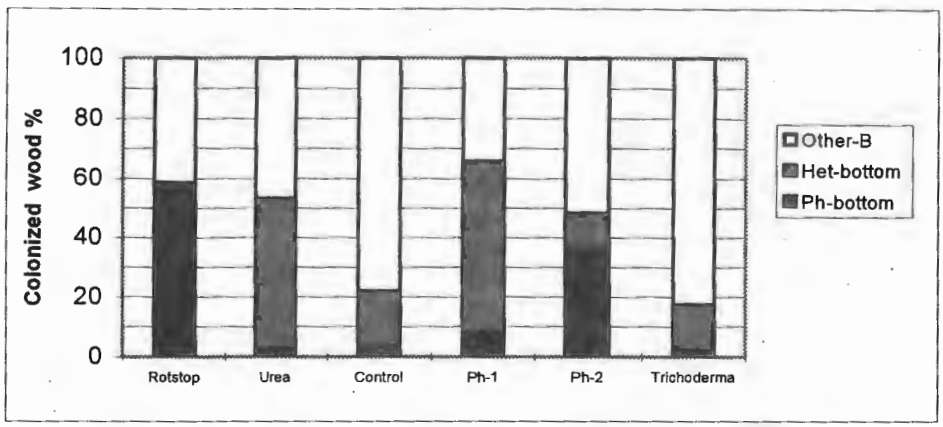

Figure 2. Percentage of colonized areas by *H. annosum* and *P. gigantea* on the bottom side of the discs.

Surprisingly, the urea treatment as well as Ph-1, contained higher infections of *H. annosum* than those of the control. To try to explain these surprising results it is necessary to take into account that the rainfall of 16.2 mm occurred on 29th May, just a few hours after the treatment. It is reasonable to presume, especially with the urea treatment, that dilution of the material took place due to the rainfall, which compromised its efficacy. Despite its ineffectiveness in terms of protection, the urea treatment gave rise to a nitrogen enrichment of the substrate, that may have favoured the development of *H. annosum*. It is well known that

nitrogen is one of the main limiting factors in *H. annosum* growth into the inner wood (Piri, 1998). The behaviour of Ph-1 may be explained by a selective pressure of the treatment that, nevertheless, was not able to protect the stump from spore germination, but allowed the more aggressive *H. annosum* strains to penetrate and grow faster into wood.
On the basis of the present results it seems that the stump treatment with *P. gigantea* was confirmed as an effective biological control method also under conditions of very high inoculum in Norway spruce stand. Urea can also have an opposite effect in controlling *H. annosum*, especially when it is used under unfavourable rainfall conditions. Moreover, in spite its origin, Rotstop controlled *H. annosum* under high inoculum density in the southern latitudes, much more effectively than other intra- and interspecific candidates. However, additional studies are needed to further clarify the unexpected results from this work. These data suggest that diversity exists in biocontrol ability among strains of the same species of *P. gigantea*. The influence of biodiversity in controlling *H. annosum*, and the effect of environmental conditions must be considered in future field experiments.

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How do basidiomycetes interact in conifer wood?

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Annosum root rot of conifers caused by *Heterobasidion annosum* is causing losses to European forest owners of about 1 billion Euro every year. Control measures include stump treatment with the basidiomycete *Phlebiopsis gigantea*. The mode of action has been claimed to be competition for substrate and space, but also hyphal interference and parasitism have been advocated to play a role. The aim of our project is to develop new and enhanced biocontrol of annosum root rot. For this purpose we want to elucidate the mechanism of biocontrol. We therefore have conducted a study on the effects of *P. gigantea* and other potential biocontrol fungi on physiology, gene expression and survival of the target fungus *H. annosum*.

Interacting fungal cultures alter their oxidative metabolism as compared to non-interacting ones. Both enhanced and suppressed phenoloxidase activities have been detected. Highly reactive radicals are produced in the interactions. The results so far indicate a changed pattern in gene expression, several of the differentially expressed genes are being identified. We have also studied gene expression following exposure to some of the fungitoxic metabolites that are produced by wood inhabiting basidiomycetes.

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Potential of wood-decaying fungi to control *Heterobasidion annosum*

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Root rot disease of conifers causes losses in the range of 800 million Euro each year in Europe (Woodward *et al.*, 1998). Chemical treatment of these pathogens has negative environmental effects (Pratt *et al.*, 1998). Thus, there is a need to develop biological methods of control that are more environmentally acceptable. Today, biological control of the major root rot pathogen, *Heterobasidion annosum*, is carried out by spraying a spore suspension of the antagonistic fungus *Phlebiopsis gigantea* onto stump surfaces at the time of felling. However, in Sweden only one isolate of *P. gigantea* is in commercial use which could be environmentally hazardous. There is therefore a need to discover more isolates effective against the pathogen. Although new pathogen infections are largely prevented by this stump treatment, it cannot eliminate the pathogen once it has established in the forest, e.g. root to root spread is not controlled. Using preinoculated woodblocks or biodegradable capsules, fungi antagonistic against root rotting pathogens could be inserted directly into roots and stumps of trees. Once established, the treatment would be self sustaining since the biocontrol fungi create an environment that is suppressive to root rot spread.

Eighty species of wood-decaying species and 100 isolates of *P. gigantea* were tested in the lab for their competitive ability against the root-rotting fungus *H. annosum*. Nine species of wood-decaying fungi and five isolates of *P. gigantea* were chosen for further tests in the field. Wood blocks inoculated with five species of cord-forming fungi were buried in the soil beside newly thinned spruce stand. No significant effect on the growth of *H. annosum* in the roots was found compared to the control. Four species of wood decaying fungi and five isolates of *P. gigantea* were mixed in water and sprayed on the surface of newly cut spruce stumps. All five isolates of *P. gigantea* showed significant effect against *H. annosum* and all five were as effective as, or better than, the Swedish commercial product of *P. gigantea*, Rotstop.

Dissolvable capsules filled with *P. gigantea* and *Resinicium bicolor,* respectively were inserted into stumproots in a clear-cut area. No significant control of *H. annosum* was observed.

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Involvement of population levels of *Penicillium oxalicum* in the biocontrol of tomato wilt

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Abstract: The effect of timing and method of application of *Penicillium oxalicum* on population levels of the biological agent in soils and in tomato rhizosphere were investigated and related to disease control. Experiments were carried out in the growth chamber and glasshouse. Tomato plants treated with a conidial dry power of *P. oxalicum* 14 days after sowing, showed a significantly reduction in Fusarium wilt in all the experiments. In these cases, population of the antagonist in the substrate was highest. A 10-fold decrease in rhizosphere populations of *P. oxalicum* was observed at the end of assays, independent of the treatments applied.

Key words: Antagonist, biological control, induced resistance, rhizosphere, *Fusarium oxysporum* f.sp. *lycopersici*

Introduction

Fusarium wilt of tomato caused by *Fusarium oxysporum* f.sp. *lycopersici* (FOL) is an economically important disease without effective control measures. Biological control could be a good alternative use in a disease management programme. De Cal *et al.* (1997a) reported that a strain of *Penicillium oxalicum* (PO) induced systemic resistance in tomato plants to Fusarium wilt. This resistance was related with morphological changes in tomato plants (PO prevents the distortion of cambial cells and the total differentiation of cambium in infected plants, and limited the secondary distribution of the pathogen within the vascular tissues (De Cal *et al.*, 2000), but was not correlated with a reduction in the population of FOL (the pathogen was present in the rhizosphere but did not induce severe symptoms (De Cal *et al.*, 1997a). *P. oxalicum* colonized the tomato rhizosphere but was not detected inside stems (De Cal *et al.*, 1997a). The result was a reduction in disease severity of 20-50% after application of *P. oxalicum*. Variation in efficacy of control depends on disease severity (De Cal *et al.*, 1997b, 1999, 2000). But other factors can be related with biocontrol. The objectives of the present study were to evaluate the efficacy of the biocontrol agent PO to reduce Fusarium wilt and the relationship with the population levels of PO in soil and tomato rhizosphere.

Materials and Methods

Plant material

Seeds of tomato cultivars Precodor (susceptible to race 1 and 2 of FOL) and Josefina (susceptible to race 2 of FOL) were sown in trays, containing an autoclaved mixture of vermiculite and peat and maintained in a grown chamber at 28°C with fluorescent light ($125 \mu Em^{-2}s^{-1}$, 13 h photoperiod) and 80% humidity for 4 weeks (growth chamber assays) or 3 weeks (glasshouse assay). For glasshouse assay, trays of seedlings were transferred to bags (80 l, 10 plants/ bag) containing sterile peat (autoclaved for 1 h, three consecutive days).

Growth chamber assay

Seedlings of cv. Precodor with two true leaves were transferred from seedbeds to flasks described by De Cal *et al.*, 1997b. Flasks were inoculated with a microconidial suspension of FOL (10^4 microconidia ml $^{-1}$) in water just before transplanting. Conidia of PO obtained by solid fermentation, was applied to tomato seedbeds giving a population of $6x10^6$ conidia per g substrate. Different methods of PO applications were used: 1) a conidial suspension was watered in the seedbed substrate 5 days before sowing, 2) a conidial suspension was watered 14 days after sowing, 3) a conidial dry powder was applied 14 days after sowing. Control treatment were flasks inoculated with FOL and plants not treated with PO. The assay was carried out twice, although only treatments 1 and 2 were applied in assay 1. Five replicate flasks, each containing four plants, were used per treatment. The flasks were placed in a randomized block design in a growth chamber at 28ºC with fluorescent light and 80% humidity for about 3 weeks. The area under the disease progress curve (AUDPC) was calculated using the disease percentage data. Populations of PO in the rhizosphere and substrate (estimated as colony forming units (cfu) g^{-1} of fresh root or dry substrate weight were recorded as described in De Cal *et al.* (1997b) in three samples per treatment just before transplanting and/or at the end of assay.

Glasshouse assay

Bags (80 L) containing sterile peat were placed in a glasshouse at 20-30ºC and inoculated with a microconidial suspension of FOL in Czapek Dox broth (giving a final concentration of 10^5 microconidia per g substrate). Bags were inoculated with FOL. Ten seedlings of tomato Cherry cv. Josefina with 2-4 true leaves were transplanted into each bag and five bags per treatment were used. PO was applied to tomato seedbeds giving a population of $6x10^6$ conidia per g substrate. The methods of application of PO were similar to those described in growth chamber assays. Control treatment were bags inoculated with FOL and plants not treated with PO. Treatments were arranged in a randomized block design with 5 blocks. Disease severity was assessed 10, 30, 40, 50 and 60 days after transplanting. All plants was transferred to moisture chamber at the end of the experiment and the presence or absence of the pathogen in the crown of plants was determined after 48 h of incubation at 25ºC. Disease incidence was then calculated on the basis of plants with symptoms from which the pathogen was re-isolated. Populations of PO in the rhizosphere and substrate in seedbeds were estimated in three plants per treatment just before transplanting. Populations of PO in the rhizosphere were recorded per plant at the end of the assay as described above.

Statistical analysis

Data for CFUs was subjected to log transformation when homogeneity of variances was needed. All data were then analyzed using analysis of variance. When data were significant at $P \leq 0.05$, means were compared by Student-Newman-Keul's multiple range test.

Results

In growth chamber assays, seedlings infected with FOL showed symptoms of Fusarium wilt, however, disease severity was lower in assay 1 (ca. 18%) than in assay 2 (ca. 36%). Treatments 1 and 2 reduced the AUDPC in assay 1 (by a 71% and 61% respectively) ($P \leq 0.05$), while in assay 2 only treatment 3 reduced AUDPC (ca. 25%). In the glasshouse assay tomato plants showed a 76% of disease incidence, and treatments 1,2 and 3 controlled disease by 52.6, 33.1 and 28.3%, respectively ($P \leq 0.05$).

PO was isolated from treated seedbed substrate before transplanting at levels lower than those that were applied (1,48-3,67x 10^6 cfu g^{-1}dry substrate), except with treatment 3 were the

population of PO in the substrate was similar to initial population (6,79x 10^6 cfu g^{-1}dry substrate) (Table 1).

The populations of PO in the rhizosphere of tomato seedlings in growth chamber assays were similar for all treatments prior to transplanting (Table 1). Rhizosphere populations of PO in all treatments decreased about 10-fold, 21 days after transplanting. The size of populations of PO in the rhizosphere of tomato plants was not related to the presence or absence of FOL (data not shown).

Table 1: Populations of *Penicillium oxalicum* (PO) in seedbed substrate and rhizosphere of tomato plants cv. Precodor inoculated with the pathogen and treated with the antagonist in growth chamber [x].

PO[y] treatments	Assay 1			Assay 2		
	cfu g^{-1}dry substrate x10^6	cfu g^{-1} fresh root x10^6		cfu g^{-1}dry substrate x10^6	cfu g^{-1} fresh root x10^6	
		Before transplanting	21 days after transplanting		Before transplanting	21 days after transplanting
1	2.48 a	11.11 a	0.68 a	1.48(6.1)a	2.55(6.28)a	1.26(5.81)a
2	2.68 a	12.70 a	1.27 a	3.67(6.4)ab	10.00(6.67)a	0.52(5.38)a
3	-	-	-	6.79(6.8)b	5.66(6.65)a	0.55(5.61)a

[x] Data are mean of three replicates. Data in parentheses have been converted to log transformation. Means followed by the same letter in each column are not significantly different by Student Newman Keul's multiple range test ($P \leq 0.05$).

[y] *P. oxalicum* treatments applied to tomato plants were: 1) a conidial suspension was watered in the seedbed substrate 5 days before sowing, 2) a conidial suspension was watered 14 days after sowing, 3) a conidial dry powder was applied 14 days after sowing.

Table 2: Populations of *Penicillium oxalicum* (PO) in seedbed substrate and rhizosphere of tomato plants cv. Josefina inoculated with the pathogen and treated with the antagonist in glasshouse [x].

PO treatments [y]	cfu g^{-1}dry substrate x10^6	cfu g^{-1} fresh root x10^6	
		Before transplanting	80 days after transplanting
1	5.38(6.71) a	1.53 a	0.17 a
2	5.36(6.61) a	5.0 b	0.23 a
3	18.8(7.19) b	1.92 a	0.10 a

[x] Population of PO data are the means of three replicates. Data in parentheses have been converted to log transformation. Means followed by the same letter in each column are not significantly different by Student Newman Keul's multiple range test ($P \leq 0.05$).

[y] *P. oxalicum* treatments applied to tomato plants were: 1) a conidial suspension was watered in the seedbed substrate 5 days before sowing, 2) a conidial suspension was watered 14 days after sowing, 3) a conidial dry powder was applied 14 days after sowing.

In glasshouse assays, population of PO in seedbed substrate treated with Treatment 3 was significantly higher before transplanting than that applied thereafter. (Table 2). At 14 days after sowing the populations decreased except in seedbeds treated with Treatment 2.

Thereafter, populations decreased in all seedbeds to about 10-fold lower, 80 days after transplanting. The size of populations of PO in the rhizosphere of tomato plants was not related to the presence or absence of FOL (data not shown).

Discussion

A relationship between the timing and method of application of PO and the population levels of the antagonist in seedbed substrate and rhizosphere of tomato plants was observed in this study. Application of PO as a conidial dry powder 14 days before transplanting (treatment 3) provided the highest PO population in seedbed substrate, but this was not maintained in rhizosphere of tomato plants before transplanting. The size of populations of PO in the rhizosphere of tomato plants was not related to the presence or absence of FOL, as described previously (De Cal *et al.*, 1997b, 1999).

All PO treatments significantly reduced the development of Fusarium wilt on tomato plants in the glasshouse and growth chamber (assay 1). Only tomato plants treated with a dry conidial powder 14 days after sowing to flasks in growth chamber (assay 2) showed a significantly reduction in the development of disease. The enhanced populations of PO observed in the seedbed substrate of this treatment (treatment 3) may be responsible for the increased control observed. In many biological control systems lack of consistency has been associated with low populations of the antagonist. Raaijmakers *et al.* (1995) showed that the rhizosphere population of two *Pseudomonads* strains was an important determinant of their efficacy in suppressing *Fusarium* wilt radish.

PO could be applied as a dry conidial powder 14 days before transplanting or as another treatment that could maintain enough antagonist population in the seedbed substrate. A formulation enhancing the performance of PO in the rhizosphere may be a way to improve the biocontrol of FOL.

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Biological Control of Fungal and Bacterial Plant Pathogens
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A screening system for identifying biological control agents of *Sclerotium cepivorum*

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Abstract: Onion white rot caused by the soilborne fungus *S. cepivorum* is a serious disease of onions worldwide and the absence of resistant cultivars or efficient chemical control makes this pathogen a good target for biological control. A three stage screening system was developed to identify potential fungal biological control agents (BCAs). Initially degradation of *S. cepivorum* sclerotia was assessed on agar and then in soil, followed by an onion seedling bioassay test to examine suppression of white rot on plants in the glasshouse. In the agar test, 65 (45%) of the 154 fungi tested degraded or infected sclerotia and 39 out of 63 (62%) of these BCAs degraded more than 30% of sclerotia in the soil assay when applied as a wheat bran culture. In the onion seedling bioassay, 9 out of 39 (23%) of the BCAs controlled white rot by more than 50%. BCAs applied as spore suspensions in the last two assays were less effective.

Key words: biocontrol, sclerotia, *Sclerotium cepivorum*, screening, white rot

Introduction

Allium white rot caused by the soilborne fungus *Sclerotium cepivorum* is a serious disease of *Allium* crops worldwide (Entwistle, 1990) and at present, conventional methods of control are limited. In the UK there are currently no approved fungicides for white rot after dicarboximide fungicides such as iprodione were shown to rapidly lose efficacy due to enhanced degradation in the soil (Walker *et al.*, 1986). Commercial *Allium* varieties resistant to *S. cepivorum* are not yet available although there has been considerable effort in searching for resistance (Brix & Zinkernagel, 1992). Finally, control by field scale fumigation is unreliable and is being phased out in the UK.

This situation makes white rot a good target for biological control and while studies have been carried out for the last 30 years, results have been variable (Kay & Stewart, 1994), perhaps due to inappropriate screening systems (Jackson *et al.*, 1991). The efficacy of biological control will also vary with environmental conditions, soil type and the origin of both BCA and *S. cepivorum* isolate. This paper describes a three phase screening program for evaluating fungal biological control agents (BCAs) for their ability to degrade sclerotia and control white rot disease in a UK soil.

Materials and methods

Fungal isolates

The isolate of *S. cepivorum* used in the screening tests was obtained from a field site in Kirton, Lincolnshire, UK. Cultures were obtained by surface sterilising sclerotia and squashing on potato dextrose agar (PDA). Sclerotia of *S. cepivorum* were obtained by inoculating autoclaved spawn bags containing maizemeal sand (1920 g sand, 80 g maizemeal, 175 ml water) with a mycelial macerate of the pathogen and incubating for 6 weeks at 20 °C.

Sclerotia were then harvested by wet sieving and dried in a stream of air for 24 hrs before being used immediately, stored at 5 °C or conditioned by burial in field soil for 12 weeks.

Potential BCAs were obtained from onions grown in white rot infested fields and from a collection of antagonists from previous studies (Jackson *et al.*, 1991). Some known antagonists of *S. cepivorum* and other sclerotial pathogens were also tested. These included *Coniothyrium minitans* (Gerlagh *et al.*, 1996), *Trichoderma virens* (Lewis *et al.*, 1991) and *T. harzianum* (McLean & Stewart, 2000). All fungal isolates were cultured on PDA at 20°C, and stored in liquid nitrogen. Spore suspensions were obtained by adding 20 ml sterile distilled water to a 3 week old PDA culture of the BCA and scraping gently with a spatula. Wheat bran cultures of BCAs were obtained by inoculating 250 ml flasks containing 12 g wheat bran +42 ml H_2O with spore suspensions (5 ml) and incubating for 3 days at 20°C.

Agar drop test

Drops of PDA (five drops per 9cm Petri dish) were inoculated with a mycelial macerate of a one-week-old culture of *S. cepivorum* and incubated for 5 days at 20°C (sclerotial initiation). An agar plug from an actively growing culture of each test fungus was added to each of the five precolonised agar drops and the plates incubated for 5 weeks at 20°C after which 100 sclerotia on each agar drop were assessed for degradation (soft or collapsed) by squeezing with forceps.

Sclerotial degradation assay

Sclerotia of *S. cepivorum* were placed in netting bags with 10 g 50:50 v/v soil/sand mix (silty clay, 100 sclerotia per bag). BCAs were then added either as wheat bran inoculum (1g in 100 g soil/sand) or spore suspensions (to give $1x10^7$ spores per 100 g soil/sand). The bags were then tied and buried in clear plastic boxes containing 175 g 85:15 v/v soil/sand mix and water added to give a moisture content of 75 % field capacity. Boxes were then incubated at 20°C for 8 weeks after which sclerotia were retrieved by wet sieving and assessed for degradation. Where possible, a sample of 30 hard (undegraded) sclerotia from each bag were also surface sterilised and squashed on agar drops to detect any infection by BCAs after 7 days incubation at 20 °C. Three replicate box tests were set up for each BCA. Control treatments consisted of sclerotia with only bran or water added.

Onion seedling bioassay

Conditioned sclerotia of *S. cepivorum* were added to soil (3 g sclerotia per 100 g soil) and BCAs added either as wheat bran cultures (1 g in 100 g soil) or spore suspensions (to give $1x10^7$ spores per 100 g soil/sand). The amended soil was added to 9 cm pots (220 g per pot) and onion seed planted (cv. White Lisbon), one seed in each pot. There were 10 pots set up for each BCA in each of 5 replicate blocks in a glasshouse (min. temperature 15 °C). Inoculated control treatments consisted of pots containing soil and sclerotia only, and uninoculated controls of soil only were also included. All watering was from below and the emerging onion plants were assessed for symptoms of white rot every week up to 12 weeks.

Results and discussion

From 154 test fungi, 65 (45 %) degraded more than 80 % of sclerotia formed on the agar drops and some inhibited sclerotial formation completely or suppressed the number formed. These fungi were selected for further testing as potential BCAs of *S. cepivorum*.

The sclerotial degradation test was then carried out with 63 selected isolates. More than 50 % of the sclerotia buried in soil were degraded or infected by 3 % of these BCAs while 39 (62 %) of the BCAs degraded or infected more than 30 % of the sclerotia when applied as a wheat bran culture (Table 1). This was in contrast to the results where spore suspensions were

used, as none of the 44 BCAs tested degraded or infected more than 30 % of the sclerotia and only 11% degraded or infected more than 20 % of the buried sclerotia.

Table 1: Percentage of BCAs degrading sclerotia of *S. cepivorum*.

% sclerotia degraded/ infected by BCA	% BCAs affecting sclerotia (bran culture)	% BCAs affecting sclerotia (spore suspensions)
>50%	3.2	0.0
41-50%	11.1	0.0
31-40%	23.8	0.0
21-30%	23.8	11.4
<21%	38.1	88.6

When 39 selected BCAs were assessed in the onion seedling bioassay, 9 (23%) resulted in more than 50% white rot control when applied as a wheat bran culture compared to only 4 (11%) of 35 BCAs which were tested as a spore suspension (Table 2).

Table 2: Percentage of BCAs controlling white rot.

% disease control	% BCAs controlling white rot (bran culture)	% BCAs controlling white rot (spore suspensions)
>50%	23.1	11.4
41-50%	10.3	8.6
31-40%	23.1	11.4
21-30%	20.5	8.6
<21%	23.1	60.0

When results from the sclerotial degradation test and onion seedling bioassay were compared (wheat bran inoculum), 9 BCAs were common to the best 20 isolates in each test, and one of these was an isolate of *T. harzianum*, one of the BCAs obtained which had controlled white rot in a previous study (McLean & Stewart, 2000). The other top isolates also appeared to be *Trichoderma* species.

The three stage screening program was successful in selecting BCAs that degraded sclerotia of *S. cepivorum* and suppressed white rot. BCAs applied as wheat bran cultures were also far more effective than spore suspensions and this former formulation allowed large populations of *Trichoderma* to develop in non-sterile soil (Lewis & Papavizas, 1984) and was used successfully in the biological control of white rot in other studies (Kay & Stewart, 1994). Generally, the ability of BCAs to degrade sclerotia was a good indicator of efficacy of white rot control but even so, there were BCAs that were relatively poor sclerotial degraders but gave good disease control. This suggests that different mechanisms play a part in the biological control of *S. cepivorum*. Some BCAs may parasitise and degrade sclerotia hence decreasing inoculum and reducing disease whereas others may protect plants through production of antibiotics, competition or inducing resistance in the host (Whipps, 2001).

Current work is now looking at the effect of different soils and isolates of *S. cepivorum* on the efficacy of BCAs.

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Biological control of damping-off on pine (*Pinus* spp.) with a new fungal species, *Ceratobasidium albasitensis* isolated in Albacete (Spain)

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Abstract: A new species, *Ceratobasidium albasitensis* V. González & V. Rubio, belonging to the *Rhizoctonia s. l.* species complex, has been found in Albacete (south-east of Spain) mainly in saffron fields. This new species is a potent biological control agent against several fungal diseases of many crops and also acts as a plant growth promoter. Results on the identification of the new species and also protection experiments using several isolates of *C. albasitensis* against damping-off on pine seedlings are included in this work.

Key words: biological control, binucleate (BNR) or non-pathogenic (np-R) *Rhizoctonia*, pine, damping-off, new *Ceratobasidium* species

Introduction

The form-genus *Rhizoctonia* is currently considered to be a heterogeneous assemblage of fungal taxa, which do not form asexual spores, but has certain significant vegetative characteristics in common. The genus has considerable ecological and economical importance, because it occurs worldwide and different isolates of the complex may live as saprophytes or as symbionts, like in terrestrial orchids (i.e. *Goodyera*, *Platanthera*, *Calipso*, *Coeloglossum*, etc.) and in some other plant species. Also, many isolates are effective plant protectors of several fungal diseases, promoting plant growth, in general (Sneh *et al.*, 1986). Many other isolates cause significant losses in agriculture and forestry, currently Rhizoctonia diseases have been described in more than 200 plant species. At least 120 epithets referring to *Rhizoctonia* have been reported. Nevertheless, only few attempts have been made in order to suggest natural and profound concepts of genera and species currently included in *Rhizoctonia s.l.* (Sneh *et al.*, 1991; Andersen & Stalpers, 1994; Roberts, 1999).

A major taxonomic feature to discriminate among taxa is the presence of: multinucleate, binucleate or uninucleate cells in young vegetative hyphae, although the nuclear status for many taxa still remain unknown. Binucleate and uninucleate were reported to belong mainly to the teleomorph genus *Ceratobasidium* Rogers. Another group of binucleate *Rhizoctonia*, *Epulorhiza* Moore (= *Rhizoctonia repens* Bernard) belong to the teleomorph genus *Tulasnella* Schroeter. Multinucleate *Rhizoctonia* belong basically to the teleomorph genus *Thanatephorus* Donk. A basic major obstacle in studying the genetics and taxonomy of *Rhizoctonia s.l.* is the difficulty, or frequent inability to obtain progeny of the sexual mating of many isolates. Therefore, most studies were carried out with cultures of the anamorph where the sexual reproductive structures were usually not or hardly observed, and determination of species was thus based on the morphological and physiological features of the anamorph. Taxonomy of the different species has been based mostly on grouping by

means of anastomosis reactions (where a number of AG's have been recognized for multi- and binucleate taxa) (Sneh *et al.*, 1991). To date, it is generally accepted to differentiate populations within the taxa into a complex of at least seven or eight (depending upon the authors) different genera (Moore, 1987; Andersen & Stalpers, 1994). Rhizoctonia diseases of vegetable, ornamentals, trees, nursery and greenhouse crops cause large economic losses in agriculture. Many isolates of *R. solani* Kühn and other taxa from the *Rhizoctonia* complex attack a wide range of hosts, producing several symptoms such as seed decay, damping-off of seedlings, stem cankers, root rots, fruit decay, etc. Some nonpathogenic isolates within the form-genus, usually denominated binucleate *Rhizoctonia* (BNR) or non-pathogenic *Rhizoctonia* (np-R), are highly effective biocontrol agents. In most studies to date, all the known BNR isolates capable of protecting several plants from diseases belong to the genus *Ceratobasidium* Rogers (anamorph *Ceratorhiza* R.T. Moore). The results presented here deal with the identification of a new species of the genus *Ceratobasidium* and protection tests in pine using isolates of the new species against damping-off produced by *R. solani* AG 4.

Materials and methods

Taxonomical analysis

In order to characterize and determine the BNR isolates used in this study belonging to the new species, morphological and molecular approaches were carried out. For morphometrical purposes, all the BNR isolates from saffron and pine in Spain were induced to produce perfect states in the laboratory, using two undescribed methods. The first method consisted of a modification of the one described by Hietala *et al.* (1994). In this first procedure, sterile radish seedlings (ethanol 70% 2'; sodium hypochloride 5% with Tween 0.005% 15' and five washes with distilled water), were pre-germinated by incubating on 1.5% water-agar plates at 24°C in the dark. After two days, radish seedlings were transferred to Petri dishes containing 20 ml sterile water with two 5 x 5 mm blocks of a 7-day-old colony of the fungus, and incubated at room temperature with natural lighting. Teleomorph production started between 3 and 10 days after fungal inoculation. A second new method was developed in our laboratory, consisting of a modification of some previously described methods (Flenje, 1956; Murray, 1982) based on transferring isolates from high nutrient agar to low nutrient agar. Briefly, basidiomes were obtained by growing cultures of the several isolates on PDA (Potato dextrose agar) for 5-6 days and then transferred to plates of 15% water agar containing small pieces (two blocks of 10 cm^2 approx. per plate) of leaves or twigs from several plant species such as *Nerium oleander* L., *Prunus laurocerasus* L., *Ligustrium vulgare* L., *Pinus halepensis* Miller, etc., previously surface sterilized as described above for the first method. Hymenial production started (depending upon the strain) between 7 and 15 days after transfer of the colonies. On the other hand a phylogenetic analysis was carried out using the entire nucleotide sequence of the ITS regions (including 5.8 S). DNA isolation, PCR amplification and DNA sequencing procedures used in this work have been described (Boysen *et al.* 1996). Alignment of the sequences from the different strains were performed using CLUSTAL W software (Thompson, 1994). Subsequently phylogenetic reconstruction was performed using the maximum parsimony analysis with PAUP v.3.1.1 software (Swofford, 1991).

Protection

Prior to protection tests, both the supposedly protective isolates and the pathogens selected were assayed for pathogenicity. All experiments were performed at two stages; Petri dishes (or flasks) and greenhouse trays. At the laboratory stage, pine seeds were pre-germinated and thus, disposed in Petri dishes and flasks containing 1.5% WA as nutritive medium. Greenhouse tests were performed in trays containing sterile substrate peat/vermiculite (3:1).

The pathogenic isolate selected for the experiment described in Table 1 was: *R. solani* pin-JR (AG 4). The protective isolate used in this experiment was Eab-F2 (*C. albasitensis*).

Results and discussion

Our phylogenetic study (data not shown) demonstrates that molecular methodology constitutes a powerful tool to characterize and identify taxa belonging to the *Rhizoctonia* complex. The separate clustering of *Ceratobasidium albasitensis sp. nov.* based on rDNA-ITS sequences, suggests the possibility of a new species. To determine if this separate clustering reflected differences at species or subspecies level, the isolates were fructified *in vitro* and the morphological data found were not previously reported for any species of the complex.

On the other hand, protection effects and growth promotion responses, generally depend on the plant species assayed, as well as on the pathogenic fungi selected for each experiment. In general terms, all the protective isolates were considered as hypovirulent, as a result of the previous pathogenicity tests performed. Moreover, although they represented isolates of the same taxa, protection rates were different among them.

Among all the pathogens assayed, the best rates of protection were obtained when *R. solani* was used as pathogen. This could be explained due to the close phylogenetic relationships among genera *Thanatephorus* and *Ceratobasidium* (Stalpers & Andersen, 1996), together with the fact that BNR isolates may play an important role in suppressing patch extension in soils infested with *R. solani* by antagonizing interactions (Sneh, 1996).

Table 1 shows that the timing for inoculation of the pathogen with respect to the protective isolate, was also an important parameter for the improvement of the protection effects. Thus, consecutive inoculation of the pathogen two weeks after the BNR isolate allowed colonization of the root and stem surfaces by the protective fungi, preventing the attack of the pathogen, probably by differential competition for infection sites or by activating plant defense responses, as suggested by some authors (Cardoso & Echandi, 1987)

Table 1. Protection test in greenhouse

Inoculum	Number of pine seedlings
none	120
R. solani pin-JR (AG4)	9
C. albasitensis Eab-F2	122
pin-JR and Eab-F2 simultaneus	43
Eab-F2 two weeks before pin-JR	119

Growth promotion was tested only in potato and garlic assays, and estimations of biomass (aerial and radicular) in terms of dry weight, showed a considerable increase of biomass in general, especially in the radicular mass of the plant, as previously reported in other studies (Escande & Echandi, 1991; Bandy & Tavanzis, 1990). In addition, an improvement of flowering and formation of microtubers with respect to controls were observed in potato as well as a higher yield (25% increase) in garlic production. Over the past years we have demonstrated the use of *C. albasitensis* to protect against several diseases caused by *Rhizoctonia* spp., *Alternaria* spp., *Fusarium* spp. *and Penicillium* spp. over a wide range of plant species, including wheat *(Triticum aestivum)*, alfalfa *(Medicago sativa)*, rape *(Brassica napus)*, carrot *(Daucus carota)*, tomato *(Lycopersicum esculentum)*, several grasses

(Poaceae), onion *(Allium cepa)*, garlic *(Allium sativum)*, saffron *(Crocus sativus)*, potato *(Solanum tuberosum)* and radish *(Raphanus sativus)*.

In summary, the use of molecular techniques to identify new BNR isolates, on pathogenicity and protection assays, could constitute a useful methodology to explore and exploit new sources of biocontrol agents within *Rhizoctonia* group.

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Biological Control of Fungal and Bacterial Plant Pathogens
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Biocontrol of white root rot on avocado plants using rhizobacterial strains

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Abstract: *Rosellinia necatrix* Prill. is the causal agent of the white root rot on avocado trees. In this work we studied the potential use of rhizobacterial strains as biocontrol agents of white root rot. From roots of healthy avocado trees, antagonistic rhizobacterial strains against *R. necatrix* were isolated and characterized. Some of them showed good biocontrol ability in tomato/*Fusarium* and avocado/*Rosellinia* tests systems, especially the strain *Pseudomonas fluorescens* PCL1606. To our knowledge, this is the first approach to biocontrol white root rot in avocado using rhizobacterial strains.

Key words: *Rosellinia necatrix*, *Pseudomonas* sp., *Bacillus* sp., *Persea americana*, phytopathogenic fungi, antifungal metabolites

Introduction

Rosellinia necatrix Prill. (anamorph: *Dematophora necatrix* Harting) is the causal agent of the white root rot disease in avocado (*Persea americana* Mill.) and many other crops (Sztejnberg & Madar, 1980). The symptoms are rotting of roots, yellowing and fall of leaves, wilting, and finally, death of the tree. Various approaches are possible for the control of white root rot, for instance soil fumigation, soil solarization, or biological control using *Trichoderma* (Lopez Herrera *et al.*, 1998, Sztejnberg *et al.*, 1987) but usually without sufficient disease suppression. Positive results of solarization are related to induction of suppressive soils and the increase of microbial activities in the soil (Freeman *et al.*, 1990; Greenberger *et al.*, 1987). Microbial mechanisms by which rhizobacteria may protect plants against soilborne pathogens include competition for niches, parasiting or predating, antibiosis and induction of systemic resistance (ISR) (Thomashow & Weller, 1995). In this study, we have isolated bacteria from avocado roots with antagonistic activity against *R. necatrix* and evaluated their biocontrol ability of avocado white root rot.

Material and methods

Bacterial and fungal strains

Rosellinia necatrix strains were isolated from roots of naturally diseased avocado orchards in southern Spain, as well as *Phytophthora cinnamomi* strains used in this study. These strains were kindly provided by R. Perez (C.I.D.A.-Malaga, Spain). Fungal strains of *Fusarium oxysporum* f. sp *radicis-lycopersici*, *Verticillium dahliae*, *Rhizoctonia solani*, *Sclerotium rolfsii* and *Pythium ultimum* were obtained from the collection of the IMP (Leiden University, The Netherlands). Bacterial strains were isolated from roots of healthy avocado trees growing in Southern Spain. The roots were blended in phosphate buffered saline, and 10-fold dilutions were plated onto TSA, KB and PI media. After 48 h at 24°C, the numbers of colonies were

counted, and some colonies were selected to perform an antagonistic test on plates (Chin-A-Woeng *et al.*, 1998).

Strain identification

Characterization of bacterial strains was performed using API20NE® (BioMerieux, Mercy L'etoyle, France) and BIOLOG® (BIOLOG Inc., California, USA). Strains were typed after amplifying the 16S ribosomal DNA by PCR, and the sequences were determined and compared with those in a GenBank (Williams *et al.*, 1990).

Detection of antifungal metabolites

Detection of antifungal metabolites was performed by TLC analysis of phenazine (Chin-A-Woeng *et al.*, 1998), phloroglucinols, pyrrolnitrin and pyoluteorin (Sarguinet *et al.*, 2000). Strains were tested for production of HCN (Castric, 1975), proteases (Chin-A-Woeng *et al.*, 1998), lipases (Howe and Ward, 1976), β-glucanases (Walsh *et al.*, 1995) and cellulases (Hankin & Anagnostakis, 1977).

Biocontrol tests

Tomato/*Fusarium oxysporum* f. sp. *radicis-lycopersici* bioassays were performed using the experimental design described previously (Chin-A-Woeng *et al.*, 1998). Briefly, seeds were coated with bacteria and sown in pots containing potting soil infected with spores of *F. oxysporum* f.sp. *radicis-lycopersici*. Avocado/*Rosellinia necatrix* bioassays were performed using plants obtained by germination of embryos as described by Pliego-Alfaro *et al.*, 1987. The bacterized plants were sown in vermiculite with wheat seeds infected with *R. necatrix* as inoculum (Bakkers *et al.*,1987; Freeman *et al.*, 1986). In both cases, plants were grown in a greenhouse, and after 16 days the plants were examined for disease symptoms. Data were analyzed for significance using analysis of variance followed by Fisher's least significant difference test (α=0.05), with SPSS software (SPSS Inc., Chicago, Il., USA). All experiments were performed at least twice.

Results and discussion

Bacterial antagonistic ability

Screening of nine hundred strains resulted in the identification of twelve isolates antagonistic to *R. necatrix* (named PCL1601 to PCL1612). Based on amplified 16S ribosomal DNA, the API20NE® and BIOLOG® tests, all of them belong to the genera *Bacillus* and *Pseudomonas*. The strains showed *in vitro* growth inhibitory activity against *F. oxysporum* f. sp. *radicis-lycopersici*, and also against other soilborne phytopathogens to avocados, including *Phytophthora cinnamomi*, *Sclerotium rolfsii*, *Pythium ultimun*, *Verticillium dhaliae* and *Rhizoctonia solani* (Table 1).

Biocontrol ability of selected antagonistic rhizosphere strains

The strains *P. chlororaphis* PCL1601, PCL1604 and *P. fluorescens* PCL1606 possess the strongest biocontrol activity in the tomato/*Fusarium oxysporum* system (Figure 1B), in comparison with the biocontrol strains *P. chlororaphis* strain PCL1391 (Chin-A-Woeng *et al.*, 1998), producer of phenazine-1-carboxamide, and *P. fluorescens* WCS365 (Geels and Schippers, 1983) which act by ISR. The strains *P. fluorescens* PCL1606 also have the strongest biocontrol activity in the avocado/*Rosellinia* test system (Figure 1A), followed by *P. chlororaphis* PCL1607.

Production of compounds related with the biocontrol ability

TLC analysis to detect antifungal factors in extracts of used growth medium of our selected strains, showed that *P. chlororaphis* strains produce phenazine-1-carboxamide. *P. fluorescens* PCL1606, with the highest biocontrol ability (Figure 1) does not produce any of the most common antibiotics produced by biocontrol bacteria. The structure of the antifungal

metabolite compound(s) is being elucidated. The production of HCN was detected in all the *P. chlororaphis* strains and in *P. fluorescens* PCL1606. Indicator assays showed that the *Pseudomonas* strains produced protease and lipase activity, whereas β-glucanase activity was detected, produced by the *Bacillus* strains.

Table 1. Characterization of the selected *Pseudomonas* and *Bacillus* strains and antagonistic ability against various fungal strains by plate assay on King B medium.

Strain	Identification	Fungal strains[1]						
		Rn	Pc	Vd	Rs	Pu	Sr	Forl
PLC1601	*P. chlororaphis*	++[2]	++	+	+	+	++	++
PCL1602	*P. fluorescens*	+	+	V	-	-	+	-
PCL1063	*P. putida*	+	+	V	+	+	++	+
PCL1604	*P. chlororaphis*	++	++	V	++	+	++	++
PCL1605	*B. subtilis*	++	-	V	+	-	-	+
PCL1606	*P. fluorescens*	++	++	V	++	++	++	++
PCL1607	*P. chlororaphis*	+	+	+	++	+	++	++
PCL1608	*B. subtilis*	+	-	+	++	+	+	++
PCL1609	*P. putida*	+	+	+	-	-	++	-
PCL1610	*B. subtilis*	+	-	+	+	-	+	+
PCL1611	*P. chlororaphis*	++	++	V	++	+	++	++
PCL1612	*B. subtilis*	+	-	+	+	+	+	++

[1] Rn: *Rosellinia necatrix* (4 strains); Pc: *Phytophthora cinnamomi* (3 strains); Vd: *Verticillium dhaliae*; Rs: *Rhizoctonia solani*; Pu: *Pythium ultimum*; Sr: *Sclerotium rolfsii*; Forl: *Fusarium oxysporum* f.sp. *radicis-lycopersici*.

[2] ++: very strong inhibition; +: strong inhibition; -: no inhibition; V: doubtful response.

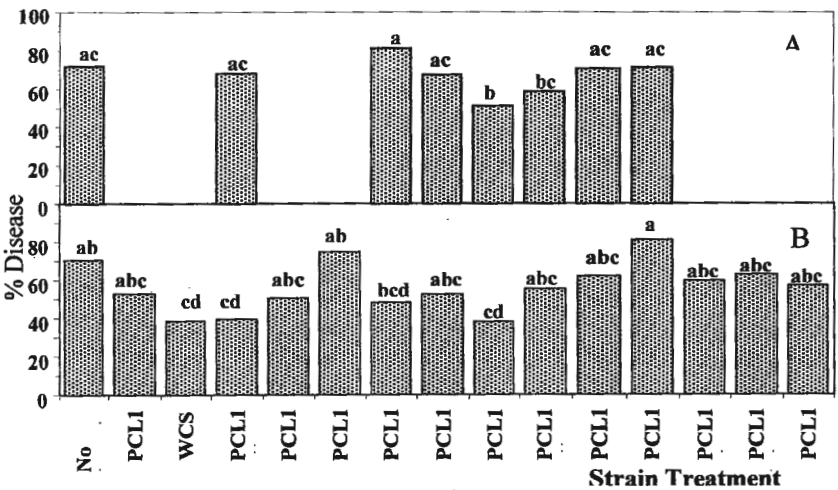

Figure 1. Biocontrol ability of selected rhizobacterial isolates: A) in a avocado/*Rosellinia* system; B) in a *Fusarium oxysporum* f.sp. *radicis-lycopersici* in a tomato/*Fusarium* system. Values with different letter indications denote a statistically significant difference.

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Biological Control of *Sclerotinia sclerotiorum* in glasshouse lettuce

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Abstract: *Coniothyrium minitans* isolate Conio applied as maizemeal perlite solid substrate soil incorporation at full application rate (10^{12} cfu m^{-2}) gave significant control of Sclerotinia disease in three glasshouse lettuce crops equal to the fungicide iprodione. This treatment also resulted in a reduction in the number and viability of sclerotia and increased infection of sclerotia by *C. minitans*. All *C. minitans* treatments [maizemeal perlite at full and reduced rates (10^{12} and 10^{8} cfu m^{-2}) and isolate Conio and a commercial preparation (Contans WG) spore suspensions at 10^{8} cfu m^{-2}] significantly reduced sclerotial germination and apothecial production compared with the untreated control in a glasshouse box bioassay. C. minitans isolate Conio maizemeal perlite at full rate compared with the untreated control also significantly reduced percentage recovery of sclerotia and percentage viability of sclerotia.

Keywords: *Coniothyrium minitans*, biological control, *Sclerotinia sclerotiorum*, lettuce

Introduction

The sclerotial parasite *Coniothyrium minitans* has potential to control *Sclerotinia sclerotiorum*, a major pathogen on a number of important commercial crops (Whipps & Gerlagh, 1992). *C. minitans* when applied as maizemeal perlite solid substrate soil incorporation at 0.6 l m^{-2} (10^{12} cfu m^{-2}) gave equivalent control to the fungicide iprodione (McQuilken *et al.*, 1995). However, this level has been stated as uneconomical for commercial purposes (Whipps & Gerlagh, 1992). The effect of *C. minitans* inoculum concentration and inoculum type (solid substrate and spore suspension soil incorporations) on control of Sclerotinia disease in three sequential glasshouse lettuce crops, and on sclerotial germination and apothecial production in a box bioassay, was investigated.

Materials and methods

The glasshouse trial was done using soil naturally infested with *S. sclerotiorum*. There were 3 plots (1.4 x 2.6 m) per treatment. Six treatments were applied to soil prior to each of three lettuce crops: i) No treatment control; ii) Fungicide spray control iprodione (Rovral); iii) *C. minitans* Conio full rate maizemeal perlite (MP) (10^{12} cfu m^{-2}); iv) Conio reduced rate MP (10^{8} cfu m^{-2}); v) isolate Conio spore suspension (10^{8} cfu m^{-2}); vi) Contans WG spore suspension, (commercial product) (10^{8} cfu m^{-2}). MP inocula of *C. minitans* isolate Conio were prepared using the method developed by McQuilken *et al.* (1995). Spore suspensions were prepared by adding 2 l tap water to 2 l of MP inoculum in a bag, agitating the contents for 1 minute, then passing the supernatant through a 180 µ m sieve. Spore suspensions of Contans WG (granular material containing 1 x 10^{9} conidia g^{-1}; Prophyta GmbH, Malchow/Poel, Germany) were prepared by adding 0.1 g dry material to 1 l tap water and stirring for 30 min. Appropriate dilutions for application were then prepared on the basis of haemacytometer counts.

Each crop was harvested and assessed for the number of diseased plants, leaving diseased material on plots. Subsequently, the number of sclerotia on the soil surface was counted in five

randomly positioned quadrats (500 cm^2) in each plot. Twenty sclerotia from the first, third and fifth quadrats were collected, surface sterilised and assessed for viability and infection with *C. minitans* (Williams *et al.*, 1998). At intervals throughout the trial, soil was taken from the top 3 cm in each plot. *C. minitans* survival assessed by soil dilution plating (McQuilken *et al.*, 1995).

A box bioassay was also set up to investigate the effect of soil incorporation of the same *C. minitans* treatments on sclerotial germination and apothecial numbers. MP or spore suspension inocula were mixed thoroughly with batches of sieved (5 mm) air dried glasshouse soil and 300g placed in each of 12 clear plastic boxes. Soil treated with MP inocula or spore suspensions and reduced rate MP received approximately 10^7 or 10^3 cfu cm^{-3} soil respectively. These concentrations were chosen to reflect application rates in the glasshouse. Twenty sclerotia produced on wheat grain (Mylchreest & Wheeler, 1987) were buried in each box of soil approx. 1 cm below the surface. Soil moisture content was adjusted to 75% field capacity, and the boxes incubated in the glasshouse. At weekly intervals, the boxes were watered to maintain soil moisture content and examined for apothecia. Control treatments consisted of boxes containing sclerotia buried in uninoculated soil. At harvest the sclerotia were recovered and assessed for viability and infection with *C. minitans* as previously described.

Results and discussion

Percentage disease in the first crop was low with no significant difference between any of the treatments. Both fungicide and *C. minitans* isolate Conio MP at full application rate treatments significantly reduced disease in comparison with the control in crops two and three (Fig 1). However, none of the other *C. minitans* treatments significantly reduced disease. *C. minitans* isolate Conio MP at full application rate significantly reduced sclerotial viability and increased infection of sclerotia compared with the untreated control, and other *C. minitans* treatments in crop three (Table 1). *C. minitans* was recovered from the soil throughout the trial at levels of between 10^5 to 10^7 cfu cm^{-3} in full rate MP inoculum treated plots and 10^1 to 10^4 cfu cm^{-3} in all other inoculum treated plots.

Table 1. Effect of soil incorporation of spore suspension and MP inocula on the number of sclerotia (per 2500 cm^{-2}) recovered from the soil surface and their viability and infection with *C. minitans* after three sequential lettuce crops. Means of 5 reps per plot, with 3 plots per treatment.

	1st crop	2nd crop			3rd crop		
Treatment	N°	N°	%	%	N°	%	%
Zero Control	1 a	34 a	82 a	27 b	260 a	98 a	2 c
Fungicide	1 a	0 c	-	-	19 c	98 a	1 c
Conio MP full	0 a	12 b	48 b	83 a	31 c	75 b	41 a
Conio MP	0 a	19 ab	69 ab	52 ab	193 a	95 a	15 b
Conio spore	1 a	22 ab	81 a	42b	116 b	98 a	2 c
Contans WG	1 a	18 ab	71 ab	50 ab	176 ab	99 a	3 c

Means within columns followed by the same letter are not significantly different ($P \leq 0.05$) as tested by ANOVA.

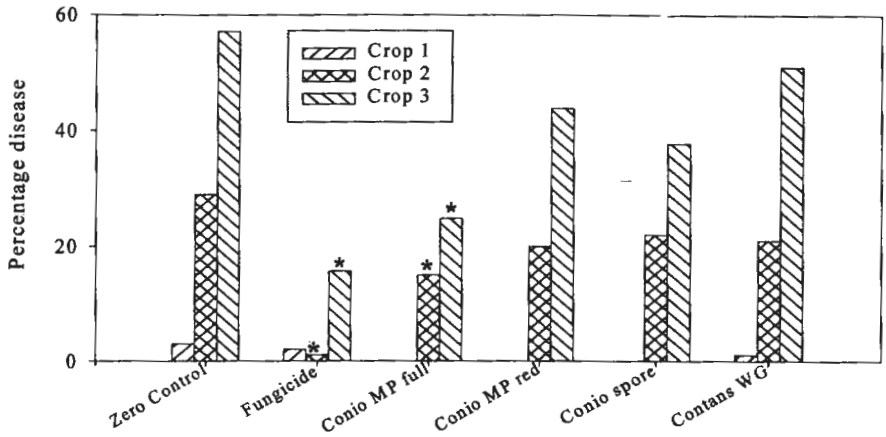

Figure 1. Effect of soil incorporation of *C. minitans* as MP or spore suspensions on percentage of Sclerotinia diseased lettuce plants in three consecutive crops. Treatments columns marked with an asterix are significantly different ($P < 0.05$) from untreated control.

In the box bioassay, all *C. minitans* treatments significantly reduced sclerotial germination and reduced apothecial production (Fig 2, Table 2). *C. minitans* isolate Conio MP at full application rate also significantly reduced sclerotial recovery and viability compared with the untreated control, confirming the results of the glasshouse trial. This may reflect the greater level of *C. minitans* applied to the soil from the full rate MP inoculum (10^{12} cfu m^{-2}) relative to the spore suspension inoculum and reduced rate MP inoculum (10^{8} cfu m^{-2}).

Table 2. The effect of maizemeal perlite (MP) and spore suspension inocula of *Coniothyrium minitans* on the number of sclerotia recovered and their viability and infection with *C. minitans* in a glasshouse box bioassay. Means from 10 replicate boxes containing 20 sclerotia each.

Treatment	Cumulative apothecial number	% Recovery	% viability	% infection
Zero Control	90 a	16 b	14 bc	0 b
Conio MP full	11 b	5 b	3 c	1 b
Conio MP reduced	28 b	50 a	36 ab	10 a
Conio spore (MP)	22 b	51 a	46 a	3 ab
Contans WG spore	35 b	16 b	16 bc	1 b

Means within columns followed by the same letter are not significantly different ($P \leq 0.05$) as tested by ANOVA.

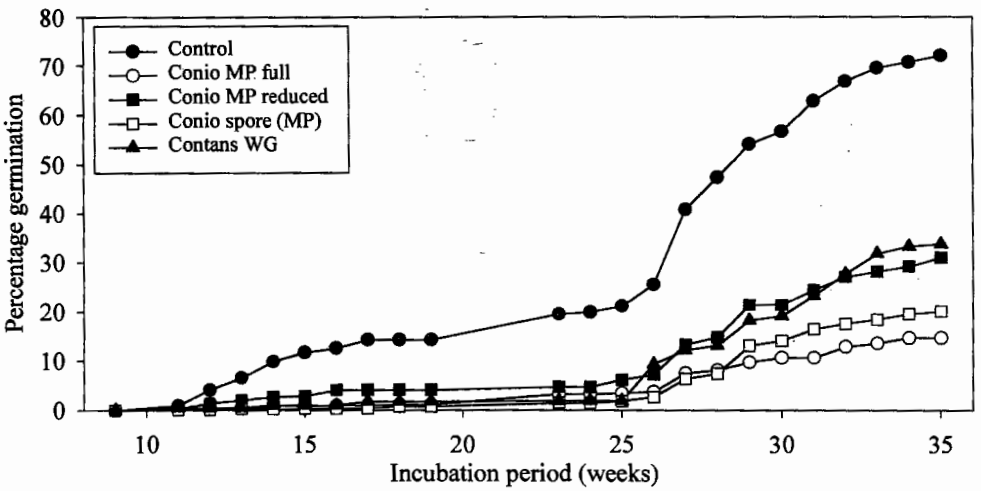

Figure 2. Effect of soil incorporation of MP or spore suspension inocula of *C. minitans* on germination of *S. sclerotiorum* sclerotia (mean 10 boxes, 20 sclerotia per box).

In the control treatment, the low value for viability (14%) may be due to degradation of sclerotia following apothecial production. However, in the low rate *C. minitans* treatments, the relatively greater levels of viability (16 - 46 %) may reflect a combination of factors. *C. minitans* at this low rate may have prevented apothecial production without killing the sclerotia. This would provide an enhanced viability relative to both controls which have a high level of apothecial production and consequent low viability and the high *C. minitans* application rate treatment where rapid destruction of sclerotial integrity follows infection.

Conclusions

Inoculum level of *C. minitans* in terms of cfu m^{-2} of soil appears to be a key factor in the control of both Sclerotinia disease and apothecial production by sclerotia.

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Biological Control of Fungal and Bacterial Plant Pathogens
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Biological Control of Pythium root rot of tomato

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Abstract: Several bioagents were tested against *Pythium aphanidermatum* in closed cycle NFT (Nutrient film technique) and rockwool cultured greenhouse tomato. These bioagents include *Pseudomonas chlororaphis*, a local isolate of *P. fluorescens* and BTM (a commercial compost starter) that consisted of *Rhizobium, Bacillus, Clostridium, Entrobacter,* and *Pseudomonas* spp. The experiments were conducted on 'trust' tomatoes 2-weeks after being transplanted to rockwool bags or NFT troughs by introducing the laboratory cultured bioagents into the feeder barrels so that a bioagent was kept at a population of 10^5 cfu/ml in the nutrient solution in the feeder barrels. In one experiment, the treatments were: (1) control; (2) inoculated with *P. chlororaphis* (Pc); (3) inoculated with BTM; (4) inoculated with Pc and BTM; (5) inoculated with *P. aphanidermatum* (Pa); (6) inoculated with Pa and Pc; (7) inoculated with Pa and BTM; (8) inoculated with Pa and Pc and BTM. In the other experiment, the local isolate *P. fluorescens* (Pf) was compared with Pc. The inoculum concentration for Pa was 10^2 cfu/ml in initial feeder barrel of nutrient solution. Each experiment was repeated twice. The results were summarized: (a) All bioagents had some antagonistic effect against Pa. Their efficacy ranged from approximately 5 to 35% reduction in root rot disease severity; (b) Pc was most effective at 35% reduction, Pf was moderately effective at 10-20% reduction, and BTM was least effective at 5-10% reduction; (c) Tomato plants grown in closed cycle rockwool system had significantly better growth and less severe disease than those grown in closed cycle NFT system. The rockwool appeared to provide better aeration and acted as a filter that trapped the bioagents; (d) None of the bioagents showed any adverse effect on plant growth.

Key words: Pythium root rot, *Pythium aphanidermatum*, tomato, bioagent, *Pseudomonas chlororaphis*, *Pseudomonas fluorescens*

Introduction

Pythium root rot caused by *Pythium aphanidermatum* (Pa) is one of the most important diseases of greenhouse tomatoes in Ontario (Huang & Tu, 1998). Hydroponic culture exacerbates the problem. Both nutrient film technique (NFT) and recirculating growing systems pose a challenge in the control of this disease, because the pathogen, especially the zoospores, can spread easily in the solution to the whole recirculating system (Jenkins & Averre, 1983; Stanghellini *et al.*, 1984). Ideally, the best way to manage the disease is prevention for which clean healthy transplants should be used with a sound, clear greenhouse infrastructure as well as hydroponic instruments free from contamination.

Unfortunately, Pythium always manages to get into the system and quickly spreads through the recirculating solution. Various research efforts have been made to alleviate the spread of the pathogen and also to control the disease. To control the spread of the pathogen, several sterilization methods have been developed. These include UV light, ozone, filtration, heat, sonication, H_2O_2, etc. (Menzies & Belanger, 1996; Runia, 1995; Tu & Zhang, 2000). However, treatment of recirculating solution can only prevent the spread of the disease but cannot control the disease that already resides in the roots of the infected plants.

In fact, sterilizing the recirculating solution in some cases exacerbates the disease because sterilization removes not only the pathogen but also the beneficial rhizobacteria in the recirculating solution. For example, Tu *et al.* (1999) showed that Pythium root rot was less severe in a closed recirculating system than an open system where run-off nutrient solution was not recirculated. They concluded that the increased rhizobacteria population in the recirculating system was attributable for the reduced disease severity. This phenomenon is a clear indication that beneficial rhizobacteria can reduce disease severity and yield loss.

This paper reports the findings of various bioagents that were tested against Pythium root rot disease and their relative effectiveness.

Materials and methods

Commercial rockwool plugs (2 cm in diameter and 4 cm high) in a multi-hole styrofoam tray were used to raise tomato transplants, one tomato (*Lycopersicon esculentum* cv. Trust, a cultivar susceptible to *Pythium aphanidermatum* (Pa)) seed/plug. After sowing, the trays were left on a greenhouse bench at 20-25 ^{0}C, irrigated daily with a nutrient solution consisting of N:100, P:30, K:200, Ca:50, Mg:30 and Fe:0.2 ppm. Two weeks later, each plug plant was transferred to 10X10X8 cm rockwool cubes and grown on rockwool cubes for 42 days before transplanting to either rockwool or NFT recirculating system. The pH of the solution was maintained at 6.0 throughout the experiments. The experiments were conducted in the greenhouse when 2-week-old tomato plants in the cubes were transplanted to rockwool bags or NFT troughs. Bioagents were introduced into the feeder barrels.

Three different types of bioagents were used, i.e., *Pseudomonas chlororaphis* (Pc), *P. fluorescens* (Pf) and BTM. BTM was a concentrated commercial product consisting of *Rhizobium, Bacillus, Clostridium, Entrobactor* and *Pseudomonas* spp. Both Pc and Pf were grown in nutrient agar for 3 days and washed off the agar plates before using. For each bioagent, the amount added to a barrel was calculated to derive a final population of 10^5 cfu/ml.

Pythium inoculum was that of zoospores of Pa. The zoospores were produced as described previously (Zhang & Tu, 1999). The inoculum concentration of Pa was 10^2 cfu/ml in the initial feeder barrel of nutrient solution. The experiments were continued for 120 days after transplanting. Tomato fruits were harvested and weighted. Total plant fresh weight was taken at the end and root rot severity was rated at the end of the experiments by a 0-9 scale where 0 = less than 10% root rot, 1 = 10-20%, ... and 9 = 90-100% root rot. All experiments were repeated once. Data were analysed statistically using ANOVA.

In one experiment, the treatments were: (1) control; (2) inoculated with Pc; (3) inoculated with BTM; (4) inoculated with Pc and BTM; (5) inoculated with Pa; (6) inoculated with Pa and Pc; (7) inoculated with Pa and BTM; (8) inoculated with Pa, Pc and BTM.

Since Pc appeared to be highly effective, a separate experiment was conducted to compare it with an effective local isolate of Pf in the laboratory and in the greenhouse.

Results and discussion

The results of experiment I, summarized in Figure 1, showed (a) all bioagents had some antagonistic effect against Pa and their efficacy ranged from approximately 5 to 35% reduction in disease severity; (b) Pc was most effective at 35% reduction, Pf was moderately effective at 10-20% reduction, and BTM was least effective at 5-10% reduction; (c) Tomato plants grown in a closed cycle rockwool system had significantly better growth and less

severe disease than those grown in the closed cycle NFT system. The rockwool appeared to provide

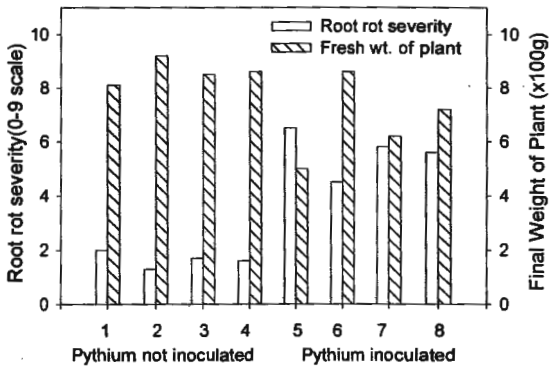

Figure 1. Fresh weight and root rot severity of tomato at the conclusion of the experiment. The treatments were: (1) control, (2) inoculated with *Pseudomonas chlororaphis* (Pc), (3) inoculated with BTM, (4) inoculated with Pc and BTM, (5) inoculated with *Pythium aphanidermatum* (Pa), (6) inoculated with Pa and Pc, (7) inoculated with Pa and BTM, (8) inoculated with Pa and Pc and BTM.

better aeration and acted as a filter that trapped the bioagents; (d) None of the bioagents showed any adverse effect on plant growth.

Statistical analysis revealed that treatments not inoculated with Pythium (i.e., 1 to 4) had significantly lower root rot severity than the inoculated treatments (i.e., 5 to 8). Similarly, fresh weight of plants was significantly higher than the inoculated treatments. Within treatments 1 to 4 for non-inoculated and 5 to 8 for inoculated with Pythium only treatments 2 and 6 were significantly different from the others in respective treatment sets. It was apparent that Pc was highly efficient in controlling Pa when used solely by itself, however, its efficacy appeared to be attenuated when combined with BTM.

In a separate experiment where Pc and Pf were compared, the former produced a larger inhibitory zone (2.5 cm) than the latter (1.0 cm) suggesting that the former might produce a more Pythium-inhibitory antibiotic. This laboratory result suggests that efficacy of Pc is based on high antibiotic production. In another greenhouse experiment root rot severity of Pythium inoculated rockwool slabs was examined to compare four different treatments (i.e., Pc, Pf, Ridomil (metalaxyl), non-treated and non-inoculated control). The results indicated that Pc was more efficient in controlling Pa than the chemical Ridomil (Figure 2).

In conclusion, it can be said that Pc is a highly effective bioagent against Pa in the greenhouse rockwool environment. Further studies are being undertaken to use Pc as an element in an integrated control measure.

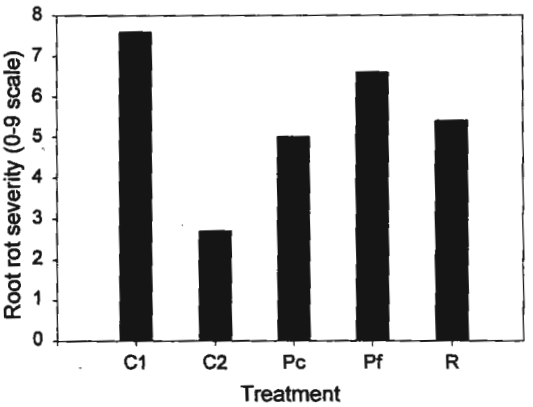

Figure 2. Root rot severity in Pythium inoculated tomato: not treated (C1), treated with *Pseudomonas chlororaphis* (Pc), *P. fluorescens* (Pf), and Ridomil (R), against non-inoculated control (C2).

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Biological Control of Fungal and Bacterial Plant Pathogens
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pp.93 - 95

Biological control of plant fungal diseases

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Abstract: A collection of spore-forming bacteria from different samples, which inhibit different kind of phytopathogenic fungi have been isolated. The fungicidal strains belong to different groups. The antagonistic strains are being assessed for their ability to control fungal diseases of potato and wheat in field conditions and postharvest storage. The treatment of plants by bacterial strains result in the decrease of infection and symptoms of diseases. The application of fungicidal bacteria increase potato and wheat harvest by 20%. The antagonistic strains produce different kinds of biological active compounds.

Key words: bacteria, fungi, diseases, potato, wheat, post-harvest

Introduction

Many fungal pathogens reduce the quality and quantity of agricultural plant production. The losses of crops due to action of plant diseases may reach 20-30%. Current practices for plant disease control are based on chemical pesticides, but their use is currently controversial. Chemical pesticides can cause environmental pollution and induce pathogen resistance.

To minimize negative consequences of chemical pesticides for human health and the environment biological control of pests can be developed. The application of spore forming bacteria, such as *Bacilli*, as agents in the protection of some commercially important crops is promising. The *Bacilli*-based biological pesticides have some advantages: they are easy to produce and use, and do not require special equipment for handling or storage.

In the course of our screening program for new biological active compounds, we have isolated a collection of spore forming microorganisms from different samples. Here we report results of a laboratory study and small-scale field trials of a few biological agents found naturally for plant disease control.

Potato is economically a very important crop in Russia. Potato cultivation occupies about 3.5 millions ha. There are two principal tasks for protection of potato. First, combat of the Colorado potato beetle. Second, the protection from fungal diseases. Earlier we had developed the *B. thuringiensis*-based insecticide "Colorado" that effectively protected against the beetle. This insecticide obtained the bronze medal at an International Exhibition Eureka in Brussel. Now, we are trying to solve the problem of protection of potato from fungal diseases. The most serious fungi that affect potato are *Phytophthora infestans, Fusarium solani, Rhizoctonia solani, Alternaria.*

Results

A few strains were preliminarily identified for their ability to suppress late blight of potato caused by *P. infestans*. The isolates with fungicidal activity belong to two taxonomic groups, *B. subtilis* and *Brevibacillus* spp. To broaden the range of use of these strains we explored their

fungicidal potential. The zoospores or conidia of the pathogenic fungi were spread on potato agar, and cultural broth of the biocontrol strains were introduced into wells cut in the agar.

Potato tubers infected by *R. solani* were tested. When treated with *Brevibacillus* spp. 1, the tubers were healthy and germination was effective. Untreated tubers were diseased and the germination of tubers delayed. *B. subtilis* strains have a broader spectrum of fungicidal activity than *Brevibacillus* spp. In the laboratory, fungal suppression by most of *Brevibacillus* spp. cultures is associated with cellular fraction (spores). In contrast, the inhibition activity of *B. subtilis* strains is connected with extracellular fractions of fully sporulated cultures.

To determine the mode of fungicidal action *Brevibacillus* spp. 1 and *B. subtilis* K16 cultures were tested against *F. solani* conidia. The treatment with *Brevibacillus* spp. 1 delayed conidial germination and germ tube growth rate was lower than in the control. In contrast, the treatment by *B. subtilis* K16 led to the lysis of conidia and complete inhibition of germ tube growth.

Furthermore, the fungicidal effect of *Brevibacillus* spp. 1 was tested on zoospores of *P. infestans* and conidia of *A. tenuis*. Cultural broth of *Brevibacillus* spp. 1 caused the mortality of dormant forms of both fungi. In the laboratory, germination of some antagonistic strains were evaluated for their effect on the yield of few cultivars of potato over five seasons at three Moscow regional field sites where the phytopathogenic fungi *P. infestans*, *R. solani*, *F. solani*, and *A. tenuis* were naturally present. Potato tubers were treated with spore-based formulations and applied to the seed.

Certain treatments provided a significant yield benefit for potato in all growing seasons. The yield of potato in the treatment was significantly greater than the yield of the untreated potato, which suggests that fungal infection was a factor in reducing yield of untreated potato. Significant yield benefits were observed with *Brevibacillus* spp. 1 in 1994 and *B. subtilis* 16 in 1996 (Table 1).

Table 1. Total potato yield and yield of healthy tubers (1996).

Variant	Total Yield		Yield of healthy Tubers,	
	Ton per ha	Diseased tubers (%)	Ton per ha	% of untreated
Untreated	21.9	12	19.3	100
1	22.8	9.8	20.6	106.7
7	24.1	11.9	21.2	109.8
16	25.8	7.0	24.0	124.3
Tecto	25.9	6.2	24.3	125.9

Thereafter, for potato protection the formulation "FUNLAT" was developed, which consists of *Brevibacillus* spp. 1 spores and a component that provides adhesion and allocation of the biological fungicide. The potato tubers were treated with different concentrations of "FUNLAT" and stored at special condition. The best results were obtained using "FUNLAT" at 80 g per ton of tubers and treated with 3L water per ton. Under those conditions part of the diseased tubers and waste in treated and untreated samples after 240 days storage was 9.2 and 6.3, and 17.0 and 11.3 %, respectively, for cultivar "Zhukovsky" (Table 2).

The degree of tuber protection of the cultivar "Sante" under different conditions was lower. The decrease of prevalence of fungi infection on potato tubers was demonstrated by using "FUNLAT".

Table 2. The effect of *Brevibacillus* Spp. 1-based formulation, "FUNLAT" on tubers of potato cultivar "Zhukovsky" during storage in the year 2000.

Number	Storage							
	60 days		120 days		180 days		240 days	
	Diseased tubers %	Waste %	Diseased tubers %	Waste %	Diseased tubers %	Waste %	Diseased tubers %	Waste, %
3	7,6	4,1	8,5	5,5	8,9	6,0	9,2	6,3
4	7,7	4,6	9,2	6,2	9,6	6,5	10,3	7,2
5	8,8	3,0	9,8	5,3	10,6	5,8	11,6	6,7
6	9,6	2,9	10,9	5,4	11,3	6,2	11,4	6,2
7	12,1	4,4	12,4	5,3	13,0	6,0	13,2	5,3
8	13,1	6,5	16,0	10,2	16,8	11,2	17,0	11,3

3- FUNLAT - 80g per ton of tubers, 3L water per ton
4- FUNLAT- 80g per ton of tubers, 6L water per ton
5- FUNLAT- 160g per ton of tubers, 3L water per ton
6- FUNLAT- 160g per ton of tubers, 6L water per ton
7- TECTO- (Novartis) 90 ml per ton of tubers, 3L water per ton
8- Untreated

Wheat is an important crop worldwide. Fungal diseases of wheat decrease yield of wheat by about 30-50%. Besides, about 80% of the crop may be contaminated by mycotoxins. Mycotoxins decrease the edible potential of wheat to 25-30%. Annual loss of the crop worldwide amounts to about $2–2.5 billion. The fungal disease known as wheat scab (head blight) is an increasing danger to sustainable wheat production in the world. The causal agent of wheat scab is *Fusarium graminearum.*

B. subtilis 16 inhibited the growth of *F. graminearun* when the fungi were spread on potato agar and when cultural broth of *B. subtilis* 16 was introduced into wells cut in the agar. A direct observation of the killing effect was made by a microscopic study of *F. graminearun* conidia. The treatment by *B. subtilis* 16 led to total lysis of conidia. A small-scale field trial of *B. subtilis* 16-based preparation supports the high efficacy reported for this stain.

Conclusions

- The spore forming bacteria *Brevibacillus* spp. and *B. subtilis* had good fungicidal activity.
- The mode of fungicidal activity of both bacteria is different.
- The treatment of potato tubers by *Brevibacillus* spp. protected them against fungal diseases in storage.
- The treatment by *B. subtilis* protected wheat against head blight.

Biocontrol of fungal pathogens in wheat and barley with bacterial seed dressings – possible mechanisms

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We have screened several hundred plant-associated bacteria for biocontrol activity against phytopathogenic fungi on seeds. The screening was done in greenhouse bioassays under conditions similar to those in the field. The biocontrol activity for specific isolates recorded in the greenhouse agreed with that in field trials. Several effective biocontrol strains have emerged from the screenings, and we have chosen to study the biocontrol mechanisms of two strains, *Pseudomonas chlororaphis* strain MA 342, and another root-associated pseudomonad. Strain MA 342 was subsequently developed into a commercial biocontrol product and has proved to be as effective as conventional fungicides in preventing the development of seedborne diseases caused by *Drechslera teres* (barley net blotch) and *Tilletia caries*. It produced antifungal metabolites, and one of these, DDR is involved in biocontrol of barley net blotch. DDR-deficient transposon-induced mutants of the strain were not effective in biocontrol. DDR is a macrocyclic lactone whose structure was recently published. The amount of DNA required for the synthesis of DDR is estimated to be at least 100 kb, based on the number of enzymes required in a proposed biosynthetic pathway. Part of this DNA has been sequenced. We have also constructed mutant libraries of MA 342 and the other biocontrol strain, using miniTn*5-Gus* and miniTn*5CatGus*, with the aim of finding mutants that are defective in their ability to suppress barley net blotch and *Fusarium culmorum*, respectively, and identifying seed-inducible genes *in vivo* in the bacteria.

Biological Control of Fungal and Bacterial Plant Pathogens
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Effect of soil moisture and planting depth on *Rhizoctonia* root rot of beans and its control by *Trichoderma harzianum*

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Abstract: The effect of soil moisture and planting depth on bean root rot caused by *Rhizoctonia solani* and its control by *Trichoderma harzianum* was studied under greenhouse conditions. *R. solani* was grown on rice grains and *T. harzianum* on wheat bran. Both fungi were inoculated before planting. Disease severity, percentage of emerged plants, plant height and dry weight were evaluated three weeks after planting. Emergence rate and growth of plants inoculated only with *R. solani* were not affected by soil moisture. Nevertheless, dry weight was higher in moist soil when both fungi were inoculated. Deep planting significantly reduced the emergence rate and growth of plants inoculated with *R. solani* alone. However, in the presence of the antagonist, the effect of plant depth was not significant. At a planting depth of 6.0 cm the percentage of emerged plants was 50% in the presence of *T. harzianum*, but only 6.7% when the pathogen was inoculated alone. The antagonist protected bean seedlings from preemergence damping-off, reduced disease severity and increased plant growth in the presence of *R. solani*.

Key words: *Rhizoctonia solani*, *Trichoderma harzianum*, biological control, soil moisture, planting depth

Introduction

Soil moisture and planting depth are known to affect bean root rot severity caused by *Rhizoctonia solani* (Abawi & Pastor-Corrales, 1990). Bean production has substantially declined in the last number of years in Brazil due to soilborne pathogens such as *R. solani*, apparently due to the combination of soil moisture and cooler temperatures observed in these areas under irrigation regimes (Cardoso, 1994). No single treatment provides a satisfactory control of the disease. Shallow planting can be effective in reducing the disease (Leach & Garber, 1970). *Trichoderma harzianum* is one of the more intensively investigated species that may inhibit activities of *R. solani* (Lewis & Kulik, 1996). *Trichoderma* species may be also influenced by soil moisture (Klein & Eveleigh, 1998). Our objective was to study the influence of soil moisture and planting depth on bean root rot caused by *R. solani* and its biological control by *T. harzianum*.

Material and methods

Greenhouse experiments were conducted at Hannover, Germany, to study the effects of soil moisture at four levels (50, 75, 100 and 125%) of saturation and planting depth (1.5, 3.0, 4.5 and 6.0 cm) on *Rhizoctonia* root rot development in absence and presence of *T. harzianum*.

R. solani (AG 4) was grown on rice grains and *T. harzianum* on wheat bran for 7 days in 200 ml-Erlenmeyer flasks at 25°C. Soil-sand (2:1) was sterilized at 150-170°C for 24 hours. Immediately before the planting, the content of each pot (300 ml of soil-sand) was poured on a tray and carefully mixed with inoculum of both fungi at 3% (w/w). About 10 seeds of the

cultivar 'Dufrix' were sown per pot. Control treatments received non-inoculated rice grains and wheat bran, respectively. The pots were maintained at 23/18°C (day/night), weighted to monitor water loss and irrigated once a day. A planting depth of 3 cm was used in the soil moisture experiments while in the planting depth experiments, soil moisture was maintained at approximately 100% saturation. The plants were removed three weeks after planting and hypocotyls were evaluated to determine the disease severity according to a 1-9 scale adapted from Van Schoonhoven & Pastor-Corrales (1987). Percentage of emerged plants, plant height, and dry weight were also determined. The following combinations were tested: no *R. solani*-with *T. harzianum* (nRwT), with *R. solani*-no *T. harzianum* (wRnT), with both fungi (wRwT) and without both fungi (nRnT).

Results and discussion

The percentage of emerged plants and plant growth (Figs. 1 and 2A) as well as disease severity (data not shown) were not significantly affected by soil moisture in the treatment wRnT. A general agreement whether high or low soil moisture favours *R. solani* does not exist in the literature (Shehata et al., 1984; Teo et al., 1988; Van Bruggen et al., 1986). Contradictory results are often found because ambiguous terms have been used to quantify the soil water status (Ploetz and Mitchell, 1985). Moreover, the results may differ depending of *R. solani* anastomosis groups (Teo et al., 1988). Plant growth increased with higher soil moisture in the treatment wRwT (Fig. 2A). *Trichoderma* species are known to be more prevalent under high moisture conditions (Klein and Eveleigh, 1998).

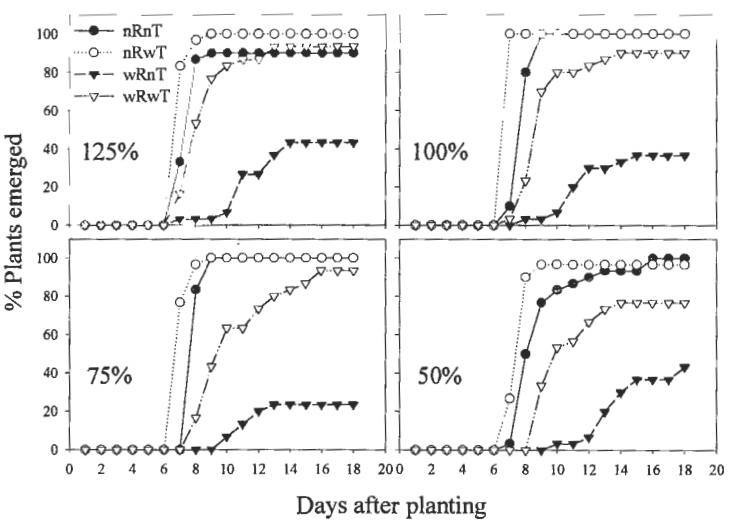

Figure 1. Effect of soil moisture on emergence of bean sown in a soil inoculated with *Rhizoctonia solani* and/or *Trichoderma harzianum*.

Deep planting significantly reduced the emergence rate and plant growth in the treatment wRnT, but not in the treatment wRwT (Figs. 2B and 3). Plant height and dry weight of aerial parts of plants in the treatment wRnT were higher at 1.5 cm and disease severity was lower compared to other planting depths (data not shown). Preemergence damping-off was most

severe at 6.0 cm (Fig. 3). At this planting depth, 50% of the plants emerged in the presence of *T. harzianum*, but only 6.7% in the absence of the antagonist. Deep planting extends the period of seedlings emergence which favours the seedlings contact with *R. solani* and increases the probability of damping-off and root rot (Leach & Garber, 1970).

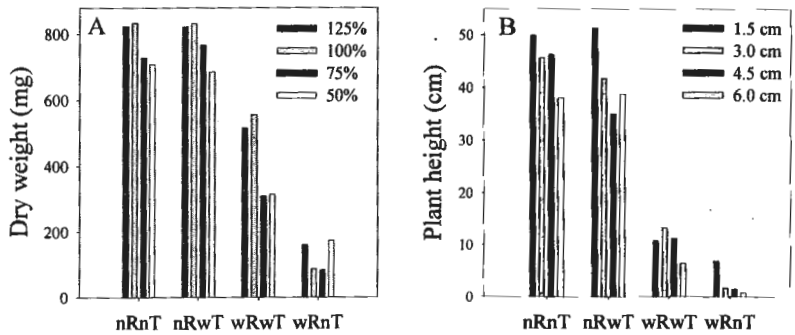

Figure 2. Effect of soil moisture on dry weight (A) and of planting depth on plant height (B).

Differences in emergence rate, plant height, and dry weight between the treatments wRnT and wRwT were dramatic (Figs. 1, 2 and 3). The antagonist protected bean seedlings from preemergence damping-off, reduced disease severity and increased emergence rate and plant growth in the presence of *R. solani*. These results are in accordance of those published by Elad *et al.* (1980), who reported that *T. harzianum* significantly reduced disease severity, delayed the progress and incidence of *R. solani* damping-off, increased the yield of beans under field conditions and reduced disease incidence in greenhouse experiments.

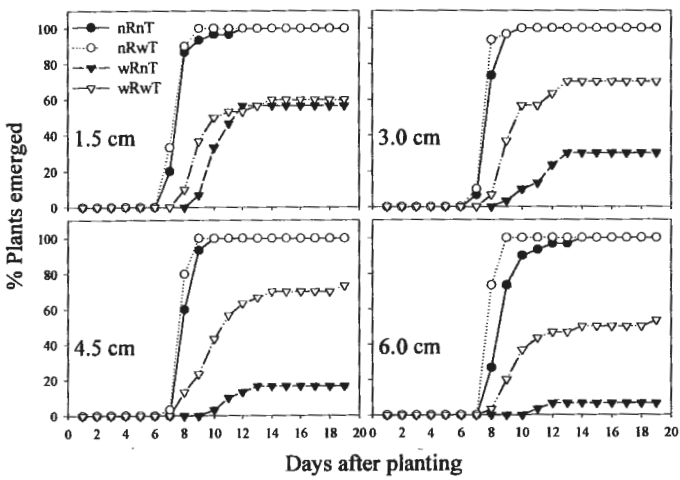

Figure 3. Effect of planting depth on emergence of bean sown in a soil inoculated with *Rhizoctonia solani* and/or *Trichoderma harzianum*.

Plants inoculated with *T. harzianum* alone emerged faster than those of nRnT treatment (Fig. 1).

The commercial use of antagonists to control *Rhizoctonia* root rot on beans in Brazil, where large areas are planted with this crop, could be feasible, for example, for fields with high inoculum potential. However, the erratic and inconsistent results in fields due to climatic and edaphic variables have limited the introduction and acceptance of commercial biocontrol agents (Lewis & Kulik, 1996). Moreover, *Trichoderma* species do not appear to be very competitive in non sterilized soil (Adams, 1990). For a successful application of these fungi, it is essential to use highly efficient isolates and an inoculum carrier that permits the antagonist to become established in the soil. The potential of *T. harzianum* was demonstrated as a component of an integrated disease management system for *Rhizoctonia* root rot of beans, which may also include shallow planting and the adjustment of soil water content to permit a rapid emergence of seedlings to favour *T. harzianum*.

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Biological Control of Fungal and Bacterial Plant Pathogens
IOBC wprs Bulletin Vol. 24 (3) 2001
pp.103 - 107

Biological control of *Phytophthora parasitica* in greenhouse tomatoes using *Trichoderma harzianum*

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Abstract: Tomato is the main vegetable crop in Chile, both in terms of acreage and farm-gate value. Among the diseases affecting this crop, stem canker caused by *Phytophthora parasitica* (PP), affects plants grown under monoculture conditions. New strategies are being developed to replace methyl bromide for control of soilborne pathogens, including biological control. The effectiveness of *Trichoderma harzianum* (TH) for biocontrol of PP was tested *in vivo*, using three TH strains that had been previously selected *in vitro* (TH11, THV and TH291), using the standard soil fumigation system (methyl bromide:chloropicrin, 98:2) as control treatments. Two inoculation techniques were tested for each TH strain: a) tomato transplants were inoculated with TH in styrofoam trays, using peat moss plus wheat bran with the inoculum in the pot mix; b) the soil was inoculated with pellets containing TH at transplanting. In both cases, soil inoculated with PP (IMI # 382780) was compared with fumigated and inoculated soil, fumigated but not inoculated soil and untreated inoculated soil. Plants were grown in pots under greenhouse conditions (12-30°C). Disease incidence was evaluated using a relative scale of 0-3 (no symptoms to plant mortality), plant height, stem diameter, root dry mass, and shoot dry mass. Methyl bromide had the highest control efficacy in PP-inoculated seedlings, followed by TH-protected plants, and both could be distinguished from the non-fumigated control, when comparing plant height, stem diameter and above-ground biomass. Inoculation with pellets of TH291 was as effective as methyl bromide, with similar levels of damage, plant height and stem diameter. These results show the potential of TH as a tool in stem canker management of greenhouse tomatoes.

Key words: *T. harzianum*, tomato, *P. parasitica*, stem canker, biological control

Introduction

Tomato (*Lycopersicon esculentum* Mill.) cultivation has intensified over the past few years, reaching a monoculture status, inducing the appearance of pests and diseases in the greenhouses. Among the soilborne pathogens, crown rot caused by *Phytophthora parasitica* has caused substantial economic damage. Control methods are based on selective fungicides and soil fumigation. Nevertheless, the search for an alternative to methyl bromide has lead to the use of a long-known biological control: *Trichoderma harzianum*. Several groups have validated its use as a control for soilborne diseases (Elad *et al.*, 1980; Harman, 2000). Similarly, a variety of soil inoculation techniques have been developed. This paper describes the *in vitro* selection of *Trichoderma* spp. isolates with high control efficacy of *P. parasitica*, and the subsequent evaluation under greenhouse condition, comparing two soil inoculation methods.

Materials and methods

Isolation and selection of strains

T. harzianum (TH), *T. polisporum* and *T. piluliferum* isolates used, were obtained from suppressive soils, and soils from tomato monoculture. Isolation was carried out from soil and fine roots, using PDA and MSF media (Nash & Snyder, 1962). *P. parasitica* (PP) isolates were obtained from diseased plants, purified and identified (IMI#382780). Three isolates were selected, and labeled as Poc 1, Poc 2 and Poc 3. In order to select TH having the best efficacy against PP, dual cultures were performed on PDA, plating PP against isolates of 14 *Trichoderma* spp.

***Seedling inoculation with* Trichoderma**

Seedling inoculation with TH was conducted according to Sivan *et al.* (1987), incorporating the fungus into potting mix as follows. Ten mycelial plugs (5 mm diameter) were taken from the margins of colonies actively growing on PDA plates, previously purified and identified, and placed in SM liquid medium. After 96 hr of growth at 25°C, propagules were homogenized, counted, and 200 ml were used to inoculate flasks containing 500 ml of a substrate mixture of wheat bran and peat moss (1:1) [which was autoclaved for 3 consecutive days (1 hr at 121°C)]. Three TH strains (TH-11, TH-V and TH-291) were used for inoculation purposes. The flasks were maintained in a growth chamber at 27° with 6,000 lux (12 h photoperiod) for 2 weeks. These cultures were incorporated into the seedling potting mix (10% v/v).

Preparation of* Trichoderma *pellets

Pellets were prepared using the methods described by Fravel *et al.* (1985), Montealegre & Larenas (1995). Twenty g of moist mycelium (developed on liquid medium for 15 days), plus 98 g of wheat flour, sodium alginate and 150 ml of distillated water (dH_2O), were added drop by drop onto 3% (w/v) $CaCl_2$, obtaining pellets that were subsequently dried at 30°C for 8 hr.

Treatments

The soil was inoculated with 3 PP isolates, adding 11.8 g of inoculum per kg of mix. The inoculum was grown on wheat seeds (which were previously soaked in dH_2O and autoclaved twice). Each gram of inoculum consisted of $0.5x10^5$ propagules of PP. Inoculated soil was kept under field conditions for 3 months. Treatments in the *in vivo* assays are listed in Tables 1 and 2. Three treatments were inoculated with TH in two ways: Experiment 1, 10% v/v in the potting mix; experiment 2, 4 g of pellets per plant. These treatments were compared to soil fumigated with methyl bromide plus chloropicrin (98:2) at a dose of 70 g/m^2, both inoculated and not incoculated with PP. Ten replicates were used, with one tomato plant per sample. The experiment was carried out in a completely randomized design, using ANOVA and comparing the means with Tukey's method ($P \leq 0.05$). Plants were grown in a greenhouse with temperatures fluctuating between 12 and 30°C.

PP symptoms were evaluated using a modified scale described by Blaker & Hewitt (1987), as follows: 0= symptomless plant, 1= 1-25% of tissue damage, 2=26-50% damage, and 3=51-100% damage or death of the plant. In addition, root dry weight, above-ground dry mass and stem diameter (below the first flower cluster) were recorded.

Results and discussion

The strains chosen for the experiment were TH 291 and TH V, both *T. harzianum* possessing the best growth inhibition percentage, and TH 11, due to good results shown recently in biocontrol of *Pyrenochaeta lycopersici* (Pardo, 1999).

Results are shown in Tables 1-2 and in Figs 1-2. Virtually no symptoms of disease were detected in methyl bromide alone or inoculated and fumigated treatments. Inoculation of the potting mix at the seedling stage with TH was not effective in controlling PP, when compared using the disease index. Nevertheless, plant growth was improved with the TH inoculation, without significant differences compared to the methyl bromide treatment. Plant height, stem diameter and above-ground dry mass were consistently lower in the PP treated plants, less than the fumigated and TH-inoculated plants. TH 291 applied as pellets was as effective as methyl bromide in preventing disease development (using the disease index). This effect was also detected when comparing plant height, stem diameter, root dry mass, and above-ground biomass, in some cases without statistically significant differences among TH strains. Other authors working with TH could not control *Phytophthora* spp. (Harman, 2000).

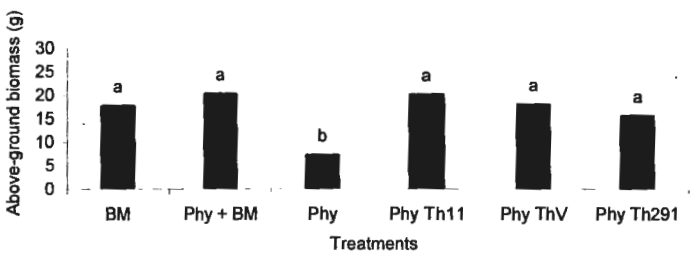

Figure 1. Plant growth measured as above-ground dry mass with *Trichoderma* incorporated into the potting mix.

Table 1. Results of inoculation of *Trichoderma harzianum* in the potting mix.

Treatment	Damage index	Plant height	Stem diameter	Root dry mass
Phy	2.4 b	60.88 c	0.48 b	1.23 b
Phy Th11	1.4 b	94.00 ab	1.12 a	2.21 ab
Phy ThV	1.8 b	98.33 a	1.04 a	2.17 ab
Phy Th291	2.3 b	84.00 ab	0.98 a	2.26 ab
BM	0.0 a	82.50 ab	1.06 a	2.39 ab
Phy + BM	0.0 a	81.30 b	1.21 a	3.04 a

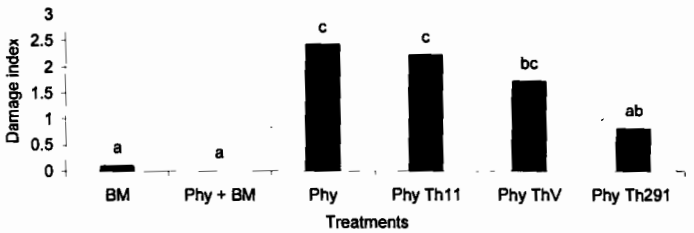

Figure 2. Effectiveness of biological control of *P. parasitica* with *Trichoderma* pellets.

Table 2. Results of inoculation of *Trichoderma harzianum* with pellets.

Treatment	Plant height(cm)	Stem diameter(cm)	Root dry mass(g)	Foliage dry mass (g)
Phy	60.88 c	0.48 b	1.23 c	7.18 c
Phy Th11	75.25 bc	0.45 b	3.42 abc	7.85 c
Phy ThV	85.64 b	0.72 ab	3.38 abc	12.10 bc
Phy Th291	101.63 ab	0.71 ab	2.94 bc	10.41 bc
BM	99.70 ab	0.81 ab	4.92 ab	15.80 ab
Phy + BM	107.3 a	0.98 a	6.05 a	20.53 a

It is important to mention that the PP inoculum was rather high compared with other experiments (Mitchell, 1978), much higher than that in naturally infested soils. Hence, *in situ* effectiveness could be increased. We are currently studying the effects of TH treatments in greenhouses and under field conditions.

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Biological Control of Fungal and Bacterial Plant Pathogens
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Selective isolation of antagonistic microorganisms to *Fusarium oxysporum* f.sp. *melonis*

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Abstract: A study of *Fusarium oxysporum* f.sp. *melonis* suppressiveness by antagonist bacteria and fungi isolated from soils, fresh vegetable material or compost samples is reported. Preliminary plate screening allowed the isolation of 25 bacteria and 44 fungi with suppressive activity against *F. oxysporum* f.sp. *melonis*. Dual liquid cultures of each selected strain with *F. oxysporum* f.sp. *melonis* were incubated and sampled every 8 days for propagule plate counts. A complete inhibition of the pathogen in dual cultures was obtained with 8 strains after 16-32 culture days. These strains were selected as potential *F. o.* f.sp. *melonis* biocontrol agents.

Key words: *Fusarium oxysporum* f.sp. *melonis*, antagonism, biocontrol

Introduction

Plant pathogen suppression using biological processes is considered a need of modern agriculture. In recent years the adverse environmental effects of hazardous pesticides, have promoted the development of environmental control alternatives, such as biological control (Cook & Baker, 1983). Mechanisms involved in biocontrol include parasitism or lysis (Elad & Chet, 1995), antibiosis (Baker & Griffin, 1995) and competition (Sivan & Chet, 1989) against other microorganisms.

During the last years, soil microorganisms have been tested for soilborne plant pathogen biocontrol. Several control strategies have been developed based on the introduction of these agents. However, such strategies have not been widely adopted because most of them do not attain a complete control of disease (Weller, 1988).

Recent advances in biological control of Fusarium wilt by suppressive soils have shown a low incidence of these diseases around the world (Alabouvette, 1989). Interesting results of many studies reflect a microbiological basis for suppression of Fusarium wilts in soils. Microbial interactions between the pathogen and the saprophytic microbiota (Scher & Baker, 1980) seem to be implied in this effect.

The aim of this study was to investigate the *in vitro* suppression of antagonist bacteria and fungi from several sources against *Fusarium oxysporum* f.sp. *melonis*.

Material and methods

Fusarium oxysporum f.sp. *melonis*

The pathogen used was a *F. o.* f.sp. *melonis* strain (*Fom*) isolated from a diseased melon plant (*Cucumis melo* L.), showing typical symptoms of Fusarium wilt, collected in June 1997 from a greenhouse located in Almería (Spain).

Antagonist isolation

Microorganisms were screened from several sources such as mature compost, soil and fresh plants of melon, tomato, pepper and cucumber. Bacteria were isolated on nutrient agar (NA) (Oxoid) and fungi on potato dextrose agar (PDA) (Rapilly, 1968). All plates were incubated at 30°C for 24-48 hours for bacterial growth and 4-5 days for fungal growth. Representative colonies growing on the plates were selected and isolated.

Preliminary test

Each bacterial isolate was cultured on nutrient broth for 48 hours at 30°C. Three drops of bacterial culture and a 6-mm plug from the leading edge of a 5-days-old culture of *Fom* on PDA were inoculated at opposite sides of a Petri dish. Inhibitory effects were confirmed by absence of contact between bacterium and *Fom* after 5 days of incubation. Plates without inoculated bacterium were used as controls.

Fungal isolates were incubated in Potato Dextrose Broth (PDB) for 7 days at 30°C. Cell-free cultures were obtained and concentrated by freeze-drying. A suppressive effect of these extracts towards *Fom* was demonstrated using the modified techniques of Landa *et al.* (1997) and Kirby *et al.* (1966), by measure of clear inhibition zones.

Dual cultures

Bacteria showing antagonistic activity from preliminary test were pre-cultured in nutrient broth and cultivated at 30°C and 120 rev min^{-1} during 48 hours. *Fom* was pre-cultured in PDB to reach a stationary culture (10^7 CFU ml^{-1}). Bacterial and *Fom* cultures were mixed (1:1 ratio) in nutrient broth and incubated at 30°C and 120 rev min^{-1}. *Fom* growth was analysed by plate counts after 4, 8, 16 and 32 days. *Fom* cultures were used as positive controls.

Spore suspensions of aproximately 10^6 fungal propagules ml^{-1} were prepared from 5-days-old slants of both *Fom* and fungi showing antagonist activity in the preliminary test. Each fungal spore suspension was mixed with *Fom* propagules (1:1 ratio). Media, incubation and sampling was performed as mentioned above.

Results and discussion

The preliminary screening revealed that 25 of 126 bacterial isolates (19.8%) were capable of interfering with *Fom* conidial germination and/or growth. Only 17 bacterial isolates sharply reduced pathogen growth after 5 days of incubation. With respect to fungal isolates, 44 of 123 isolated (35.7%) inhibited growth of *Fom*. Only 32 of them exhibited clear inhibition zones in plates.

Bacterial and fungal cultures that showed phytopathogen growth inhibition were screened for this activity in dual liquid cultures. This test showed that only 8 isolates (7 fungi and one bacterium) inhibited *Fom* growth after 16 or 32 days of incubation (Figures 1 and 2). Antagonistic strains used and their source are as follows: B17, H1, H6 (compost), M1.1 HRV100 (fresh melon residues), Si3H1 (greenhouse), Sj2H20, Sj5H17 (gardens) and Sa2H2 (dry soil).

In most cases, effective bacteria used as biocontrol agents of plant diseases belong to the genera *Bacillus*, *Pseudomonas* and *Streptomyces* (Edwards *et al.*, 1994). Landa *et al.* (1997) showed that approximately 32% of 74 bacterial isolates from the chickpea rhizosphere inhibited *in vitro* growth of *F. o.* f.sp. *ciceris* in dual cultures. In contrast, Myatt *et al.* (1992) obtained a lower proportion of antagonistic bacteria against *Phytophthora megasperma* Dreschsler f.sp. *medicaginis* from a similar environment. Furthermore, probably the degree of disease suppression changed with both the antagonist and the host as well as with the inoculum concentration of the pathogen (Landa *et al.*, 1997).

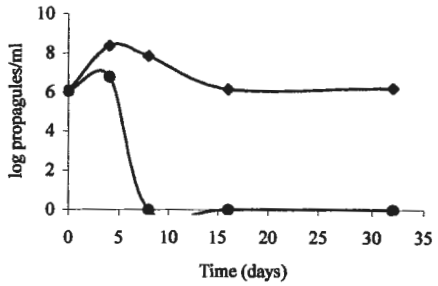

Figure 1. Effect of dual cultures (bacterium/fungus) on *Fusarium oxysporum* f.sp. *melonis* growth (control ◆, B17 ●).

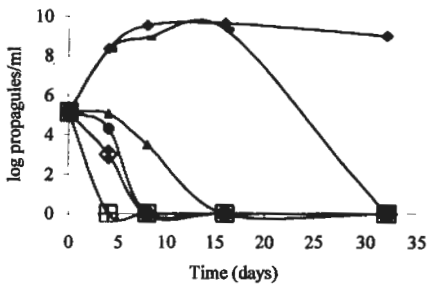

Figure 2. Effect of dual cultures (fungus/fungus) on *Fusarium oxysporum* f.sp. *melonis* growth (control ◆, Sa2H2 -, Si3H1 ▲, Sj5H17 □, H1 +, Sj2H20 ●, H6 ▽ and M1.1HRV100 *).*

Observations on the role of direct contact between organisms in biocontrol situations are relatively scarce. Backhouse and Stewart (1989) described a strain of *Paenibacillus polymyxa* that was weakly antagonistic to *Sclerotium cepivorum*, the causal agent of Allium white rot. Faull & Campbell (1979) studied the interaction between *Gaeumannomyces graminis* and *Bacillus mycoides* by means of electron microscopy and concluded that hyphae were lysed in the presence of bacteria. Polar attachment is also observed during the initial stages of lysis of cyanobacteria by a gliding myxobacterium (Daft and Stewart 1973).

Recently, it was shown that *Paenibacillus polymyxa* is able to antagonize *F. oxysporum* growth in a liquid medium by means of an interaction process in which the presence of the living bacterium is a prerequisite for continuous suppression of fungal growth (Dijksterhuis *et al.*, 1999).

Although antagonistic microorganisms from different sources only accounted for 3.2% of the total, these strains appeared to be effective for *Fom* biocontrol.

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Biological Control of Fungal and Bacterial Plant Pathogens
IOBC wprs Bulletin Vol. 24 (3) 2001
pp.113 - 116

Antagonistic effect of a *Trichoderma* formulation against *Sclerotinia sclerotiorum* in lettuce

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Abstract: Potential biocontrol strains of *Trichoderma* spp. were selected against a range of *Sclerotinia sclerotiorum* isolates pathogenic to lettuce. Field trials were carried out in different locations of the Valencia region (Eastern Spain), in farms with historical damping-off and drop of lettuce, across a wide range of cultural practices. The biocontrol agents were applied in a liquid formulation through the irrigation system. *Trichoderma* applications were also compared to other means of control and chemical fungicides. The statistical analysis of the results showed a good control of disease by the *Trichoderma* formulation designed in our laboratory.

Key words: *Trichoderma*, *Sclerotinia*, damping-off, lettuce, biocontrol

Introduction

The species of the genus *Trichoderma* are very effective biological control agents of soilborne fungi and *T. atroviride* has been demonstrated as a biological control species after its separation from the *T. harzianum* aggregate (Hermosa *et al.*, 2000).

Interactions between this aggregate of the species and *Sclerotinia sclerotiorum* are well known in lettuce (Wells *et al.*, 1971; Budge and Whipps, 1991; Inbar *et al.*, 1996). However other species such as *Coniothyrium minitans* and *T. virens* have also been used to control lettuce drop (Budge *et al.*, 1995).

In the present work, we have tried to demonstrate that *T. atroviride* can be an useful biological control agent of *Sclerotinia sclerotiorum* and *S. minor* in lettuce, across a wide range of cultural practices, being also compatible with other beneficial *Trichoderma* spp. which can be applied with this biofungicide in order to improve its efficacy.

Materials and methods

We have studied the ability of *Trichoderma atroviride* to control *Sclerotinia sclerotiorum* and *S. minor*, casual agents of lettuce drop. Field trials were carried out during spring and autumn cycles in 1998 and 1999, in two different locations of the Valencia region (Carcaixent and Benicarló), with historical *Sclerotinia* problems.

Carcaixent: Trial I, Trial II and Trial III

Three trials were performed: April 1998 (Trial I), October 1998 (Trial II) and April 1999 (Trial III), in a greenhouse naturally infested with *S. sclerotiorum*, under plastic covering. Experimental design: three plots with 100 plants (distributed in two rows of 50) per treatment. Two different types of lettuces were used: romaine (Inverna cultivar) and crisphead

(Trocadero cultivar). Biocontrol agent: *T. atroviride* supplemented with other *Trichoderma* spp. was the active agent of a formulation in which the final concentration was 10^8 conidia/ml. After corresponding dilution, this suspension was applied to lettuce at a concentration of $4x10^6$ conidia/ml. Application of the *Trichoderma* formulation was through the irrigation system at three different times: 15 days before transplanting, transplanting and 15-20 days after transplanting. Two different types of irrigation were assayed: furrow irrigation and drip irrigation.

Benicarló: Trial IV

This trial was carried out in April 1999 in natural environment in a soil infested with *S. sclerotiorum* and *S. minor* with furrow irrigation. Experimental design: four plots with 100 plants per each treatment; The lettuces used in this case were romaine type, Pisuerga cultivar. Biocontrol agent: *T. atroviride* suplemented with other *Trichoderma* spp. applied to the lettuce at a concentration of $4x10^6$ conidia/mL. Application of the *Trichoderma* formulation was through the irrigation system at three different times: transplanting, 15 days after transplanting and one month after transplanting; Chemical treatments: *Trichoderma* was compared with foliar applications of two chemical fungicides: Procimidona (0.1%) and Azoxystrobin (0.1%).

Results and conclusions

Trial I. Carcaixent. April 1998

This trial was sown in the spring of 1998 (Table 1). Statistically significant differences ($P \leq 0.05$) were detected between the lettuce cultivars used, romaine showing more resistance than crisphead to *Sclerotionia* disease. Contrary to Subbarao (1998), drip irrigation increased development of *Sclerotinia* disease compared to furrow irrigation. When *T. atroviride* and other accompanying *Trichoderma* spp. were applied, disease reduction of 47.5% was observed.

Table 1. Percentage of plant mortality of two lettuce cultivars under different types of irrigation with and without *Trichoderma* biocontrol treatment. Carcaixent, April 1998.

Cultivar, irrigation and treatment	Dead plants (%)
Inverna, drip, *Trichoderma*	1.00 a
Inverna, drip, control	1.66 a
Inverna, furrow, *Trichoderma*	0 a
Inverna, furrow, control	2.33 a
Trocadero, drip, *Trichoderma*	9.67 bc
Trocadero, drip, control	18.33 d
Trocadero, furrow, *Trichoderma*	7.33 b
Trocadero, furrow, control	12.00 c

Within each column, the numbers followed by the same letter are not significantly ($P \leq 0.05$) different according to the LSD test.

Trial II. Carcaixent. October 1998

This trial was sown in the autumn of 1998 (Table 2). No significant differences were found between different treatments (lettuce type, irrigation and biological control formulation), nevertheless, the incidence of lettuce drop was decreased after *Trichoderma* application.

Trial III. Carcaixent. April 1999

This trial was established in the spring of 1999 (Table 3). Significant reductions in disease incidence were obtained with *Trichoderma* applications. No significant differences were observed when different cultivars or irrigation systems were considered.

Table 2. Percentage of plant mortality of two lettuce cultivars under different types of irrigation with and without *Trichoderma* biocontrol treatment. Carcaixent, October 1998.

Cultivar, irrigation and treatment	Dead plants (%)
Inverna, drip, *Trichoderma*	0.33 a
Inverna, drip, control	0.67 a
Inverna, furrow, *Trichoderma*	0 a
Inverna, furrow, control	2.33 a
Trocadero, drip, *Trichoderma*	0.33 a
Trocadero, drip, control	2.00 a
Trocadero, furrow, *Trichoderma*	0.33 a
Trocadero, furrow, control	2.00 a

Numbers followed by the same letter are not significantly ($P \leq 0.05$) different according to the LSD test.

Table 3. Percentage of plant mortality of two lettuce cultivars, under different types of irrigation with and without *Trichoderma* biocontrol treatment. Carcaixent, April 1999.

Cultivar, irrigation and treatment	Dead plants (%)
Inverna, drip, *Trichoderma*	0a
Inverna, drip, control	0 a
Inverna, furrow, *Trichoderma*	0 a
Inverna, furrow, control	2 d
Trocadero, drip, *Trichoderma*	0.67 ac
Trocadero, drip, control	1.00 bc
Trocadero, furrow, *Trichoderma*	0 a
Trocadero, furrow, control	1.00 bc

Numbers followed by the same letter are not significantly ($P \leq 0.05$) different according to the LSD test.

Trial IV. Benicarló. April 1999

Since biological control was effective in the three previous trials, a comparative study between two chemical fungicides and *Trichoderma* was performed in Benicarló in April 1999 (Table 4). *Trichoderma* gave similar results to Azoxystrobin (without significant differences), but Procimidona was the best treatment. Nevertheless, it was the first time that this chemical was applied to this field and a rapid development of *Sclerotinia* resistance to this fungicide has been detected.

Table 4. Percentage of plant mortality after treatment with chemical fungicides and *Trichoderma*. Benicarló, April 1999.

Treatments	Dead plants (%)
Procimidona	0.25 a
Azoxystrobin	4.50 b
Trichoderma	6.00 b
Control	10.75 c

The numbers followed by the same letter are not significantly ($P \leq 0.05$) different according to the LSD test.

In the four trials carried out from Spring 1998 to Autumn 1999, the *Trichoderma* protected lettuce developed well compared to untreated controls. The *Sclerotinia* disease reduction ranged between 44.2 and 85.7%.

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Biological Control of Fungal and Bacterial Plant Pathogens
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Effects of antagonistic rhizobacteria on plant health, yield and the bacterial rhizosphere community of strawberry

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Abstract: The antagonistic *Pseudomonas fluorescens* strain DSMZ 12501 and the chitinolytic *Serratia plymuthica* strain DSMZ 12502 were evaluated for plant growth promotion of strawberries and biological control of Verticillium wilt. In two different vegetation periods, field trials were carried out in soils naturally infested by Verticillium in two different locations. In the field trials, the relative reduction of Verticillium wilt by the *Pseudomonas* treatment compared with the untreated control ranged from 15 to 59%, whereas the relative increase of yield ranged from 24 to 174%. The relative reduction of Verticillium wilt by the *Serratia* treatment compared with the untreated control ranged from 7.5 to 54%, whereas the relative increase of yield ranged from 62 to 296%. A synergistic effect was shown using a combination of both biological control agents. The results of the field trials were strongly influenced by the pathogen inoculum density, the cropping history of the field site, and the weather conditions. Additionally, the influence of different biological control agents on the bacterial communities of the strawberry rhizosphere was analyzed. Therefore, effects of introduced bacteria on the indigenous microbial community were monitored by analysis of PCR-amplified fragments of the 16S rRNA genes of the whole bacterial community after separation by denaturing gradient gel electrophoresis (DGGE). At any sampling time, the DGGE pattern of rhizosphere communities did not show differences between the different treatments and the nontreated strawberries. The strains introduced by root dipping did not become dominant members of the bacterial community. Nevertheless, they could be recovered from the rhizosphere at a level of 3-5 $\log_{10}$ CFU g^{-1} root fresh weight. Otherwise, there was no influence of the treatments on the whole bacterial community.

Introduction

Strawberry (*Fragaria x ananassa* Duch.) is an important crop with an increasing cultivation area worldwide (FAO, Statistical Databases). The target pathogen for antagonism studies reported here was *Verticillium dahliae* Kleb. The fungus can cause a dramatic loss of strawberry crop (Maas, 1998). Microsclerotia which develop in the senescing tissues of the diseased plant may persist in soil for several years in the absence of a susceptible host (Maas, 1998). In the coming years, the loss of methyl bromide as a control measure for *V. dahliae* will have a great impact on the accumulation of microsclerotia in soil. An interesting alternative to protect roots against fungal pathogens is rhizobacteria-based biological control (Whipps, 1997). Many biocontrol agents produce a long list of antibacterial and antifungal antibiotics (Thomashow *et al.*, 1997). A hypothetical risk has been raised that these organisms can cause changes in naturally occurring bacterial communities. There is currently much debate concerning risks of biological control and the potential harmful effects of non-indigenous species especially antibiotic producing organisms introduced for controlling diseases. However, due to this, methods for evaluating the potential environmental impacts of biocontrol need to be developed further through appropriate ecological research. In addition, knowledge about the ecological behaviour of the biocontrol agents can enhance and stabilize

the biocontrol effect (Whipps, 1997).

The objective of our study was to analyze the impact of the introduced biocontrol agents *Pseudomonas fluorescens* strain DSMZ 12501 (Pf-12501) and *Serratia plymuthica* strain DSMZ 12502 (Sp-12502) on (i) plant development and health in two different field trials naturally infested by *Verticillium* and, (ii) the indigenous rhizosphere bacteria by fingerprinting the bacterial communities using denaturing gradient gel electrophoresis (DGGE) of 16S rDNA amplicons.

Material and methods

Bacterial strains

Pseudomonas fluorescens Pf-12501 (= DZMZ 12501; Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany) was isolated from the rhizosphere of strawberry (Berg *et al.*, 2000). *Serratia plymuthica* DZMZ 12502 was isolated from the rhizosphere of oilseed rape (Kalbe *et al.*, 1996). Spontaneous mutants of the strains resistant to rifampicin *Rif* (100 μg ml^{-1}) were used. There were no differences in colony morphology, antifungal properties, and growth rate between the mutants and wild types. The strains were stored in nutrient broth containing 15% glycerol at -70°C.

Biological control of Verticillium wilt in field trials

Field trial I (1997, location Stuthof) and II (1999, location Rövershagen) were carried out in Germany (Mecklenburg-Western Pomerania) in areas of close proximity, both naturally infested by *V. dahliae*. Density of microsclerotia of *V. dahliae* in soil was determined according to the soil dilution method of Termorshuizen *et al.* (1998). In Stuthof, 40 microsclerotia g^{-1} soil on average, and in Rövershagen, 21 microsclerotia g^{-1} soil on average, could be determined. In trial I and II strawberry plants cv. Elsanta were grown in trials with ca. 2000 plants in complete block design (CBD) with six replicates and 1200 plants per treatment in randomized CBD with three replicates, respectively. The strawberries were planted as frigo-plants (in autumn harvested as runner plants, during winter stored at a temperature of -2°C) in May. Bacteria (4×10^9 CFU ml^{-1}) were diluted with standing, potable water (v/v) 1:1. The application was implemented via a 15-minute dipping bath treatment of the roots in the rhizobacteria suspension immediately prior to planting. As a control, potable water was used instead of bacterial suspension. In July of the same year, the fruits were harvested and their weight was measured. Additionally, percentages of wilted and dead plants were monitored. Root samples were collected and CFUs of spontaneous rifampicin resistant mutants on nutrient agar 2 containing 100 ppm rifampicin were determined.

Molecular analysis by DGGE

Fingerprinting of the bacterial rhizosphere communities by DGGE was carried out as described (Heuer & Smalla, 1997). Briefly, 16S rDNA fragments (positions 968 to 1401 [*Escherichia coli* rDNA sequence]) were amplified by PCR from DNA extracts with the primer pair F984GC-R1378. The amplicons were separated at 60°C in a denaturing gradient of 40% to 58% of 7 M urea and 40% (v/v) formamide. Acid silver staining was used for the detection of DNA in DGGE gels.

Statistical analysis

Statistical analyses were determined by U-Test "Mann-Whitney" ($P \leq 0.05$) by Statistical Product and Service Solutions for Windows, Rel. 9. 0. 1. (SSPS Inc., Chicago, Illinois).

Results

Two field trials at different locations were carried out to evaluate the effects of *P. fluorescens*

Pf-12501 and *S. plymuthica* Sp-12502 under natural conditions. In field trial I, significant (P = 0.004) reductions in infection were observed in plots treated with Pf-12501 (59%) and Sp-12502 (54%). In field trial II, the relative reduction of the pathogen by the bacterial *Pseudomonas* treatment ranged from 1.3 to 17.9%, with an average of 15%. The relative reduction of Verticillium wilt by the *Serratia* treatment increased percentage of healthy plants (7.5%). The treatment with both rhizobacterial strains showed synergistic effects and enhanced the percentage of healthy plants to 42.5%.

There was a significant increase in the percentage of marketable fruit (P = 0.011) trial I. Pf-12501 and Sp-12502 resulted in 74% and 196% higher yield, respectively. These results were confirmed in trial II. The *Pseudomonas* and the *Serratia* resulted in a 24% and 62% higher yield, respectively. Although lower increases, the treatment with both rhizobacteria showed a synergistic effect - 184% higher than the nontreated control.

Both rhizobacterial strains were able to colonize the strawberry rhizosphere under field conditions. At three different times during the vegetation period, abundance of spontaneous rifampicin resistant mutants *Rif*in the rhizosphere of treated strawberry plants were determined. The introduced *P. fluorescens* strain 12501 colonized the roots of strawberry resulting in population densities between 4.8 and 4.1 (trial I) and 6.0 and 5.3 (trial II) $\log_{10}$ CFU root fw g^{-1}. The *S. plymuthica* strain 12502 was able to colonize the roots of strawberries resulting in population densities between 6.9 and 3.7 (trial I) and 5.7 and 3.3 (trial II) $\log_{10}$ CFU root fw g^{-1}. In both field trials I and II, populations of both strains decreased at each sampling period. The strains could be identified using the API and BIOLOG systems. In both field trials I and II, the total average numbers of aerobic bacteria that could be recovered from the rhizosphere on nutrient agar showed a nonsignificant difference between the nontreated control and the plots treated with rhizobacterial strains.

In 1999, total DNA was extracted from all rhizosphere samples. A fragment of the 16S rRNA gene was amplified by PCR and separated in DGGE according to sequence-dependent melting differences. For each sampling, the DGGE fingerprints were compared between inoculated and uninoculated roots. Pf-12501 and Sp-12502 were not detectable in any DGGE fingerprint. No differences became apparent between DGGE fingerprints of inoculated and uninoculated roots, both showing the same evident seasonal community shifts.

Discussion

In our study, a general correlation between the yield and biocontrol activity was found. In both field trials disease incidence was high and and the biocontrol effect caused by *P. fluorescens* Pf-12501 and *S. plymuthica* Sp-12502 was significant. In general, the incidence of Verticillium wilt is influenced by the initial inoculum density of microsclerotia, the cropping history of the field site, the abiotic conditions such as weather conditions and stress, soil parameters and other pathogens such as nematode infestation (Harris & Young 1994). In our study, different contents of microsclerotia in the different soils of the field trials were established. In field trial I the potential of infectious microsclerotia was much higher than in field trial II. An influence of weather conditions on the experiments was also shown. The infection data of field trial I reflects the fact that warm, dry summers are known to favour wilt in strawberry. Soil parameters in the both trials were similar (sand, good nutrient substance).

A very important requirement for an efficient biological control agent (BCA) is the competence to survive and to establish in the rhizosphere (Lugtenberg & Dekkers, 1999). The rhizosphere competence of both strains was shown by reisolation of the spontaneous rifampicin resistant mutant from the rhizosphere. Structure of the bacterial rhizospheric community as revealed by molecular fingerprinting (DGGE) was not significantly changed in

inoculated plants. Due to target competition in PCR, the relative abundance of a 16S rRNA gene must exceed 1% of the total targets to give a clear band in the DGGE fingerprint (Heuer & Smalla, 1997). Therefore, the strain disappeared from the fingerprints, in accordance with the selective plate counts. For the same reason, only prominent populations could be monitored for inoculation effects. On the basis of the presented results it was possible to patent the biocontrol candidates *P. fluorescens* Pf-12501 and *S. plymuthica* Sp-12502 (Berg *et al.*, 1999). The development of a commercial formulation of DSMZ 12502 is in progress, and in the near future RhizoStar® will be commercially available.

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Biological Control of Fungal and Bacterial Plant Pathogens
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Efficient biocontrol of fungal plant diseases by Rhizovit® on the basis of *Streptomyces* sp. DSMZ 12424

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Abstract: The actinomycete *Streptomyces* sp. DSMZ 12424 (HRO71) was originally isolated from the rhizosphere of strawberry and shown to be an effective antagonist *in vitro* toward several plant pathogenic fungi. When *Streptomyces* spores were used to coat sugar beet seeds, the emergence rate of treated plants control was enhanced in soil infested by *Rhizoctonia solani* and *Pythium ultimum* in comparison to the untreated. The bacterium was able to suppress plant pathogenic fungi in different pathosystems: (1) sugar beet - *Rhizoctonia solani*, (2) lambs lettuce - *Phoma valerianellae* and, (3) cress – *Alternaria* spp. DSMZ 12424 was added using seed coating (1) and seed infiltration techniques (2, 3). The biocontol agent was formulated as water dispersible granules. The spores are conserved in the granules and they can be stored at least one year without significant loss of activity. Finally, on the basis of *Streptomyces* spb. DSMZ 12424, a microbial product was developed and named Rhizovit®.

Introduction

Biological control, using microorganisms to suppress plant diseases, offers a powerful and environmentally friendly alternative to the use of pesticides (Emmert & Handelsman, 1999). One problem is the difficulty to formulate rhizobacteria, especially Gram-negative bacteria as products, without losing antifungal activity. Gram-positive bacteria, such as species belonging to *Bacillus* or *Streptomyces*, produce spores, which can be formulated readily into stable products (Emmert & Handelsman, 1999). Unfortunatly, only a few biocontrol products on the basis of Gram-positive bacteria are currently on the market. In this study we investigated (i) the effect on four fungal pathogens *(Alternaria* spp., *Phoma valerianellae, Pythium ultimum, Rhizoctonia solani)* in three different pathosystems in greenhouse trials, and (ii) the formulation of the plant-associated strain *Streptomyces* sp. DSMZ 12424.

Material and methods

Biocontrol agent

The antagonistic *Streptomyces* species (HRO71 = DSMZ 12424; deposited at the Deutsche Sammlung für Mikroorganismen und Zellkulturen GmbH [Braunschweig, Germany]) was isolated from the rhizosphere of strawberry (Berg *et al.* 2000). Characterisation of *Streptomyces* species was performed using the 41 morphological and physiological diagnostic characters contained in the probabilistic identification matrix of Williams *et al.* (1983). The strain was identified as *Streptomyces rimosus* (identification probability of 95%). Additionally, the complete 16S rDNA was sequenced and aligned with the reference 16S rRNA gene sequence using BLAST algorithm (Altschul *et al.*, 1997). According to the sequence, it was identified as *S. kasugaensis* (97%).

Biocontrol experiments

Two different bioassays were used to evaluate the efficacy of *Streptomyces* sp. DSMZ 12424. Bioassay 1: Soil samples were infested with *Rhizoctonia solani* or *Pythium ultimum* (6 g kg^{-1} soil dried sclerotia of *R. solani* or 20 g kg^{-1} dried oogonia and mycelia of *P. ultimum* grown for 4 weeks in Erlenmeyer flasks on rye grains). Seeds of sugar beet (SUET, Eschwege, Germany) were treated with a mixture of hydroxyethylcellulose (1.5%) and NaCl (0.85%) and bacterial suspension (48 h-culture, nutrient broth II [Sifin]) at a concentration of 5 x 10^{10} ml^{-1}. For 500 seeds, 2 ml of this mixture were used. After this process, the seeds were treated with talcum powder and dried. For the control pots, seeds were treated with sterilized tap water. Multipot-polypropylene boxes containing 96 places were filled with 20 ml per place infested potting substrate (Einheitserde 00, Balster Einheitserdenwerk, Fröndenberg, Germany) and planted 1 seed per pot, 2 cm deep. The experiments were conducted under greenhouse conditions at a temperature of 20°C with daily irrigation and 14 h day^{-1} natural light. Two weeks after planting, the disease incidence was determined.

Bioassay 2: 50 g of lambs lettuce seed (JULIWA Julius Wagner GmbH, Heidelberg, Germany) naturally infested by *Phoma valerianellae* and seed of cress naturally infested by *Alternaria* spp. was treated with a suspension of spores and mycelium of *Streptomyces* sp. DSMZ 12424 (72 h-culture, nutrient broth II [Sifin]) after centrifugation at 150 min^{-1} and resuspension in sterile NaCl-solution (0.85%). Five g of seed were soaked in 10 ml of a spore suspension at a concentration of 5 x 10^8 CFU ml^{-1} and allowed to swell for about 4 h at 20°C to infiltrate bacteria into the seed. After infiltration, the seed were separated from the suspension and dried in a laminar flow cabinet for 2-3 h. Control seed were treated with sterile tap water. Four x fifty seeds were planted into Einheitserde 00 (Balster Einheitserdenwerk) in polypropylene pots and maintained under conditions at a temperature of 20°C with daily irrigation and 14 h day^{-1} natural light in a greenhouse. Four weeks after emergence, the number of plants infected by *P. valerianellae* and *Alternaria* spp. were monitored. All the experiments were repeated twice.

Formulation of the product

Oat-extract-broth (self made: 100 g l^{-1} oat, cooked 5 min in 1 l distilled water, filtered, 8 g l^{-1} NaCl, 2 g l^{-1} $MgSO_4$, distilled water added 1 l, pH 6.5) 30 l, pH 6.5 was used for the cultivation of antagonistic *Streptomyces* strain in a 50-litre-submerged-fermenter. After 72 h fermentation at a temperature of 26°C with 150 l h^{-1} ventilation and stirring at 500 g min^{-1} the biomass was separated from culture broth by centrifugation (CEPA, Lahr, Germany, 10.000 g min^{-1}, 10 min, 15°C). The spore-mycelium-pellet was resuspended in 3 l sterile NaCl-solution (0.85%) homogeneously amended with 1.5% hydroxyethylcellulose and than mixed using Ultra-Turax (IKA, Staufen, Germany). During this process most of the *Streptomyces* mycelium was destroyed. The spore suspension was dried for 20 min in a fluidized bed dryer (MLW, Halle, Germany) at 28°C using crystalline glucose as carrier. The final product was a water dispersable granule with a dry matter content of 93%. At a concentration of 3 x 10^{10} spores g^{-1} content of *Streptomyces* sp. DSMZ 12424 in the formulation was verified. At a temperature of 4°C, the granules were stored. Once per month, a 1 g sample of from the granular formulation was taken and diluted in series in 9 ml sterile NaCl-solution (0.85%) and plated on nutrient agar II (Sifin). The viability of the formulated antagonist was detected by counting colony forming units (CFU). Antifungal activity of the bacteria was evaluated in dual culture assays.

Statistics

Differences between the treatments in the biocontrol experiments were determined by a two-sided test of binomial proportion or the Student's t-Test ($P \leq 0.05$; $P \leq 0.1$) by Statistical Product and Service Solutions for Windows, Rel. 8. 0. 0. (SSPS Inc., Chicago, Illinois).

Results

Greenhouse trials were carried out to determine the potential of *Streptomyces* sp. DSMZ 12424 to suppress diseases in soils artificially inoculated by *P. ultimum, R. solani, P. valerianellae,* and *Alternaria* spp. Additionally, the strain was evaluated for plant growth promotion and resulting yield enhancement in different pathosystems. The first trials were carried out with test plants and pathogens that could be used easily and rapidly for testing purposes, such as *P. ultimum* and *R. solani* on sugar beet. In the combined results from two independent experiments, there was a statistically significant enhancement ($P \leq 0.05$) of 17.8% on average (40.4% in maximum) of the emergence rate of sugar beet using soils artificially infested by *R. solani* for seeds coated with *Streptomyces* compared to the untreated control. Damping off caused by *P. ultimum* was also significantly reduced up to 47.3% when seeds were coated with the *Streptomyces* antagonist.

In greenhouse trials, the bacterial treatment reduced the relative percentage of different pathogens. In pathosystem sugar beet –*R. solani*, the relative reduction of the pathogen by the bacterial treatment compared with the untreated control ranged from 0 to 37.7%, with an average of 9.2%. In the pathosystem lettuce - *P. valerianellae*-, statistically significant (P=0.004) reductions in infection were observed in plots treated with strain DSMZ 12424 compared with untreated control plots. The greatest differences in constitution and health between the control and the plots treated with DSMZ 12424 of 61% on average could be monitored in the pathosystem cress – *Alternaria* spp. The effect of *Streptomyces* sp. DSMZ 12424 to suppress *Alternaria* spp. was statistically significant at $P \leq 0.05$ in greenhouse trials.

Furthermore, a formulation for the *Streptomyces* isolate was developed. Therefore, the microbial biomass was produced by fluid fermentation. After cultivation, the suspension was centrifuged and mixed with formulation agents before drying. The end product contained granulated spores and mycelium of the microorganism. To evaluate the formulation, survival of *Streptomyces* sp. DSMZ 12424 in the formulation was determined. The viability of spores in the granules when stored at 4°C was determined at given intervals over a period of a year. The initial density of spores in the well-mixed formulation was 3.3×10^{10} CFU g^{-1}. After one month of storage at 4°C, the density of spores in the formulation had declined to 2.7×10^{10} CFU g^{-1}. Over a period of one year, the viability was maintained at a level of 2.5×10^{10} CFU g^{-1}. After formulation, the activity of bacteria was checked in dual culture assay *in vitro*. No loss of antifungal activity was observed because the same inhibition zones were measured than before the formulation.

Discussion

The present investigation explores the potential of microbial antagonism of *Streptomyces* sp. DSMZ 12424 to control some fungal pathogens in greenhouse studies and additionally, the formulation of the agent. The success of biocontrol approaches to control plant diseases must be judged by their performance under natural conditions. Although it has been argued, that there is no general relationship between *in vitro* antagonism of a biocontrol candidate and disease suppression *in vivo*, potential biocontrol *Streptomyces* species seems to be exceptions to this rule (Turhan, 1980; Jones & Samac, 1996). In this study, *Streptomyces* sp. DSMZ 12424 selected because of its strong *in vitro* antifungal activity was evaluated as an effective biocontrol agent. The ability of *Streptomyces* sp. DSMZ 12424 to suppress pathogenic fungi was demonstrated in different systems with statistically significant effects. The efficiency of biological control and plant growth promotion varied in different pathosystems. The plant species or cultivar and especially the composition of root exudates play a key role in the

species or cultivar and especially the composition of root exudates play a key role in the diversity of rhizobacteria populations colonizing the roots perhaps the reason for differential rhizosphere competence.

For the biological control experiments an effective granule formulation was developed. In conclusion, on the basis of the presented results it was possible to patent the biocontrol candidate (Berg & Lüth, 1999). The results described here are very promising for commercial application of *Streptomyces* sp. DSMZ 12424, and in the near future Rhizovit® will be commercial available from PROPHYTA GmbH (Malchow/Poel, Germany).

Acknowledgements

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Biological Control of Fungal and Bacterial Plant Pathogens
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Biological control of tomato bacterial wilt caused by *Ralstonia solanacearum* in a rockwool hydroponic system employing *Pseudomonas fluorescens* B16

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Abstract: Biocontrol agent, *Pseudomonas fluorescens* B16, readily colonized tomato root and rock wool medium. B16 population in tomato root and rockwool was sustained above 10^6 cfu/g root from seeding till 75 days and effectively suppressed *R. solonacearum* in the same niche. Although B16 grew much faster in complete media, the antibacterial substance was produced only in minimal media. An antibiosis defective mutant 1-36 was obtained through Tn-*5* mutagenesis. Production of an antibacterial substance is the principal factor for control of the bacterial wilt of tomato by B16. Root dipping of tomato seedlings in B16 cell suspension (10^9 cfu/ml) at transplanting was sufficient to suppress tomato bacterial wilt till fruit bearing. The attributes of pruduction of an antibacterial substance in minimal conditions and colonization of plant roots and rockwool by *P. fluorescens* B16, indicates that this microorganism is a candidate biocontrol agent against bacterial wilt of tomato in rockwool hydroponic culture.

Key words: tomato bacterial wilt, *P. fluorescens* B16, rockwool, minimal condition

Introduction

Bacterial wilt caused by *Ralstonia solanacearum* is commonly encountered, and often causes serious damage to solanaceous crops such as hot pepper, tomato and eggplant in Korea, especially in soilless culture systems. *Pseudomonas fluorescens* B16 has been developed as a promising biocontrol agent because of its antibacterial activity, enhancement of plant growth and colonization ability in many plant roots (Park *et al.*, 2000). It was known that the bacterium produced antibacterial substance only in minimal conditions (Kim *et al.*, 2000). The nutritional component of root surroundings in hydroponic culture is almost the same as the minimal medium except that some organic substances are derived from plant roots. In the current study, we tried to evaluate the biocontrol activity of B16 against bacterial wilt of tomato in a hydroponic system and to elucidate the role of antibiotic production.

Materials and methods

Bacterial strains and culture conditions

P. fluorescens B16 was originally isolated and developed for biocontrol agent in our laboratory. B16 and its mutants were grown overnight at 28°C in liquid Luria Broth (LB) medium under vigorous aeration, or on solidified LB plates for routine use. When appropriate, the media were supplemented with rifampicin at final concentrations of 50 μg/ml. All strains were stored in 50% glycerol at -80 °C. *E. coli* strains, which were used for mutagenesis of *P. fluorescens* B16 were grown overnight in liquid or solidified LB.

Isolation of an antibiosis defective mutant

Mutants of *P. fluorescens* B16 were generated by random transposon mutagenesis described by Fellay *et al.*, (Fellay *et al.*, 1989). *E. coli* strain S17-1 used, carries pJFF350, the source of Omegon-Km, a vector plasmid that harbors the Tn*5* transposon. The vector behaved as a suicide plasmid in *P. fluorescens* B16 after mating. Among the 5,000 conjugants, an antibiosis defective mutant was selected in minimal medium exposed to an overlay of *R. solanacearum*.

Evaluation of disease suppression

Biocontrol agents were introduced to tomato plant by seed or root dip treatments. For seed treatment, tomato seeds (*Licopersicon esculentum* Mill. cv. 'Seokwang') were soaked in the cell suspension of B16 or mutants (10^9 cells/ml). For root dip treatments, the roots of tomato seedling at 4-5 leaf stage were dipped in bacterial suspensions of B16 or mutants then transplanted to rock wool cubes (10 ×10 ×7cm). Seven days after transplant, 100ml of *R. solanacearum* cell suspension (10^6 cells/ml) was added to each rockwool cube. The plants were kept in a greenhouse and plants were examined for disease up to 20 days after inoculation.

Analysis of population changes of B16 and R. solanacearum

The population changes of B16, and its mutants, as well as *R. solanacearum* in tomato root and rock wool pieces were analyzed. One gram of tomato root or rockwool was sampled, ground, diluted and colony forming units (cfu) of each bacteria was enumerated. King's B medium supplemented with 100ppm rifampicin was used for enumerating B16 and mutants, while tetrazolium chloride medium was utilized for the pathogen.

Results and discussion

Among the 5,000 conjugants, we selected two mutants related to production of antibacterial substance. Mutant 2-31 showed much enhanced antibiotic activity to many plant pathogenic bacteria, while mutant 1-36 lost its antibiosis. The production of antibacterial substance was achieved only in minimal media (Figure 1).

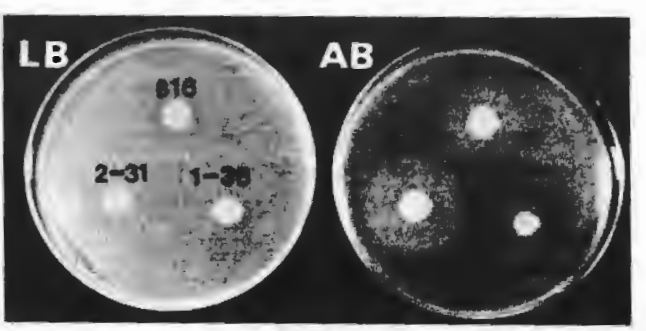

Figure 1. Antibacterial activities of *P. fluorescens* B16(wild type), mutant 2-31 and 1-36 against *R. solanacearum* in LB media and minimal media (AB).

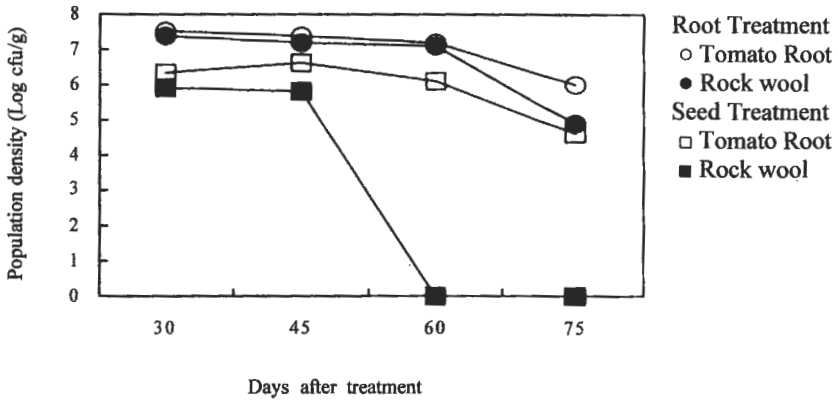

Figure 2. Population changes of *P. fluorescens* B16 in tomato root and rockwool when the bacteria were introduced by means of seed treatment or root dip treatment.

Basically, B16 readily colonized tomato roots and rockwool media in the hydroponic system. Seed and root inoculated bacteria moved to newly emerging roots and surrounding rockwool and multiplied. However, sustainability of B16 in tomato root and rockwool differed greatly with methods of inoculation. Bacterial populations were sustained above 10^6 cfu/g root introduced by the root dip treatment up to 75 days after seeding, while that of the seed treatment did not reach 10^5 cfu/g. The difference between the two treatments was more pronounced in rockwool. Root-treated bacterial populations in rockwool was calculated at 10^5 cfu/g up to 75 days after inoculation while that of seed treatment decreased sharply from 45 days to negligible amounts after 60 days (Figure 2). The population density of B16 over than is important to control the bacterial wilt of tomato. The population densities, 10^6 cfu/g, of *R. solnacearum* in tomato root and rockwool were definitely influenced by population densities of B16 in the same niche. Disease was not suppressed when the population density of B16 remained below 10^6 cfu/g.

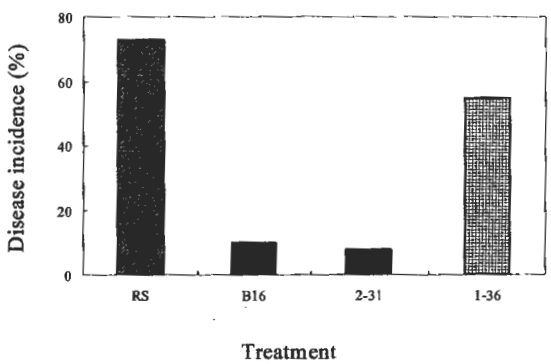

Figure 3. Suppression of tomato bacterial wilt by biocontrol agent B16, mutant 2-31 and 1-36 in rockwool culture after artificial inoculation by *R. solanacearum*.

Wild type isolate B16 and antibiosis-enhanced mutant 2-31 successfully suppressed bacterial wilt of tomato, however, antibiosis defective mutant 1-36 failed to control the disease (Figure 3). Mutant 1-36 exhibited almost the same abilities as B16 or 2-31 besides the production of the antibacterial substance. This is an indication that production of the antibiotic substance is responsible for disease suppression by biocontrol agent B16. However, disease control efficiency of antibiosis-enhanced mutant 2-31 was not significantly different than B16.

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Comparison of strains K84 and the GEM K1026 in biological control of crown gall caused by *Agrobacterium* spp.

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Crown gall disease caused by several *Agrobacterium* species is distributed worldwide and is responsible for nursery and field losses among a large variety of plants (fruit trees, nut trees, caneberries, grapevine and some ornamentals).

Biological control of crown gall has been successful in fruit trees, nut trees, caneberries and ornamentals in different countries using the non-pathogenic strain K84 of *Agrobacterium*. This biocontrol agent has been used on a commercial scale for almost 30 years all over the world. In spite of its demonstrated efficiency, there are some potential problems which could be associated with its application. The majority have not been explained, but the most important risk of failure when using strain K84 is the possibility of transferring the plasmid pAgK84 to pathogenic *Agrobacterium* strains. pAgK84 encodes production of and immunity to the anti-agrobacterial antibiotic agrocin 84, the main factor involved in crown gall biocontrol by strain K84. After this plasmid transfer, which occurs in nature, the pathogenic recipient becomes resistant and a producer of agrocin 84 and these transconjugants then threaten the biocontrol of crown gall using strain K84. A second generation of strain K84 has been obtained and the genetically engineered strain was called K1026. It contains a deletion in the transfer region of pAgK84 and keeps the genomic background intact as well as the rest of the plasmid content. Strain K1026 has been registered as a biopesticide in Australia and USA. The commercial product is sold as NOGALL®.

We have performed a considerable number of studies to compare both strains for control of crown gall, plasmid transfer, antibiotic production, root colonisation and survival in the rhizosphere. The results of biocontrol experiments in inoculated substrates and in naturally infected soils demonstrated that strain K1026 is as efficient as K84 in controlling crown gall in different hosts and countries. Comparative studies of plasmid transfer did not detect the transfer of pAgK1026 to pathogenic recipients. The abilities to produce inhibitory substances, root colonisation and survival in the rhizosphere were similar for both strains.

In conclusion, strain K1026 should be used wherever strain K84 is employed to safeguard biological control of crown gall due to equal efficacy compared to its parent strain K84 under different conditions, hosts and countries, and moreover, because its utilisation should not represent a risk either for living organisms nor for the environment.

Suppression of wheat seedling blight caused by *Fusarium culmorum* and *Microdochium nivale* using seed-applied bacteria

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A number of soil micro-organisms have been reported as efficient biocontrol agents against seedborne diseases in different crops, but for diseases in cereals caused by *Fusarium* spp. and relatives, there are few reported organisms showing high level of efficacy.

In a greenhouse bioassay, designed to enhance disease development, about 600 newly isolated plant-associated bacteria were screened for suppressive effects against seedborne *Fusarium culmorum* infecting wheat. The bacteria were applied to artificially infested seed as a cell-suspension, dried and sown in nonsterile soil in pots.

Most of the bacteria tested had no effect on test plants or disease development, a few were synergistic to the pathogen, and about one fifth of the isolates shoved 80% disease suppression, or more, under greenhouse conditions. During three growing seasons, 30 isolates selected in the bioassay were tested in field experiments against both *F. culmorum* and *Microdochium nivale*. Some of these showed disease suppression comparable to the fungicidal control, and this effect was consistent over the seasons. The levels of disease suppression obtained in the greenhouse and in the field was well correlated ($r^2 = 0.72$).

We conclude that bacterial isolates able to effectively suppress seedling blight caused by *F. culmorum* and *M. nivale* may be selected by simple screening procedures.

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Biological Control of Fungal and Bacterial Plant Pathogens
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Incidence and biocontrol of some mycotoxins in South Africa

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Abstract: Mycotoxins are fungal toxins, which have recently become recognized as serious predisposing factors in many human and animal diseases. They are usually found in contaminated grains and cereals. Many compounds are labeled "mycotoxins", but there are only five agriculturally important fungal toxins: deoxynivalenol, zearalenone, ochratoxin A, fumonisin and aflatoxin. The problem varies by region, with fumonisins and aflatoxins being serious problems in South Africa. In tropical regions, high humidity and improper storage practices pose additional problems. This is further exacerbated in that maize is the staple diet of the majority of populations in rural areas where storage conditions are primitive, and resources scarce, predisposing to ingestion of mycotoxin containing crops. This paper reviews the occurrence of these toxins in imported grains viz., wheat, rice, beans, nuts, maize, rye, pea and lentil, in local maize cultivars, and in fruit juices. The impact of reducing these toxins with certain food processing methods such as thermal processing and fruit juice clarification methods, as well as biocontrol using natural plant compounds from indigenous South African plants and spice oils was studied. A survey of grains imported to S. Africa show that wheat, maize and rice have aflatoxins and zearalenone in concentrations ranging from 1- 60 ug/g. All the local maize cultivars have Fusaria, and they all have either fumonisin or zearalenone. A survey of locally produced apple juice (143 samples) for patulin indicate that although 24% had patulin, only 5% of these were above the legally acceptable level. The levels of these mycotoxins can be reduced by processing techniques such as apple juice clarification, use of preservatives such as acetic and propionic acid, and by incorporating certain spice oils in food, such as clove oil. Some of the above methods are effective in controlling both fumonisin and aflatoxins (two mycotoxins which co-occur in many grains). A novel approach, looking at plant compounds to control these toxins, showed that flavanoids from *Virgilia oroboides* and chlorophorin from *Chlorophora excelsa*, and also some drimane-type sesquiterpenoids from the tree *Warburgia Salutaris* have potential and offer safe strategies for reducing toxin levels.

Introduction

Current control measures are aimed at controlling fungal growth and subsequent mycotoxin formation in stored grains by physical methods, viz., aeration, cooling, modified atmosphere (Paster *et al.*, 1988) or by chemical methods, viz., ammoniation, acids and bases, and food preservatives. These methods require sophisticated equipment and expensive chemicals or reagents. Moreck *et al.* (1980) evaluated effect of the food preservative sodium bisulphite on naturally contaminated maize. An initial concentration of 200 ppb of AFB_1 was degraded by over 90% by 2% bisulphite. It has not yet been demonstrated that aflatoxin contaminated commodities treated with bisulphite are biologically safe and retain their nutritional and functional properties. All the above treatments are not feasible when one considers the rural subsistence farmers since they require expensive chemicals, technology and technical expertise to monitor temperature, pressure, etc.

There are also no effective control measures to control fumonisins. Ammoniation has been successfully used to decontaminate aflatoxin-contaminated foodstuffs (Brekke *et al.*,

1977). With this treatment, however, although the fumonisin content was reduced by 90%, the effective loss was found to be <30% when tested for toxicity in rats (Voss *et al.*, 1989). Similarly, alkali treatment of maize with $Ca(OH)_2$ was found to convert FB_1 to its hydrolysed aminopentol form, but this compound had the same carcinogenic potential as fumonisin B_1 (Hendrich *et al.*, 1993). The only biological treatment evaluated for the control of fumonisins has been in beer which underwent fermentation. Even these had to be further detoxified before they could be used as animal feeds (Bothast *et al.*, 1992). The results of various physical methods of controlling fumonisins have also been investigated. Sydenham *et al.* (1994) reported that physically removing the fines or screenings from bulk shipments of maize reduced the fumonisin content by 26-29%. Bennett & Richard (1995) showed that starch prepared by wet milling of maize reduced fumonisin content, however, most of the fumonisins were subsequently found in the gluten and fibre fractions. This toxin poses a serious threat since heat treatments are ineffective as temperatures of 62^0C for 30 min are inadequate, and temperatures as high as 190-220^0C for 25 min are required to reduce the toxin by 75%. The only successful report on the control of fumonisins is a report by Scott & Lawrence (1994) that indicates that steeping maize for 6 h in 2% SO_2 was an effective treatment for decreasing fumonisin concentrations in solution.

Materials and methods

Sources of samples

A total of 101 grains comprising 60 wheat, 11 rice, 10 bean, 9 nuts, 5 maize, 2 rye grain, 2 pea and 2 lentil samples were examined. Seven agricultural pet foods and the raw ingredients, and 139 fruit juices were surveyed for mycotoxins. A 50 g portion of the samples were immediately analysed and the remaining samples were stored at room temperature.

Mycotoxin extraction analysis

Aflatoxin and zearalenone were extracted from grain samples according to the method of Howell & Taylor (1981), fumonisins according to method of Sydenham *et al.* (1991) and patulin according to Zubrick (1998). The mycotoxins were analysed by high performance liquid chromatography (HPLC) using a Merck-Hitachi (LaChromm) system. Aflatoxins were analysed according to Takahashi & Beebe (1979); zearalenone and fumonisins according to Thiel *et al.* (1993) and paulin by the method of Forbito & Babsky (1985). Mycotoxin standards were obtained from the Sigma Aldrich Chemical Company.

Control strategies

Thermal processing, preservatives, spice oils and indigenous plant compounds were investigated for their potential as control strategies for processed foods and foods in storage. Fruit juice clarification was investigated for controlling patulin levels in apple juices.

SMKY broth cultures of *Aspergillus parasiticus* (10^6 spores/ml) and maize patty cultures of *Fusarium moniliforme* (10^6 spores/ml) (Alberts *et al.*, 1993) were used to test effect of thermal processing, preservative, spice oils and plant compounds on aflatoxin and FB_1 production.

Thermal processing was investigated by heating samples at temperatures ranging from 100 to 200°C at different time intervals. The effect of preservatives was tested with sodium metabisulfite, sodium benzoate, propionic acid and acetic acid at concentrations ranging from 0,1% to 3%. Clove, cinnamon, oregano, tumeric, eucalyptus, neem, aniseed, mace and nutmeg were tested for their effect against *A. parasiticus* and *F. moniliforme* and clove oil was further analysed.

Plant compounds from the barks of *Chlorophoro excelsa, Bridelia micrantha, Wabargia salutaris*, *Lippiya javanica* and *Scenecio serratuloide*, and phytoalexins induced in fruits of

lemons (*Citrus limon* cv. Eureka) and oranges (*Citrus sinesis* cv. Valencia) by fungal infection using *Penicillium digitatum* were chemically characterised by HPLC, UV spectroscopy, infrared and ^{1}H NMR spectroscopy.

For the effect of fruit juice clarification apple pulp was spiked with patulin, pressed and clarified using four different processes, namely, fining with bentonite, enzyme (pectinase) treatment, paper filtration and centrifugation. Patulin was recovered from the clarified juice by liquid-liquid extraction and solid phase chromatography was used for sample clean-up prior to analysis by HPLC.

Results

Mycotoxin levels in South African commodities

Mycotoxin levels in South African commodities show that 17.8% of grains surveyed are contaminated. Of these 9.9% have zearalenone, 6.9% have aflatoxins and none have ochratoxins. These toxins were present in wheat, maize, rye and pea. Rice, beans, nuts and lentils were toxin-free. Although mycotoxins were present in these grains, their levels were low and ranged from 1 to 60 µg/ml. Pet and agricultural feeds were all contaminated with low levels of aflatoxins ranging from 0.2 to 39 µg/g, zearalenone was found in three samples at levels of 50 to 3300 µg/kg. Fumonisins were found in six samples at levels of 5.9 to 497 µg/kg. Patulin was found in 21% of all juices tested and these were above legally acceptable levels.

Thermal processing

Processing techniques such as heating are frequently used and this study shows that temperatures of 220°C (30 min) are needed to destroy fumonisins completely and 200°C (5 min) to eliminate 62% of the aflatoxins. These temperatures are all above normal cooking temperatures.

Preservatives

Sodium metabisulphite at 0.4% and propionic acid at 0.3% can eliminate fumonisins. Aflatoxins require a lower concentration of 0.2% and 0.1%, respectively. Propionic acid and acetic acid are less effective.

Spice Oils

Natural compounds such as clove oil are organoleptically acceptable and can be added to foods at low concentration (0.01 ppm). These compounds can be added to foods at either the processing or storing stages.

Indigenous plant compounds

Plant compounds offer a safe strategy to control mycotoxins. A phytoalexin, 6.7 dimethoxycoumarin from citrus fruit showed activity against *F. moniliforme*. Other compounds which showed activity were constitutive compounds which included furans from the bark of *W. salutaris* and chlorophorin and Iroko from *V. oroboides*

Discussion

Four identified toxins that impact health and trade are aflatoxin, fumonisin, patulin and ochratoxin. One of the serious problems of these toxins is that they co-occur in food commodities. In our endeavours to control these toxins, we have found that some food processing techniques such as thermal treatment and fruit juice clarification can be used to reduce or eliminate toxins from foods. In foods that are likely to be affected during storage or transportation, preventative measures are necessary. In this regard, we have chemically characterised some compounds from indigenous plants, phytoalexins and spices that show

potential to reduce fungal contamination and toxin production. An advantage of these methods is that natural compounds are safe and offer simple strategies for third world countries enabling safer storage practices to eliminate/reduce toxin containing foods.

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Biological Control of Fungal and Bacterial Plant Pathogens
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Biocontrol potential of *Bacillus* antagonists selected for their different modes of action against *Botrytis cinerea*

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Abstract: A screening programme is described for the assessment of potential biocontrol agents to control grey mould of tomatoes caused by *Botrytis cinerea*. Isolations were obtained from stems, leaves, fruits and seeds of tomato and Chinese cabbage plants using a variety of methods to distinguish between endophytes and epiphytes. 108 morphologically distinct isolates were tested and of these 52 belonged to the genus *Bacillus*. A range of bioassays was used to screen the *Bacillus* isolates for *in vitro* antagonism against *B. cinerea*. Those that showed strong activity *in vitro* against one or more developmental stages of *Botrytis* were further tested for their biocontrol potential *in planta* using detached tomato leaves. Further selection was made by testing the best antagonistic isolates on whole tomato plants. Three isolates were finally selected. One of these isolates produced diffusible antifungal compounds and a biosurfactant, which showed very good inhibition of *B. cinerea* conidial germination and disease suppression, presumably operating through antibiosis. The other two selected isolates (one endophyte and one epiphyte) did not produce diffusible antifungal compounds but still affected conidial germination of *B. cinerea* and exhibited improved disease suppression *in planta*. The mode of action of the latter may be via large molecular weight non-diffusible antifungal substances, competition for nutrients and/or space or other mechanisms. The three isolates were assessed in a glasshouse trial for their ability to control grey mould in tomatoes.

Key words: biological control, *Bacillus* antagonists, *Botrytis cinerea*, grey mould, tomato

Introduction

Grey mould of tomatoes is caused by the fungus *Botrytis cinerea* Pers., and is managed mainly by fungicides. Public concern with fungicide residues on fruits and the development of fungicide resistance in pathogen populations has increased the need to find alternative methods for managing grey mould. Markellou (1999) successfully reduced grey mould disease by this approach using *Bacillus* antagonists. In this study, potential *Bacillus* antagonists to *B. cinerea* were isolated from different tissues of tomato and Chinese cabbage and were tested for suppression of grey mould *in vitro*, *in planta* and in glasshouse tomato.

Material and methods

Isolation of Bacillus *spp. from tomato and Chinese cabbage tissues*

Seeds, stems, leaves, fruits and flowers of tomatoes (*Lycopersicum esculentum* cv. Moneymaker) as well as seeds and leaves of Chinese cabbage (*Brassica campestris* spp. *pekinensis* cv. Yamico) were used. Washing, sonication and stomacher homogenisation of plant material in Ringer's solution, with and without prior sterilisation, was used in order to isolate organisms with different levels of association/attachment to the plant (epiphytes, strongly attached epiphytes and endophytes).

In vitro* and *in planta* antagonism to *B. cinerea

Dual culture technique (Whipps *et al.*, 1987) of potential antagonists against *B. cinerea* was carried out on Potato Dextrose Agar (PDA, Oxoid). The aim of this assay was to study the effect of the bacterial isolates on mycelial development and growth of *B. cinerea.* Any inhibition of fungal growth by the isolates was measured visually. A seeded conidial plate assay (Edwards, 1993) was also used to test inhibition of conidial germination and/or inhibition of mycelium growth by isolates streaked onto the surface of the PDA plate. A comparison of the results by this method with those of the dual culture technique allowed confirmation of the mode of inhibition (germination, mycelial growth or both). All bacterial isolates were also tested using the filter membrane bioassay (Walker *et al,* 1998) to confirm whether the mode of *in vitro* antagonism shown by the microbial isolates was via diffusible antifungal metabolites and to provide further information regarding the details of antagonism. Inhibition of conidial germination of the pathogen by the bacterial isolates was tested on glass slides (Walker *et al.*, 1996). Using these methods, the modes of action of the biological control agents may be determined. All *Bacillus* isolates that showed good antagonistic activity against *Botrytis cinerea in vitro* were tested on detached tomato leaves (Elad *et al.*, 1994) for their efficacy to suppress disease. The isolates that exhibited the best results were then tested for disease suppression on whole tomato plants.

Field trial

Three *Bacillus* isolates that exhibited the best antagonistic activities were tested in a glasshouse trial. The bacteria were grown in Tryptic Soya Broth (TSB) medium for 14 d (150 rpm at 30 ° C) to ensure production of mature spores and antifungal metabolites (if any). At the end of the incubation period this culture preparation was used directly for mass application of the biocontrol agent in the glasshouse trial. The control treatments included a water control and sterile quarter strength TSB (¼ TSB). All the treatments were applied at 7-day intervals. Disease incidence was assessed every 7 days by determining percentage of infected leaves per plant, length of infected stems, number of infected flowers and fruits as well as number of fruits showing ghost spots.

Results and Discussion

Isolation of* Bacillus *spp. from tomato and Chinese cabbage tissues

Using the various isolation procedures described, 108 different bacterial isolates were selected on the basis of colony colour, size and morphology. Fifty-eight of these isolates were selected from Chinese cabbage and 50 from tomato plants. The principle adopted in the methods was that the surface washing and sonication procedures isolated epiphytes, whereas the maceration procedure isolated endophytes. As expected the highest number of isolates were epiphytes (72%) in both Chinese cabbage and tomato plants and only 28% were endophytes. Overall 48% (52) of the isolates were identified as *Bacillus* spp. The high number of *Bacillus* isolates suggests that these are the predominant bacteria in the tissues of tomato and Chinese cabbage plants that were sampled.

In vitro* antagonism to *B. cinerea

a) Dual culture technique. *Bacillus* spp. isolated from different tomato and Chinese cabbage tissues were all tested in dual culture plates. Fifty-one percent of the bacteria showed clear inhibition zones against *B. cinerea*, 38% inhibited the pathogen when the two organisms came into direct contact, 2% inhibited only the sporulation of *Botrytis* and 9% showed no effect on *Botrytis* development. The fact that 53% of the isolates clearly inhibited mycelial growth is typical of the genus *Bacillus* that is recognised for its ability to produce water-soluble antifungal metabolites. The bacteria that inhibited *Botrytis* when the two organisms came into

contact suggests that these isolates may produce insoluble antifungal metabolites or that the mode of action is nutrient competition and/or competition for space.

b) Seeded plates. Sixty-seven percent of the isolates showed clear inhibition zones against *Botrytis*, 18% inhibited the pathogen when the two organisms came into contact, 7% inhibited only the sporulation of *Botrytis* and 7% showed no effect on *Botrytis* development. The results of this bioassay were very similar to the that of the dual culture technique.

c) Percentage conidial germination on glass slides. All the bacteria were tested on glass slides for their efficacy to inhibit *B. cinerea* conidia germination. Results obtained generally showed little or no inhibition. Only one isolate completely inhibited conidial germination and two others by 40 and 51 % respectively. This confirms the results of tests a & b above where the majority of the isolates inhibited mycelial development.

d) Filter membrane assay. Ten bacteria exhibited clear inhibition zones, with two having some effect on conidial germination. The production of inhibition zones indicates the production of metabolites with antifungal activity against the development and growth of *B. cinerea* and suggests that the mode of action of these isolates is antibiosis. Although these antifungal metabolites were not further characterised it is known that *Bacillus* isolates produce a range of antibiotics including subtilin, iturins, gramicidin s, bacitracins and others.

In planta *antagonism to* B. cinerea

a) Detached tomato leaf bioassay. Forty bacterial isolates that showed good antagonistic activity against two or more *in vitro* tests were tested for disease suppression on detached tomato leaves. Eighteen showed good antagonistic properties when tested as both cells and spores. The results were generally poorer when spores were used. The production of antifungal compounds was one possible cause for high efficacy of *Bacillus* strains when they were screened *in planta* in the form of cells. This would indicate some slowing down in growth rate to produce antifungal metabolites that are known to be produced under these conditions. Another possible mode of antagonism could be competition for nutrients since cells are metabolically active and *B. cinerea* requires sugars and amino-acids for germination.

b) Whole tomato plant bioassay. Three bacterial isolates suppressed *Botrytis* disease when sprayed on whole tomato plants in a controlled environment. The isolates reduced percentage leaf infection by more that 80 %. The *Bacillus* strains showed high effectiveness in disease suppression *in planta* on detached tomato leaves and on whole plants. The *in planta* production of antifungal compounds was one possible cause for this. Competition for space on leaves between *Bacillus* antagonists and *Botrytis* could be an additional or synergistic mode of antifungal action of the bacteria in addition to their products. Some isolates caused the spreading of the droplets on the leaf surface suggesting production of biosurfactants that affect surface tension. Reports are available on biosurfactant production of some *Bacillus* species, such as *B. subtilis*, *B. licheniformis* and *B. brevis*. Biosurfactant production reduces periods of surface wetness and has an indirect effect on germination by limiting water availability to the pathogen. The combined use of a *Bacillus* strain that produces a biosurfactant together with a strain that produces an antibiotic compound would allow the antagonism from these two modes of action to be combined in different environmental and crop situations. This could result in more effective control of *B. cinerea* and other fungal plant diseases that in general are water dependent. The best results in all four tests were observed using three isolates. One of these was characterised by the complete inhibition of conidial germination, the production of antifungal compound(s) (antibiosis) and probably biosurfactants, the disease suppression on detached tomato leaves and on whole tomato plants. The other two (one endophyte and one epiphyte) did not produce diffusible antifungal compounds but still affected *B. cinerea* conidial germination and suppressed disease.

Glasshouse trial

The three bacteria (A, B and C) that were selected from all the *in vitro* and *in planta* tests were tested in a glasshouse trial. All of them inhibited natural *Botrytis* infection to some extent but isolate C showed the best results in inhibiting leaf and fruit infection (Table 1, Fig 1). All the treatments (except isolate B) were significantly different ($P \leq 0.05$) to the water control (WC) in disease suppression. The ¼ TSB control showed the best results in suppressing leaf infection. This might be explained by enhancement of indigenous micro-flora that were antagonistic to *Botrytis* (production of antifungal compounds, antagonism for space/nutrients). Leaf samples taken from all the treatments showed that plants sprayed with TSB were higher in numbers and diversity of microorganisms than all other treatments (data not shown). This is interesting and suggests that nutrient supplementation of natural antagonists on the leaf may play a role in suppressing disease. It is obvious that more detailed research is needed in this area.

Table 1. Percentage tomato leaf infection and number of infected fruits by *B. cinerea* in different treatments and blocks in a glasshouse trial.

	Leaf infection (%)					Number of infected fruits				
	A	B	C	¼TSB	WC	A	B	C	¼TSB	WC
BLOCK 1	32.5	26	23.5	21.1	52.3	2	2	1	5	108
BLOCK 2	37.5	50.4	49	28	41.8	7	6	1	22	44
BLOCK 3	30	48.5	26.5	19	48.6	2	13	0	16	80
BLOCK 4	32.5	50.5	27	23.5	56.5	3	5	2	9	133
Mean	33.12	43.85[+]	31.5	22.9	49.8	3.5	6.5	1	13	91.25
St. Error	1.57	5.96	5.88	1.93	3.11	1.19	2.32	0.40	3.76	19.11

[+] no significant difference from water control (WC) ($P \leq 0.05$)

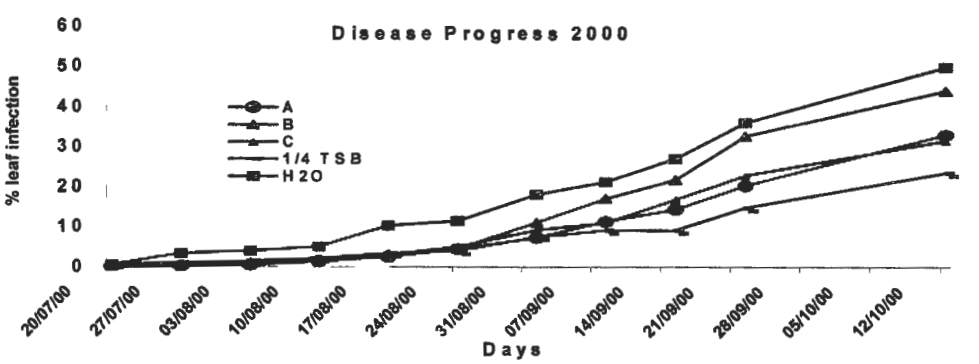

Figure 1. Disease progress and the effect of different treatments in *B. cinerea* infection.

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Biological Control of Fungal and Bacterial Plant Pathogens
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Biological control of cucurbit powdery mildew by mycoparasitic fungi

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Abstract: *Sphaerotheca fusca* (formerly *S. fuliginea*) is the causal agent of cucurbit powdery mildew in Southern Spain. In this work we have studied the biological control abilities of two mycoparasitic fungi, *Acremonium alternatum* and *Verticillium lecanii*, against cucurbit powdery mildew by *in vitro* assays on detached melon leaves. Our results suggest that both mycoparasites, when applied in early curative treatments, are interesting candidates for biological control of melon powdery mildew.

Key words: *Acremonium alternatum*, *Cucumis melo*, *Sphaerotheca fusca*, *Verticillium lecanii*

Introduction

Cultivation of cucurbits, mainly melon, is an economically important activity in Southern Spain. Powdery mildew is a devastating disease of cucurbits worldwide. Disease symptoms are characterized by the appearance of spots or patches of a white powdery fungal growth that develops on both leaf surfaces and other organs (Zitter *et al.*, 1996). In Spain, powdery mildew of cucurbits is caused by *Sphaerotheca fusca* (formerly *S. fuliginea*) (Torés *et al.*, 1990). Control of this disease lies in the use of resistant cultivars and the consecutive applications of different fungicides (Zitter *et al.*, 1996). Several races of the pathogen have been described (Pitrat & Dogimont, 1998) causing difficulties in developing absolutely resistant varieties of melon, and therefore, leaving melon production highly dependent on chemical control. Biological control of powdery mildew in melon has been poorly investigated. In this paper we show the results of *in vitro* assays using detached melon leaves to evaluate the biological control abilities of two mycoparasitic fungi, *Acremonium alternatum* and *Verticillium lecanii*, which have been previously described as hyperparasites for Erysiphales species included *S. fusca*, against cucurbits powdery mildew (Bélanger *et al.*, 1998).

Material and methods

Fungal strains

An isolate of race 1 of *S. fusca*, obtained from a powdery mildew-infected melon plant in Torrox (Málaga, Spain) and named SF26, was used in this study. The isolate was maintained *in vitro* on cotyledons of melon cv. Iran-H (Bertrand, 1988). *A. alternatum* was routinely grown on PDA plates at 25°C for 8 days (Malathrakis, 1985). *V. lecanii* was used as the commercial product Mycotal (Koppert Biological System) (Verhaar *et al.*, 1998).

Biological control tests

Melon plants of cv. Rochet were used. Prior to inoculation, second and third true leaves of 3-week-old melon plants were detached and maintained *in vitro* with their petioles immersed in 50% Hoagland's solution according to the double Petri plate system (Quinn & Powell, 1982).

Inoculum of the pathogen was preparing by rinsing powdery mildew infected melon cotyledons with water. The conidial suspension was adjusted to 10^5spores/ml with distilled water and leaves were then inoculated by spreading the suspension on the upper surface. Detached leaves were incubated at 25°C under a 16h photoperiod. Three or six days after infection, the biological control agents were applied on powdery mildew-inoculated detached leaves as a conidial suspension adjusted to $5X10^6$spores/ml. In both cases, leaves were examined for disease symptoms 12 days after infection. Symptoms were macroscopically recorded according to scales of 0 to 5, from no symptoms to abundant. Experiments were performed at least twice.

Fluorescence microscopy

Fungal development (*S. fusca* penetration sites) on the leaf surface was visualized by the calcofluor staining technique. Leaf discs were sampled, cleared in boiling ethanol for 10 min and rinsed in tap water. Discs were stained with 0.01% aniline blue in 7mM K_2PO_4 for 24 h, mounted on glass slides with 0.1% fluorescence brightener 28 (Sigma), and observed by fluorescence microscopy (Cohen & Eyal, 1988).

Light microscopy

Fungal development (*S. fusca* conidiophores, collapsed conidiophores and parasitized mycelium) on the leaf surface was also visualized by the cotton blue staining technique. Decoloured discs were stained in lactoethanol (20% lactic acid, 40% glycerol and 40% ethanol) containing 0.1% cotton blue for 30 min at room temperature. After destaining in lactoethanol, discs were mounted on glass slides and observed by light microscopy.

Results and discussion

To determine the biological control abilities of *A. alternatum* and *V. lecanii* against cucurbits powdey mildew, we used an *in vitro* assay with detached melon leaves. By way of this assay, abundant and reproducible powdery mildew symptoms were observed on the upper surface of melon leaves, inoculated with conidiospores of *S. fusca* after 12 days of incubation at 25°C. Once the *in vitro* pathogenicity assay was set up, the mycoparasitic fungi *A. alternatum* and *V. lecanii* were applied to powdery mildew inoculated melon leaves in curative treatments at 3 or 6 days post-pathogen inoculation (dpi). In table 1 results of biological control tests are shown. Pathogen development was microscopically recorded by two parameters, number of penetration sites (indirect determination of the number of haustoria) and number of conidiophores per mm^2. Best control results were obtained with a 3dpi application of *V. lecanii*, which reduced the number of penetration sites to 38% and the number of conidiophores to 42%, 8 days post-biological agent application (dpb), reducing disease symptoms considerably (disease scale 1). In contrast, a 6 dpi application of *A. alternatum* only reduced conidiophore formation to 75% 8 dpb, leading to only a slight decrease of powdery mildew symptoms (disease scale 4). Disease symptoms were reduced to 50% (disease scale 3) with 3dpi and 6dpi applications of *A. alternatum* and *V. lecanii*, respectively, with reductions of approximately 50% in number of penetration sites and conidiophores, 8 dpb.

Direct interactions between the pathogen and biological control agents are shown in figures 1 and 2, where percentages of *S. fusca* parasitized mycelia and collapsed conidiophores, were determined, respectively. In relation to parasitized mycelia (Fig. 1), 3dpi or 6dpi applications of *V. lecanii* and 3dpi application of *A. alternatum* showed that at least

Table 1. *S. fusca* development and powdery mildew disease symptoms following curative treatments, 3 or 6 days after inoculation (dpi) with *A. alternatum* and *V. lecanii*. Fungal development was recorded as number of penetration sites and conidiophores per mm^2, 4 and 8 days post-application (dpb). Percentages of both structures related to *S. fusca* untreated control are shown in brackets.

Biological agent application	Penetration sites 4 dpb	8 dpb	Conidiophores 4 dpb	8 dpb	Disease symptoms 12 dpi
3 dpi					
Untreated	128 (100)	204 (100)	10 (100)	31 (100)	5
A. alternatum	94 (73)	120 (59)	0 (0)	12 (39)	3
V. lecanii	55 (42)	79 (38)	0 (0)	13 (42)	1
6 dpi					
Untreated	281 (100)	312 (100)	70 (100)	81 (100)	5
A. alternatum	190 (67)	133 (43)	73 (100)	61 (75)	4
V. lecanii	278 (98)	180 (57)	55 (42)	38 (46)	3

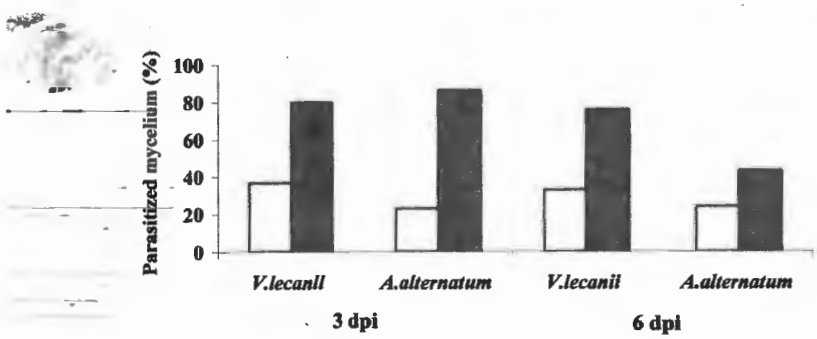

Figure 1. Percentages of *S. fusca* parasitized mycelia following curative treatments with *A. alternatum* and *V. lecanii*. Values were determined 4 (□) and 8 (■) days post-application of the biological agent. Mean values of two independient experiments are shown.

75% of *S. fusca* mycelia was parasitized, 8 days post-application. In contrast, with a 6dpi application of *A. alternatum* only 40% of the mycelia was parasitized 8dpb. Regarding conidiophore collapse (Fig. 2), 3dpi or 6dpi applications of *V. lecanii* and 3dpi application of *A. alternatum* caused collapse of at least 60% of the conidiophores of *S. fusca*, 12 days post-application. In contrast, with a 6dpi application of *A. alternatum* only 25% of conidiophores had collapsed by 12dpb. These results suggest that both mycoparasites, when applied in early curative treatments, are interesting candidates for biological control of melon powdery mildew. Greenhouse assays will reveal whether or not these hyperparasites can be included in integrated control programs for powdery mildew of melon in Southern Spain.

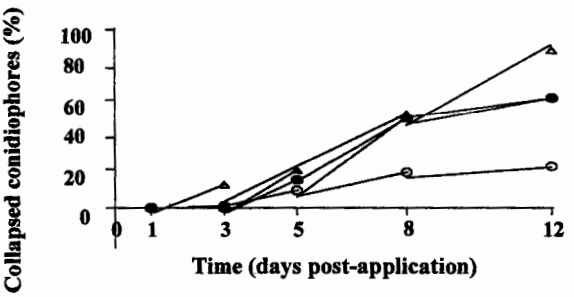

Figure 2. Time course of percentages of *S. fusca* collapsed conidiophores following curative treatments with *A. alternatum* and *V. lecanii*. Symbols denote applications of *A. alternatum* 3 dpi (●) or 6 dpi (○), and *V. lecanii* 3 dpi (▲) or 6 dpi (Δ). Mean values of two independent experiments are shown.

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Biological Control of Fungal and Bacterial Plant Pathogens
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Trichoderma spp. for biocontrol of *Colletotrichum acutatum* and *Botrytis cinerea* in strawberry

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Abstract: Isolates of *Trichoderma* spp. originated from different organs of non-infected or *C. acutatum*- and *B. cinerea*-infected strawberry plants, from soil in infected plots, and from some other plants. *T. harzianum* isolate T-39, which is the active ingredient of the biocontrol preparation TRICHODEX, was also included in this study. Seventy-four isolates of *Trichoderma* were assessed *in vitro* in dual culture with C. *acutatum* (5 isolates) and *B. cinerea* (3 isolates), respective anthracnose and gray mold pathogens of strawberry. Antagonism, involving hyphal interactions, inhibition zones due to production of antibiotics or cases of no interaction were recorded and visually rated on a scale of 0 to100. *In planta* tests were based on a bioassay using detached strawberry "petiole segments" and leaves. The petioles and leaves were inoculated at different time periods with the 74 *Trichoderma* isolates prior to pathogen inoculation. Eleven isolates from those mentioned above, as well as TRICHODEX, were selected for intact plant tests based on reduced lesions on petioles and leaves, observed in the detached bioassays. Plants were sprayed with the *Trichoderma* isolates (10^6 conidia/ml) or TRICHODEX (0.2%/ml = 10^6 conidia /ml) prior to inoculations with *C. acutatum* (10^5 conidia/ml) or mycelial plugs of *B. cinerea*. Three isolates (T-39, T-105 and T-118) and TRICHODEX controlled both diseases, two isolates (T-115 and T-166) controlled *C. acutatum* and two isolates (T-161 and T-165) controlled *B. cinerea*. The more effective isolates are finally being tested in mini-plot field experiments. In summary, *in vitro* culture tests, *in planta* detached plant organ tests and intact plant experiments were used for screening the biocontrol potential of selected *Trichoderma* isolates. Effective biocontrol agents were found for disease reduction of anthracnose and gray mold in strawberry.

Keywords: anthracnose, *Botrytis, Colletotrichum*, strawberry, *Trichoderma*

Introduction

Anthracnose and grey mould caused by *Colletotrichum acutatum* Simmonds and *Botrytis cinerea* Pers.;Fr., respectively, are two of the major fungal diseases of strawberry occurring worldwide. *C. acutatum* causes black spot on fruit, root necrosis and crown rot resulting in mortality of transplants in the field, and in addition, lesions are formed on petioles and stolons thus, limiting production of strawberry plants in the nurseries (Freeman & Katan, 1997). *B. cinerea* causes serious yield loss of fruit both in the field and after harvest, and can also attack flowers when conditions are favorable.

Selecting effective biocontrol agents for use under field conditions from isolates with no biocontrol potential is commonly based on tests such as antagonism under *in vitro* conditions; enzymatic, antibiotic or toxic activity; suppression of the target pathogen on detached plant material or biocontrol of a plant disease on whole plants exposed to the screened microorganism and to the causal pathogen. The selection of potential biocontrol agents under controlled conditions should rely on a bioassay system specifically tailored to the nature of

the target pathosystem.

Successful biocontrol agents are usually selected from a large number of isolates after a careful screening process. Variability among isolates makes it difficult to establish criteria according to which an antagonist is regarded a competent biocontrol agent. A-biotic conditions such as temperatures, vapor pressure deficit, condensation, chemicals, gases and fertilizers under which potential biocontrol agents are screened and tested affect the activity of the biocontrol agents. Moreover, biotic parameters such as the pathogen and its pathotypes, the species of the host plant, its varieties and the plant organ that is infected by the pathogen may also modify the efficacy of microorganisms that are selected for biocontrol (Paulitz & Matta, 1989; Smith & Goodman, 1999). The unexpected effect of the biotic and a-biotic factors on biocontrol should be considered during the screening process. In the present study isolates of *Trichoderma* spp., including *T. harzianum* T39 which is the active ingredient of the biocontrol preparation TRICHODEX (O'Neill *et al.*, 1996a) were collected and screened for the control of *B. cinerea* and *C. acutatum*. In addition, the study evaluated the possibility to use *in vitro* culture tests, *in planta* detached plant organ tests and whole plants for screening potential biocontrol agents.

Materials and methods

Collection of* Trichoderma *spp. isolates

Trichoderma spp. were isolated from different organs of non infected or *C. acutatum* and *B. cinerea* infected strawberry plants, from soil in infected plots and from watermelon on *Trichoderma*-selective medium (Elad *et al.*, 1981). The collection of the *Trichoderma* spp. also included isolate T39 of *T. harzianum* of the commercial biocontrol preparation TRICHODEX (20 WP of T39, Makhteshim Chemical Works, Be'er Sheva, Israel) which was previously reported to control *B. cinerea* (O'Neill *et al.*, 1996ab; Elad, 1996; Elad, 2000). The experiments to test the *Trichoderma* isolates consisted of two phases as follows: first phase in culture and detached plant parts with all the collection of isolates, and second phase with selected isolates.

a. Tests in culture: Interaction in dual culture *in vitro* on PDA was tested with *C. acutatum* (5 isolates) and *Botrytis* (3 isolates) by plating each of the pathogen isolates in front of each of the *Trichoderma* spp. isolates (Elad *et al.*, 1980). Antagonism, i.e. parasitism that involves hyphal interactions, inhibition zones due to production of antibiotics by the 74 *Trichoderma* isolates or cases of no interaction were recorded and visually rated on a scale of 0-100.

b. *In planta* tests - detached plant material: i. "petiole segments" bioassay entailed inoculating the top part of sections of detached strawberry petioles and runners standing upright in small containers. The plant sections were inoculated at different time periods with the 74 *Trichoderma* isolates prior to pathogen inoculation as previously described by O'Neill *et al.* (1996b); ii. leaf blade (leaflets) treated similarly but infected by mycelium plugs of the pathogen. The rate of disease advancement was recorded daily. *Trichoderma* and water controls did not cause necrosis on detached plant material.

c. Whole plant tests: Eleven isolates from the above-mentioned and TRICHODEX were selected for whole plant tests of the second phase of the study. The plants were spray treated by conidia (10^6 /ml) of the *Trichoderma* isolates or by TRICHODEX (0.2%/ml = 10^6 conidia /ml). The plants were infected by spraying conidia of *B. cinerea* or *C. acutatum* (10^5-10^6/ml, respectively) from 2 weeks old cultures or by mycelium plugs of *B. cinerea* from 4 days old cultures.

Results and discussion

Collection and selection of isolates

All the isolates, and isolate T-39 of the commercial TRICHODEX preparation were screened for *in vitro* activity. The following activities were observed: against *Colletotrichum,* 6 *Trichoderma* isolates possessed antibiotic activity; 56 parasites (23 very active); and 11 including T39 did not show activity; against *Botrytis* no isolates possessed antibiotic activity; 55 possessed parasitic activity (21 were very active); and 18 did not show any activity.

Control of diseases on detached plant parts and on whole plants

All the *Trichoderma* isolates, as well as T-39 and TRICHODEX were used in two strawberry plant bioassays, on petioles and leaves, for the control of *Colletotrichum* and *Botrytis in planta.* With certain *Trichoderma* isolates a significant reduction of lesion necrosis caused by both *Colletotrichum* and *Botrytis* was observed.

Based on results of the bioassays, *Trichoderma* isolates, T-105, T-115, T-116, T-118, T-126, T-129, T-147, T-165, T-165, T-166 and T-39 of TRICHODEX were used in whole plant inoculations in the greenhouse. The isolates selected represented mycoparasites, antibiotic producers and those not interacting in dual cultures that were effective in biocontrol. Isolates T-115, T-161, T-166, T-39 and TRICHODEX were effective in reducing *Botrytis* leaf mold incidence on leaves (Fig. 1). Isolates T-118, T-165, T-166, T-39 and TRICHODEX were effective in reducing anthracnose caused by *C. acutatum* in petioles (Fig. 2).

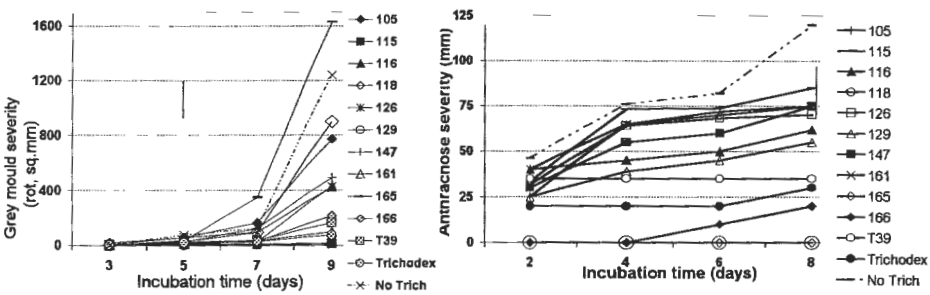

Figure 1 (left). Intact plant assays of biological control of gray mould caused by *B. cinerea* in strawberry leaves by means of *Trichoderma* spp. isolates; Figure 2 (right). Intact plant assays of biological control of anthracnose caused by *C. acutatum* in strawberry petioles by means of *Trichoderma* spp. Bars = LSD.

Out of the 74 isolates of *Trichoderma* spp. few consistently controlled *B. cinerea* or *C. acutatum* on whole plants. Two isolates (T-39 and T-166) and TRICHODEX controlled both diseases, two isolates (T-118 and T-165) controlled *C. acutatum* alone and two isolates (T-115 and T-161) controlled *B. cinerea* alone.

It can be concluded that it is possible to find biocontrol agents out of a collection of isolates that belong to the genus *Trichoderma.* However, a great variability among the isolates of this collection was observed. This confirms many earlier works dealing with the selection of biocontrol agents from this genus. Variability among isolates makes it difficult to establish criteria according to which an antagonist is regarded a competent biocontrol agent. Moreover,

biotic parameters such as the pathogen and its pathotypes, the species of the host plant, its varieties and the plant organ that is infected by the pathogen may also modify the efficacy of microorganisms that are selected for biocontrol.

Acknowledgements

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Biological Control of Fungal and Bacterial Plant Pathogens
IOBC wprs Bulletin Vol. 24 (3) 2001
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A biological control approach making use of rhizobacteria and soil fungi for soilborne post harvest infection of *Aspergillus flavus* in groundnut

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Abstract: Bacterial and fungal strains, potentially antagonistic to *Aspergillus flavus,* were isolated from the rhizosphere and geocarposphere of groundnut from India and tested for *in vitro* antagonism against *A. flavus* in a dual-culture plate method. Twelve of 102 bacterial isolates and 4 of 70 *Trichoderma* isolates were promising antagonists. The selected bacterial and fungal isolates were inoculated on groundnut plants in the greenhouse and were confirmed as non-pathogenic (absence of disease symptoms and biomass of groundnut plants unaffected), as a prerequisite for possible use as biocontrol agents. Most strains were fluorescent *Pseudomonas* (morphology, Gram-stain, physiology and molecular markers). There was no *P. aeruginosa* (comprising human pathogens) amongst the isolates, another condition for subsequent use. In pot culture experiments (greenhouse), inoculation of antagonists resulted in a significant reduction of *A. flavus* population (as cfu) in the rhizosphere of groundnut. Application of inoculum of selected bacterial strains at sowing was more effective than at pegging of the groundnut plant. However, the effectiveness of *Trichoderma* inoculation on the reduction of *A. flavus* population was similar both at sowing and pegging. The overall seed infection by *A. flavus* in groundnut was reduced by application of the antagonists in pot cultures. The potential of these antagonists, as effective field biocontrol agents, as well as the chemical nature of the active substance(s) is currently being investigated.

Keywords: *Arachis hypogaea, Aspergillus flavus, Pseudomonas fluorescens, Trichoderma* sp.

Introduction

Groundnut (*Arachis hypogaea* L.) is a major oilseed crop in the world, widely grown in tropical and subtropical regions and important to the economies of many developing countries. Aflatoxins, produced by *Aspergillus flavus* and *A. parasiticus,* are highly hepatotoxic, teratogenic, carcinogenic and immunosuppressive substances to many animal species. Aflatoxin can be transmitted through the food chain. Among several forms of aflatoxin, aflatoxin B_1 is more common in food and feed. Aflatoxin contamination in groundnut may not affect productivity of the crop, but it makes the produce unfit for consumption (Blankenship *et al.,* 1985). Amounts allowed in seeds are strictly regulated. Infection of *A. flavus* can occur pre- and post-harvest during drying and storage. Biological control by competitive exclusion of aflatoxigenic aspergilli is promising in the integrated management of aflatoxin contamination in groundnut. In this strategy, inoculation of competitive and native antagonistic microorganisms is expected to reduce the populations of

aflatoxigenic fungal strains in the soil and to subsequently reduce infection to the developing and mature (harvested) pods and seeds of groundnut.

Materials and methods

***Isolation of antagonistic bacteria and fungi against* Aspergillus flavus**

The isolation of potential bacterial and fungal antagonists to *A. flavus* was conducted using a double layer technique and further *in vitro* evaluation and selection of aggressive antagonists was done using the dual culture plate method. Using this technique, *A. flavus* spores were plated on a ground layer of glucose casaminoacid yeast extract (15 mL/plate) agar medium (GCY; Anjaiah *et al.*, 1998). A top layer of GCY (10 mL/plate) agar medium was then poured and soil (collected from geocarposphere of groundnut) dilutions were plated. The potential antagonists to *A. flavus* thus selected were purified and transferred to GCY agar slants. These strains were further screened *in vitro* in a dual culture plate technique as described earlier (Anjaiah *et al.*, 1998).

Characterization of bacterial and fungal antagonistic strains and their evaluation

The selected antagonistic strains were inoculated on groundnut plants to confirm their non-pathogenic identity (no yield/biomass reduction of groundnut) as safe biocontrol agents (no *Pseudomonas aeruginosa*). Further, the selected antagonistic strains were characterized for morphological, physiological, biochemical and molecular traits (Anjaiah *et al.*, 1998). In pot experiments, selected bacteria (*Pseudomonas fluorescens*) and fungi (*Trichoderma harzianum*) were evaluated in the greenhouse for their effectiveness in suppressing *A. flavus* populations in soil and infection to pods and kernels. The antagonists were applied at sowing (as a seed dressing) and peg formation stages (as a soil drench) of cv. J 11 and JL 24 (moderately resistant and susceptible, respectively). *A. flavus* populations in the rhizosphere and those colonizing seed were monitored (Mehan *et al.*, 1987).

Results and discussion

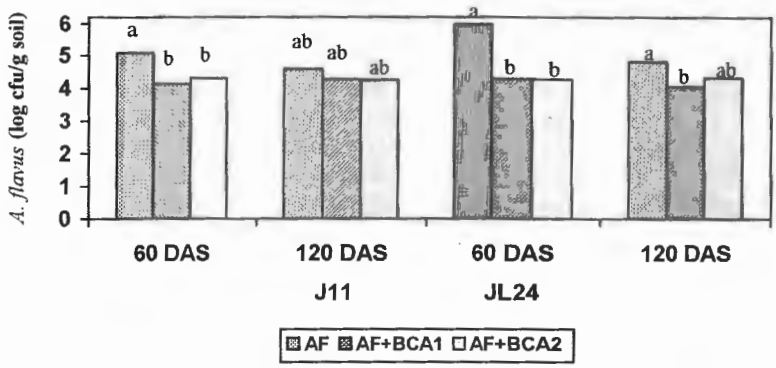
In vitro *screening and selection of antagonists to* A. flavus

In the double layer technique, *A. flavus* grew through the agar and covered the whole surface, whereas antagonistic microorganisms (bacteria and fungi) grew on the upper surface layer and did not allow the growth of *A. flavus* in their vicinity. These potential antagonists of *A. flavus* (at least one colony of each morphological type) were isolated. They were further selected based on *in vitro* antagonism in dual culture plate method against *A. flavus* on GCY agar medium. In subsequent evaluations only 12 of 102 bacterial isolates and 4 of 70 *Trichoderma* isolates were found to be promising.

***Characterization of antagonists to* A. flavus**

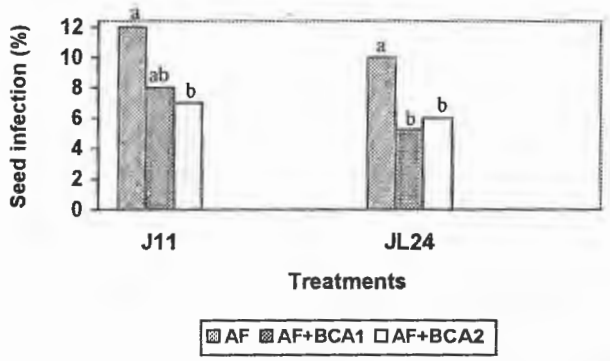
All selected isolates were inoculated on groundnut in the greenhouse (India) and confirmed as non-pathogenic (results not shown). These antagonists satisfied the first condition for their possible use as safe biocontrol agents. Morphological characterization of bacterial strains showed that they all were non-sporulating and gram-negative rods. Physiological characterization on different growth media (King's B agar, Pseudomonas agar, P-isolation agar, Casamino acid agar) indicated that more than 70% of the strains were fluorescent Pseudomonads. Multiplex PCR analysis using primers of outer membrane lipoprotein genes (*opr*I and *opr*L) of *Pseudomonas*, sequence analysis of *opr*I gene (De Vos *et al.*, 1993) and iso-electric focusing (IEF) analysis of bacterial siderophores (Koedam *et al.*, 1994) confirmed that the majority are from rRNA group I fluorescent pseudomonads, other than *P. aeruginosa*.

Since the *P. aeruginosa* group comprises human pathogens, the direct agricultural use of strains from this group is undesirable.



AF : *Aspergillus flavus* BCA1 : *Pseudomonas fluorescens* BCA2 : *Trichoderma harzianum*

Figure 1. Reduction in *A. flavus* population in the rhizosphere of groundnut at two sampling times. Values are means of four replicates. Columns followed with the same letter do not differ significantly according to Fisher's least significant difference (LSD) test as $P \leq 0.05$. DAS = days after sowing.



AF : *Aspergillus flavus* BCA1 : *Pseudomonas fluorescens* BCA2 : *Trichoderma harzianum*

Figure 2. Reduction of *A. flavus* infection in groundnut kernels. Values are means of four replications. Data represented by bars with the same letter do not differ significantly according to Fisher's least significant difference (LSD) test as $P \leq 0.05$.

Trichoderma isolates were characterized for their biocontrol ability, growth rate, *in vitro* antagonism (mycoparasitism) against *A. flavus* and the presence of chitinase gene(s). Among 26 selected isolates, 8 recorded faster growth and 4 isolates inhibited growth of *A. flavus* in dual-culture plates. The similarity in chitinase genes among the *Trichoderma* species allowed

to indicate the presence of presumed chitinase genes using PCR (degenerated primers were designed in the conserved motifs of chitinase gene sequences). Presumed chitinase genes were amplified in 11 of 17 *Trichoderma* isolates. All fluorescent *Pseudomonas* strains are compatible to *Trichoderma* species *in vitro* in dual culture; they can therefore be used together for dual application (results not shown).

Evaluation of antagonists for* A. flavus *management in greenhouse conditions

The rhizosphere population of *A. flavus* in *A. hypogaea* cv. JL 24 was higher than in cv. J 11. The bacterial inoculation at sowing was more effective than at pegging in both cultivars. The effectiveness of *Trichoderma* inoculation was similar at both stages of application. Inoculation of antagonists resulted in significant reduction of *A. flavus* population in the rhizosphere (Fig. 1). The groundnut seed infection by *A. flavus* was reduced significantly in antagonist applied treatments compared to controls in both cultivars (Fig 2). Before being evaluated in the field trials, experiments are currently being repeated in the greenhouse.

Fluorescent pseudomonads have been considered effective biological control agents against soilborne plant pathogens because of their rapid and aggressive colonization of plant roots (Anderson *et al.*, 1988). Several mechanisms involved in pathogen suppression are as follows: competition for sites and nutrients and production of antibiotics, siderophores, mycolytic enzymes and hydrogen cyanide (Thomashow & Weller, 1996). Soil fungi such as *Trichoderma* have been isolated from natural habitats of the target pathogens and identified as potential biocontrol agents for certain soilborne plant diseases. Studies on the mechanisms involved in disease reduction have revealed that *Trichoderma* can act as a mycoparasite, secrete extracellular hydrolytic enzymes e.g. chitinase, β-1,3-glucanase, protease and lipase (Knudsen *et al.*, 1991). Not only direct effects on the pathogen but also indirect effects, through induced systemic resistance, are reported mechanisms involved in biocontrol (Van Loon *et al.*, 1998). In the present study, we have isolated potential antagonists amongst fluorescent pseudomonads and *Trichoderma* spp. from the rhizosphere and geocarposphere of groundnut. In preliminary studies, bacterial and fungal antagonists applied on groundnut plants reduced groundnut seed infection by *A. flavus* and its rhizosphere population.

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Biological Control of Fungal and Bacterial Plant Pathogens
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Increasing the ability of the biocontrol product, Aspire, to control postharvest diseases of apple and peach with the use of additives

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Abstract: At least two, yeast-based, biocontrol products, are commercially available (Aspire and Yield Plus) for postharvest prevention of disease in pome, stone, and citrus fruit. Further research is aimed at improving the ability of yeast antagonists to control postharvest diseases under a wider array of conditions with less variability. Additionally, the ability to control pre-existing infections (curative activity) is also highly desired. To improve efficacy, we have explored the use of various additives, many of them commonly used in food products, in combination with Aspire, the product based on the yeast *Candida oleophila*. Among the additives, the use of calcium propionate, sodium bicarbonate, and EDTA was examined. On apple, all three additives exhibited both curative and protective activity against *Botrytis cinerea* and *Penicillium expansum* in some experiments, while in peach, only sodium bicarbonate exhibited any disease control against *Monolinia fructicola* and *Rhizopus stolonifer*. Overall, the use of 2% sodium bicarbonate was the most consistent in providing a measure of fungicidal activity when used alone.

Keywords: *Prunus persica, Malus domestica, Candida oleophila* biological control, salt solutions

Introduction

Considerable research effort has been devoted to identifying yeast organisms that effectively control postharvest diseases of fruit, vegetables, and grains (Wilson *et al*, 1996). At least two, yeast-based products are now commercially available (Aspire based on *Candida oleophila*, and Yield Plus based on *Cryptococcus albidus*), and several more are in the advanced stages of commercialization (El Ghaouth *et al.*, 2000a,b). The products Biosave-100 and Biosave-110, based on the bacterium, *Pseudomonas syringae*, are also available for postharvest disease control. Although antagonistic yeasts have been shown to protect a variety of fruit, their efficacy under semi-commercial conditions is often lower than chemical control (Droby *et al.*, 1993; Droby *et al.*, 1998, El Ghaouth *et al.*, 2000a and b). In large-scale tests the use of antagonists often needs to be supplemented with low doses of synthetic fungicides in order to provide a level of disease control equivalent to synthetic fungicides (Brown *et al.*, 1996; Wilson and Wisniewski, 1994). A principal goal in the development of "second generation" products is improving the ability of yeast antagonists to successfully control postharvest diseases under a wider array of conditions and with less variability. Additionally, the ability to control pre-existing infections, as is possible with synthetic fungicides, is also highly desired (El Ghaouth *et al.*, 2000a,b). To attain these goals, we have explored the use of various additives, many of them commonly used in food products, in combination with yeast antagonists. This was done to identify combinations that had the potential to control pre-existing infections (curative activity), as well as enhancing overall protective activity.

Materials and methods

Apples (cv Golden Delicious) were obtained from the Appalachian Fruit Research Station, Kearneysville, WV during the summer and fall of 1999. Fruit were wounded with four wounds, approximately 3mm in diameter and 3-4 mm deep, to each fruit. The product, Aspire (Ecogen Inc., Langhorne, PA) was used according to label instructions and suspended in water or one of the additive solutions and 25 µl of each treatment was pipetted into each wound. For protective tests the treatments were applied to the wounds 2 hours prior to the application of the pathogen which was applied as a 25 µl suspension of spores (1×10^4 spores/ml). For curative tests the pathogen was applied 24 hours prior to the treatment solutions. Pathogens used in the apple studies were *Botrytis cinerea* and *Penicillium expansum*. Treated fruit were stored in closed plastic trays, to maintain high humidity, at ambient temperatures Fruit were assayed for disease at 7-21 days. For the peach tests, natural infection of wounds in peach (cv Loring) by *Monilinia fructicola* and *Rhizopus stolonifer* were monitored after dipping the fruit in various treatment solutions. The fruit were placed in closed plastic trays and the percent rot in treated fruit was compared to untreated fruit after 3 - 5 days.

Results and discussion

Based on preliminary screening tests, we examined in a comprehensive manner, the use of calcium propionate, sodium bicarbonate, and EDTA, as possible additives to be used in combination with different yeast antagonists and with the commercial product, Aspire. All three of these additives had a distinct inhibitory effect on the radial growth of *B. cinerea* and *P. expansum*. In all cases, the inhibitory effect on growth increased with concentration of the additive. The inhibitory effect of sodium bicarbonate was evident at relatively low concentrations (0.3 - 0.6%) compared to the concentration of calcium propionate (2 - 5%). When tested directly on apples, the three additives exhibited principally curative activity against *B. cinerea* and *P. expansum* rather than protective activity.

The ability of *Candida oleophila*, the yeast used in the product Aspire, was not significantly affected by any of the additives except EDTA (20mM) which did have a slight inhibitory effect. It should be noted, however, that standing solutions of the additives containing the yeast were much more inhibitory, suggesting that in the wound site the additives are quickly absorbed or become bound to cell walls. Continued experiments combining the additives with Aspire revealed that the inhibitory effects of the additives was variable and that the use of 2% sodium bicarbonate gave the most consistent, positive results. This is illustrated in Figure 1 A to C, where only the use of 2% sodium bicarbonate enhanced the performance of Aspire in controlling Botrytis rot in apple. Similar variability was observed in experiments using *P. expansum* (Figure 2, A to C). The ability of the additives to enhance both protective and curative activity of Aspire against Botrytis and Penicillium rots, is illustrated in Figure 3A and B. In the tray tests conducted, Aspire exhibited such effective control of *Penicillium* that the enhancement brought about by the use of the additives was only evident in the curative studies. Similar results were obtained in peach by combining the additives with Aspire. In peach, only natural infection of wounds were monitored. The main rot organisms identified were *M. fructicola* and *R. stolonifer*. Only the use of 2% sodium bicarbonate affected the biocontrol efficacy of Aspire. In contrast to apple, application of EDTA to peaches was phytotoxic and greatly enhanced rot of the fruit (data not shown).

Smilanick *et al.* (1997, 1999) have demonstrated that treatment of citrus with warm (35 - 46 EC) solutions of sodium carbonate and bicarbonate salts is an effective method of reducing

postharvest decay caused by green mold (*Penicillium digitatum*). Our study demonstrates that the use of these salts may also be applicable to the postharvest treatment of pome fruit. Additionally, the sodium bicarbonate salt also enhances the efficacy of the biocontrol product, Aspire, which utilizes the yeast *C. oleophila*. The use of the carbonate salt, as opposed to the bicarbonate salt, was not compatible with Aspire due to the resulting high pH of the solution that was lethal to the yeast. Clearly however, further research is needed to identify the best method of utilizing these combinations (temperature, method of application, etc.). It should also be noted that recently, El Ghaouth *et al.* (2000a,b) have reported enhancing the efficacy of the biocontrol yeast, *C. saitoana*, by combining it with either glycochitosan, forming a "bioactive coating," or with the sugar 2-deoxy-D-glucose. Both approaches increased the protective and curative activity of the yeast in controlling postharvest diseases. As efforts continue to find alternatives for synthetic fungicides to control postharvest losses, biocontrol will continue to be a viable approach, especially as it becomes integrated with other approaches such as the use of additives that enhance their performance over a wider range of conditions.

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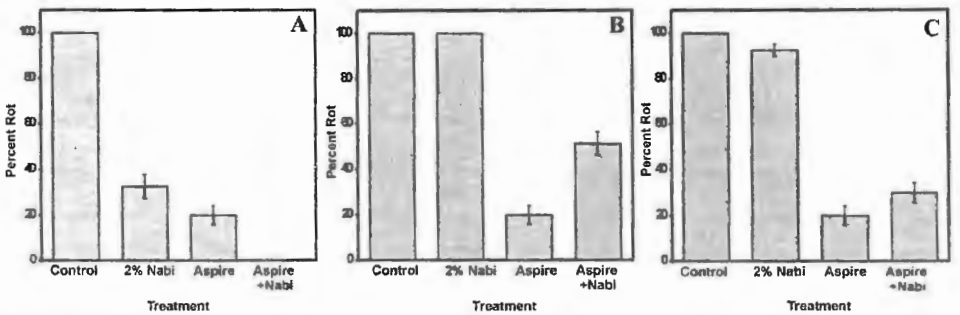

Figure 1. Effect of various additives on the efficacy of Aspire against *Botrytis cinerea* rot on apple. A. 2% sodium bicarbonate (NaBi). B. 2% calcium propionate (CaP). C. 25 mM EDTA. n = 20 " se.

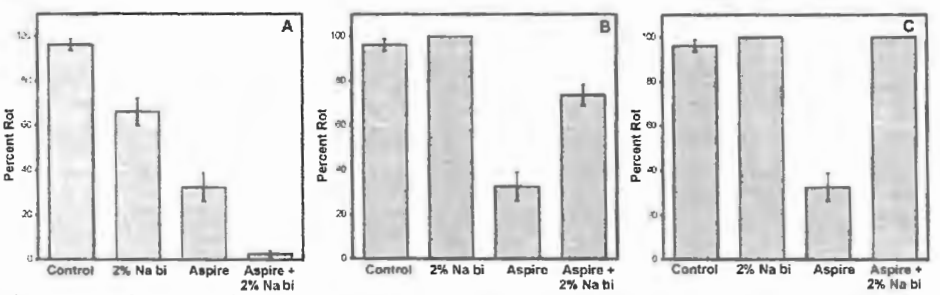

Figure 2. Effect of various additives on the efficacy of Aspire against *Penicillium expansum* rot on apple. A.2% sodium bicarbonate (NaBi). B.2% calcium propionate (CaP). C. 25 mM EDTA. n=20" se.

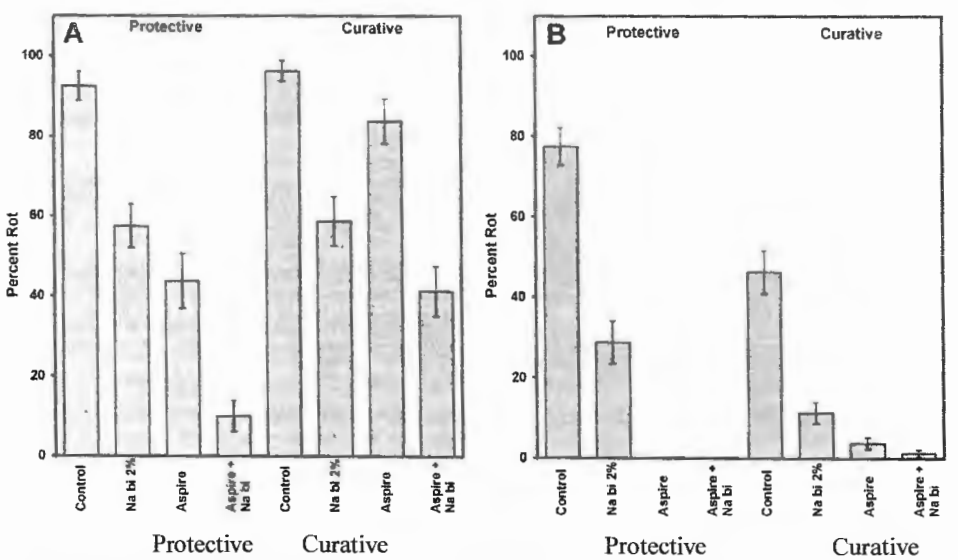

Figure 3. Curative and protective enhancement of Aspire, using 2% sodium bicarbonate as an additive, against *Botrytis cinerea* (A) and *Penicillium expansum* (B). n = 20 " se.

Biological Control of Fungal and Bacterial Plant Pathogens
IOBC wprs Bulletin Vol. 24 (3) 2001
pp.161 - 165

Evaluation of two biocontrol products, Bio-Coat and Biocure, for the control of postharvest decay of pome and citrus fruit

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Abstract: The efficacy of two postharvest biological products under commercial development was determined in laboratory and semi-commercial tests. The products consist of the yeast (*Candida saitoana*) combined with either chitosan (Bio-Coat) or an antifungal, lytic enzyme (Biocure). When applied to fruit wounds immediately or within 24 h after inoculation with target pathogens, Bio-Coat and Biocure were more effective in controlling decay of apple, orange, and lemon caused by *Penicillium expansum, Botrytis cinerea* and *Penicillium digitatum* than either *C. saitoana* or the additives alone. In semi-commercial trials, Bio-Coat and Biocure effectively controlled postharvest decay of Golden Delicious' apple. The level of control was comparable or superior to thiabendazole (600 ppm). The formulations were also effective in controlling postharvest decay of oranges (Washington navel and Valencia) and Eureka lemons. The level of decay control was equivalent to that obtained with imazalil (2,000 ppm). Efficacy of Bio-Coat and Biocure effectively controlled decay on both early and late season oranges, and was not affected by application of wax to citrus fruit. Solutions of the product remained stable and effective for at least 24 h after mixing. A commercial product is expected in the near future.

Keywords: postharvest, biological control, yeast, *Candida saitoana*, chitosan, lytic enzymes

Introduction

In recent years, research on the use of microbial biocontrol agents for the control of postharvest diseases of fruits has gained considerable attention and has moved from the laboratory to commercial application. Presently, four biological products are commercially available under the trade names ASPIRE, YieldPlus, BIOSAVE-100, and BIOSAVE-110. Currently available microbial biocontrol products, however, confer only a protective effect and often do not provide control of previously established infections (Brown & Chambers, 1996; Droby *et al.*, 1998; Wilson *et al.*, 1996). Since fruit infection can occur either prior to harvest or during harvesting and subsequent handling, biocontrol products have to protect wounds and control previously established infections to an extent similar to synthetic fungicides. Recently, we have developed two biocontrol products: Bioactive Coating (*Candida saitoana* with chitosan salts) and Biocure (*C. saitoana* with lytic enzyme). The mixtures combine the antifungal property of chitosan salts or lytic enzyme with the biocontrol activity of an antagonist. Laboratory tests on apple and citrus fruits showed that the Bioactive coating and Biocure were more effective in controlling decay than either the antagonist or the additives alone (El Ghaouth & Wilson, 1997; 1998). The results from semi-commercials tests on apple and citrus fruits show the reliability and efficacy of the Bioactive Coating and Biocure as viable alternatives to synthetic fungicides for the control of some postharvest diseases.

Materials and methods

The Bioactive Coating and Biocure were prepared by mixing the additives (chitosan salts or lytic enzyme) with wet paste of *C. saitoana* or a dried formulation of *C. saitoana* prepared by Anchor yeast (Capetown, South Africa). Apple fruits of cultivars 'Empire' and 'Red Delicious' were hand-picked at harvest maturity at the USDA-ARS, Appalachian Fruit Research Station, Kearneysville, WV. Early and late season 'Washington' Navel and Valencia oranges and 'Eureka' lemons grown in the San Joaquin Valley of California were harvested within 2 days before treatment.

Semi-commercial trials were conducted over several years at Kearneysville, WV and Lindcove, Ca. At Kearneysville, apple fruits were wounded (3 mm by 5 mm deep), placed on a dry dump conveyor-belt, and passed over rotating brushes through a washer to an air drier operating at 32°C. Subsequently, the fruits were treated with either water; Bioactive coating; Biocure at approximately 15 ml per kg of fruits; or 600 ppm of Mertec. Each treatment was applied to 5 replicates of 56 apples. Treated fruits were packed in cardboard boxes, stored at 18°C for 2-4 weeks. In Lindcove, orange and lemon fruits were placed on a dry dump conveyor-belt, high-pressure washed and dried by passing over foam rubber rollers. Lemon fruits were submerged for approximately 2 min into a 2,400-liter-capacity tank containing a solution of 3% (w/v) sodium carbonate heated to 37°C and then lifted by rollers to a washer with a series of overhead nozzles and then to foam-rubber rollers for drying. Dried lemons and oranges were treated with water; Bioactive coating; and Biocure at 2-1/2 gallon per bin of fruits; or 2000 ppm of Imazalil. Treated fruits were either waxed or non-waxed, dried by passing through a high-velocity air drier, packed in commercial cartons or plastic bins, and stored for 25-28 days at 10°C. The fruit where then transferred to room temperature for 5-7 days. All the treatments except Imazalil were applied using an on-line overhead spray system installed before the waxer. The Imazalil treatment was applied in the wax using the waxer with an overhead single nozzle. Within each experiment, the treatments consisted of 4 replicates of 2700-3000 'Washington' Navel oranges, 2000-2600 'Valencia' oranges, and 10-12 replicates of 110-140 'Eureka' lemons. Methods used for laboratory tests have been previously described (El Ghaouth *et al.*, 2000). In curative tests, fruit were pre-inoculated with the pathogen 24 h prior to treatment (either *B. cinerea* or *P. digitatum* on apple and citrus, respectively).

Results and discussion

In laboratory studies, the Bioactive coating and Biocure were very effective in controlling decay of apple, lemon, and orange fruits (Figure. 1). On apple, challenge-inoculated with *B. cinerea*, the Bioactive coating and Biocure, when applied immediately after inoculation, significantly reduced the incidence of gray mould but not as well as 600 ppm of Mertec. Significant decay control was also observed among lemon and orange fruits treated with the biocontrol formulations immediately after inoculated with *P. digitatum*. The level of control obtained was significantly better than with 500 ppm Imazalil.

Bioactive coating and Biocure, when applied 24 h after inoculation with a pathogen, were also effective in controlling decay of apple and citrus fruits, thus demonstrating curative activity (Figure. 2). The incidence of green mould among orange and lemon fruits treated with the formulations was significantly lower than that observed after treatment with 500 ppm of Imazalil. On apple fruits, the Bioactive coating and Biocure displayed a curative activity comparable to that seen with 600 ppm of Mertec. The Biocure mixture offered consistent curative control of decay on oranges, regardless of whether the solutions were used right away

or 48 h later (data not shown). The curative activity of the Bioactive coating and Biocure represents a substantial improvement over existing microbial biocontrol products and should help to overcome the variable performance of microbial antagonists.

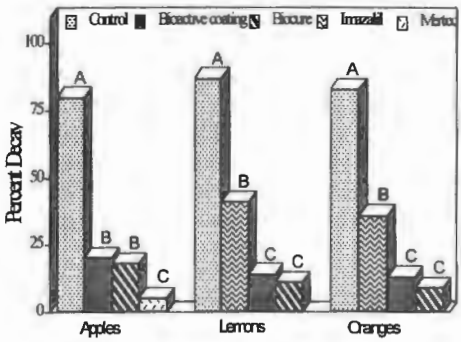

Figure 1. Protective effect of Bioactive coating and Biocure on apples, lemons, and oranges.

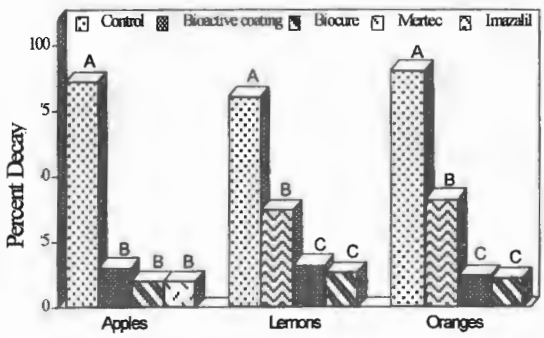

Figure 2. Curative effect of Bioactive coating and Biocure.

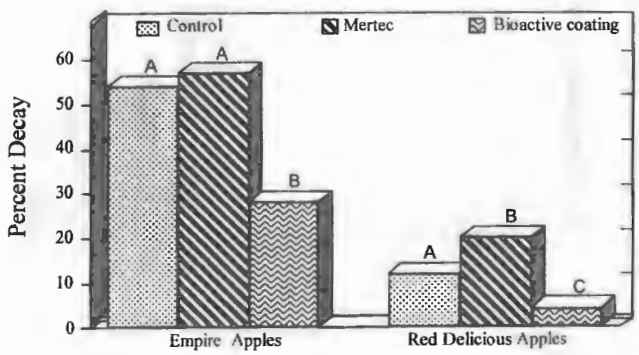

Figure 3. Semi-commercial test on the effect of the Bioactive coating on natural decay of apples.

Under semi-commercial trials, the Bioactive coating was effective in controlling natural infection of 'Red Delicious', and 'Empire' apples and the level of control was significantly higher than that obtained with 600 ppm of Mertec (Figure. 3). Mertec was ineffective against decay on 'Red Delicious', and 'Empire' apples, and in some cases the level of decay was higher than the water-treated control.

On both early and late season lemons, the Bioactive coating and Biocure were as effective as 2000 mg/ml of Imazalil in controlling green mould (Figure 4). Similar control patterns were observed with the Bioactive coating and the Biocure on oranges (Figure 5). On 'Washington' Navel and 'Valencia' oranges, the Bioactive coating and Biocure were as effective as 2000 ppm of Imazalil in controlling green mould on early and late season fruits and their efficacy was not affected by fruit waxing. Results from laboratory and semi-commercial trials on apple and citrus fruits show that the efficacy of the Bioactive coating and Biocure is equivalent to that achieved with the commercial fungicides, Mertec and Imazalil.

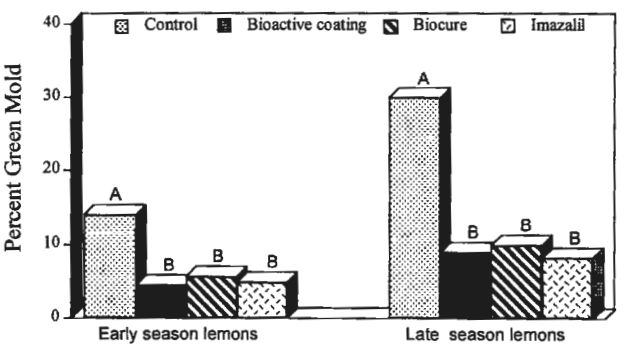

Figure 4. Semi-commercial test on the effect of Bioactive Coating and Biocure on natural decay on Eureka lemons.

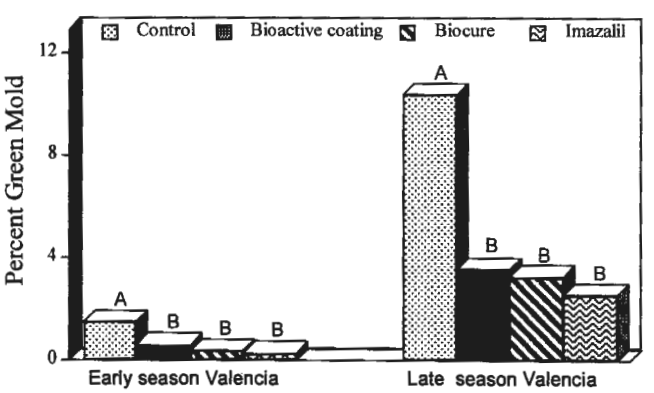

Figure 5. Semi-commercial test on the effect of Bioactive Coating and Biocure on natural decay on Valencia oranges.

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Wilson, C., Wisniewski, M., El Ghaouth, A., Droby, S. & Chalutz, E. 1996: Commercialization of antagonistic yeasts for the biological control of postharvest diseases of fruits and vegetables. J. Indust. Microbiol. Biotechnol. 46: 237-242.

Biological Control of Fungal and Bacterial Plant Pathogens
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Control of green mould by the combination of *Pantoea agglomerans* (CPA-2) and sodium bicarbonate on oranges

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Abstract: The potential of using the strain CPA-2 of *Pantoea agglomerans*, isolated from the apple surface, alone or in combination with sodium bicarbonate solutions for control of *Penicillium digitatum* on oranges was investigated under ambient (20°C) and cold storage conditions (3°C). Valencia oranges without any commercial treatments were used in all experiments. Oranges were wounded with a steel scalpel to simulate natural infection. A conidial suspension of *P. digitatum* at $1x10^5$ and $1x10^6$ spores ml^{-1} was applied at each wound. Biocontrol with *P. agglomerans* CPA-2 was tested at $4x10^7$ and $2x10^8$ cfu ml^{-1}; to improve the efficacy of this biocontrol agent other experiments with 2% sodium bicarbonate were carried out. *P. agglomerans* effectively controlled this pathogen on oranges at $2x10^8$ cfu ml^{-1}. The efficacy of *P. agglomerans* for control green mold was improved significantly when combined with sodium bicarbonate treatment, resulting in complete and 98% reduction of decay incidence at 3°C and 20°C, respectively when compared to untreated controls. This antagonist had a good capacity for growth inside wounds on oranges at both 20°C and 3°C. Sodium bicarbonate at 2% concentration did not noticeably affect antagonist population development. Use of bicarbonate treatment at 2% followed by the antagonist *P. agglomerans* CPA-2 could be a reliable alternative to substitute chemicals to control postharvest diseases on oranges.

Key words: Biological control, citrus, *Penicillium digitatum*, postharvest diseases

Introduction

Biological control using microbial antagonists has received a great deal of attention as a promising alternative to chemicals to control postharvest diseases. Recent studies at the University of Lleida, Catalonia have demonstrated that the strain CPA-2 of *Pantoea agglomerans*, isolated from the apple surface, is an effective antagonist to the major fungal pathogens of apples and pears (Viñas *et al.*, 1999). Recently, interest has been given to potential of combining microbial biocontrol agents with other chemical components for enhancing control of postharvest diseases of pome, stone and citrus fruits.

Carbonic acid salts are common food additives allowed with no restrictions for many applications. The antimicrobial activity of these chemicals has been described *in vitro* (Corral *et al.*, 1988) and in a wide range of substrates as well. Sodium bicarbonate ($NaHCO_3$) and sodium carbonate (Na_2CO_3) have been demonstrated to reduce the incidence of postharvest decay on citrus fruits (Smilanick *et al.*, 1997).

This study reports (a) the efficacy of *P. agglomerans* (strain CPA-2) for the control of *P. digitatum* rot on oranges; (b) the potential for improving its efficacy by the combination with Sodium bicarbonate treatments when stored under ambient conditions (20°C) or in cold storage (3°C) and (c) quantification of the population dynamics of *P. agglomerans* in wounded fruits.

Material and methods

Organisms (fruits, pathogen and antagonist)

Valencia oranges used in all experiments were grown in Baix Ebre and Montsià areas in Tarragona (Catalonia, Spain) following standard culture practices. *P. digitatum* PDM-1 was isolated from decayed oranges and mantained on potato dextrose agar medium. A conidial suspension was prepared by adding 10 ml of sterile water with tween 80 over the surface of 10-day-old cultures grown on PDA and rubbing the surface with sterile glass rod. The cells were counted in an haemocytometer and diluted to a concentration of $1x10^5$ and $1x10^6$ spores ml^{-1} . *P. agglomerans* strain CPA-2 was obtained from the UdL-IRTA Centre, Catalonia. It was originally isolated from apple surface (cv. Golden Delicious). Stock cultures were stored at 5°C and were subcultured on nutrient yeast dextrose agar (NYDA).

***Biocontrol with* P. agglomerans**

Oranges were wounded with a steel scalpel and a 25 µl aqueous suspension of *P. digitatum* at $1x10^5$ or a $1x10^6$ spores ml^{-1} was applied to each wound, followed by inoculation with 20 µl of the appropiate concentration of a suspension of *P. agglomerans* CPA-2 ($4x10^7$ or $2x10^8$ cfu ml^{-1}). Treated oranges were stored at 20°C and 90% RH and decayed fruits were recorded after 7 days.

Improved biocontrol using* P. agglomerans *with sodium bicarbonate

Valencia oranges were wounded and inoculated with *P. digitatum* at $1x10^6$ spores ml^{-1} about 2 h before treatments were applied. Control treatment consisted of dipping oranges in water for 1 minute. Antagonist treatment was applied by dipping oranges for 1 minute in an aqueous solution containing $2x10^8$ cfu ml^{-1} of *P. agglomerans*. For the sodium bicarbonate treatment the concentration of 2% and a 150-seconds immersion period were chosen. Finally, bicarbonate and antagonist treatment were combined by first dipping fruits in 2% sodium bicarbonate for 150 s and, after allowing to air-dry at room temperature, by dipping fruit in $2x10^8$ cfu ml^{-1} *P. agglomerans* suspension for 1 minute. Treated fruits were stored 14 days at 20°C and 90% RH or 60 days at 3°C and 90% RH. Decayed fruits were evaluated after 7 and 14 days storage at 20°C and after 30 and 60 days of cold storage.

Population dynamics on the orange surface

The populations of *P. agglomerans* CPA-2 were monitored on wounded oranges removing 25 pieces of peel surface of 2.5 cm^2 with a cork borer and shaking them in 100 ml sterile phosphate buffer for 20 minutes and sonicating for 10 minutes in an ultrasound bath. Thereafter, serial 10-fold dilutions of the washings were made and plated on NYDA medium to record the viable colonies per cm^2. Oranges were treated with *P. agglomerans* or sodium bicarbonate followed by *P. agglomerans*, as described in efficacy trials. Fruits were incubated at 20°C and 90% RH, and in long-term cold storage at 3°C and 90% RH.

Results and discussion

***Biocontrol with* P. agglomerans**

P. agglomerans CPA-2 strongly inhibited the development of *P. digitatum* on wounded oranges inoculated at $1x10^5$ and $1x10^6$ spores ml^{-1}. The efficacy of $4x10^7$ and $2x10^8$ cfu ml^{-1} of the antagonist was not statistically different when the concentration of pathogen used was $1x10^5$ spores ml^{-1}, with percentages of infected wound reductions >70%. However, when the antagonist concentrations were increased from $4x10^7$ to $2x10^8$ cfu ml^{-1}, the efficacy at $1x10^6$ spores ml^{-1} was significantly increased achieving reductions in incidences of *P. digitatum* about 71% (Fig.1).

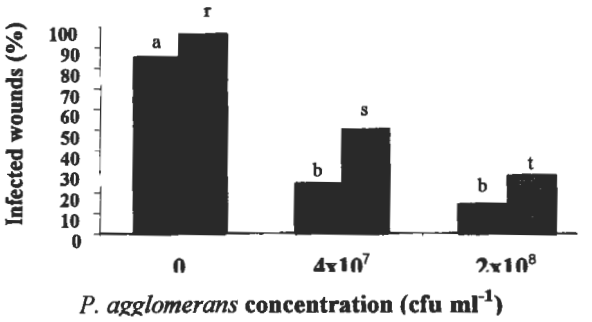

Figure 1. Incidence of green mould on wounded Valencia oranges inoculated with $1x10^5$ (■) and $1x10^6$ (▪) spores ml^{-1} of *P. digitatum*, followed by treatment with different concentrations of *P. agglomerans* after 7 days of storage at 20°C and 90% RH.

Enhancement of biocontrol by* P. agglomerans *with sodium bicarbonate

At 20°C, all assayed treatments (antagonist, sodium bicarbonate and their combination) significantly decreased decay incidence of *P. digitatum* after 7 or 14 days of incubation. Green mould incidence was reduced about 57% after 7 days with *P. agglomerans* and sodium bicarbonate treatments and no significant differences were found between them. The combination of antagonist and sodium bicarbonate were capable of reducing the incidence to a level of 98% (Fig. 2A).

All the studied treatments significantly inhibited *P. digitatum* decay under cold storage conditions (3°C). However, the best treatments were sodium bicarbonate alone, when combined with the antagonist, resulting in complete control (100%) after 30 days in cold storage (Fig. 2B).

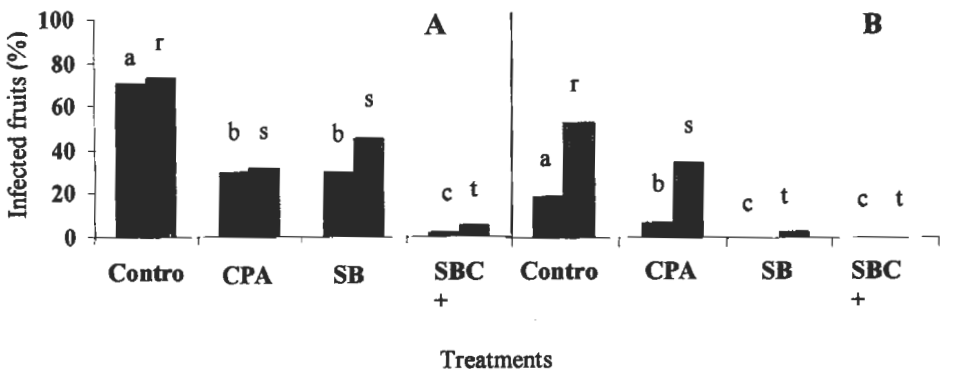

Figure 2. Incidence of green mould on Valencia oranges inoculated with 10^6 spores ml^{-1} of *P. digitatum*, followed by treatment with water, *P. agglomerans* CPA-2, sodium bicarbonate (SBC) and the combination, after 7 (Black columns) and 14 (Gray columns) days of storage at 20°C and 90% RH (A) or after 30 (Black columns) and 60 (Gray columns) days of storage at 3°C and 90% RH (B).

Population dynamics on the orange surface

The patterns of growth of *P. agglomerans* was similar (Fig. 3), whether applied alone or in combination with sodium bicarbonate. Thereafter, sodium bicarbonate at 2% did not noticeably affect the antagonist development. Moreover, the antagonist developed well either at 20°C or at 3°C. This aspect indicates excellent adaptation of the strain CPA-2 to cold storage conditions, which is an important feature for postharvest biocontrol agents.

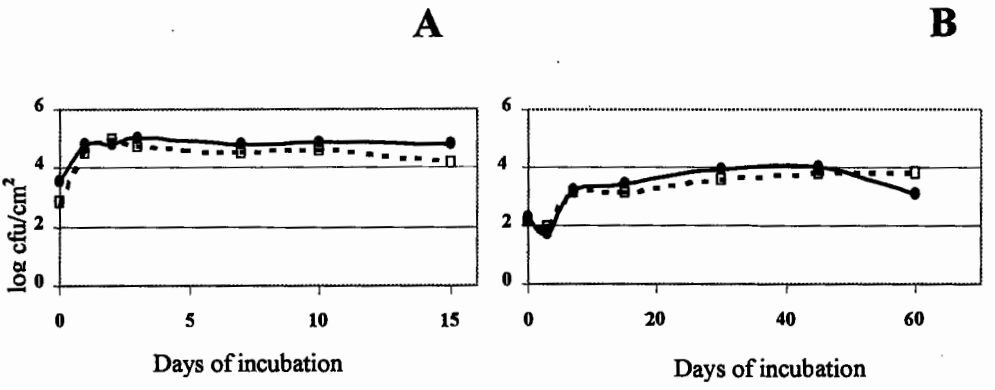

Figure 3. Population dynamics of *P. agglomerans* CPA-2 on wounded oranges treated *with P. agglomerans* (●) or the combination of sodium bicarbonate and *P. agglomerans* (□) and stored at 20°C and 90% RH (A) or 3°C and 90% RH (B).

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Biological Control of Fungal and Bacterial Plant Pathogens
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Epidemiology of *Botrytis* spp. in different crops determines success of biocontrol by competitive substrate exclusion by *Ulocladium atrum*

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Abstract: The fungal antagonist *Ulocladium atrum* was used in field and greenhouse experiments in cyclamen, strawberry, grapevine and lilies for biocontrol of *Botrytis* spp. The biocontrol effect is based on competitive exclusion of *Botrytis* spp. from necrotic plant tissues. The role of different necrotic tissues in epidemiology of *Botrytis* spp. was studied in various crops. Biocontrol was achieved when (1) a mutual substrate was present for *Botrytis* spp. and *U. atrum*; (2) *U. atrum* could out-compete *Botrytis* spp. in the mutual substrate; and (3) the mutual substrate played an important role in *Botrytis* epidemiology. Based on these results, other crops can be identified in which applications of the antagonist may result in control of diseases caused by *Botrytis* spp., and perhaps also other necrotrophic pathogens.

Key words: biological control, *Botrytis* spp., competition, cyclamen, grapevine, lily, strawberry, *Ulocladium atrum*

Introduction

The fungal saprophyte *Ulocladium atrum* can be used for biological control of *Botrytis* spp. The antagonist is a strong competitor during the colonisation of necrotic plant tissues. Such necrotic plant parts play a key role in epidemiology of *Botrytis* spp. The mode-of-action of *U. atrum* most probably is nutrient competition (Kessel, 1999). There is no evidence that metabolites inhibiting the growth of *Botrytis* spp. are produced by *U. atrum*. The objective of our study was to develop rational guidelines for identifying crops in which biological control of *Botrytis* spp. is feasible when competitive interactions on necrotic plant parts are exploited as the basic mechanism.

Material and methods

Field experiments were carried out in strawberries and lilies in the Netherlands and in grapevine in Germany (Schoene & Köhl, 1999). Experiments with cyclamen were carried out in Dutch greenhouses. In all experiments, *U. atrum* was applied as a conidial suspension. Other treatments were controls (untreated or treated with water), fungicide programmes as applied in practice, or treatments in which dead leaves were removed by hand at 3 to 7-day intervals to determine their role in the epidemiology of *Botrytis* spp. in specific crops. In all experiments, disease development was monitored at crop level but observations were also done at substrate level, e.g. on necrotic leaves, bark or flower debris, to study the competitive substrate colonisation by *U. atrum* and *Botrytis* spp. Details of the experiments are described elsewhere (Boff, submitted; Kessel, 1999; Kessel, *et al.*, in press; Köhl *et al.*, 1998, 2000;

Schoene & Köhl, 1999).

Results and discussion

In cyclamen, applications of *U. atrum* at four-week intervals resulted in disease control (Fig. 1). In the same experiment it was demonstrated that dead leaves, which had senesced naturally, are essential for disease development. Regular removal of such leaves resulted in almost complete disease control. Additional experiments showed that *U. atrum* can outcompete *B. cinerea* efficiently on dead leaves.

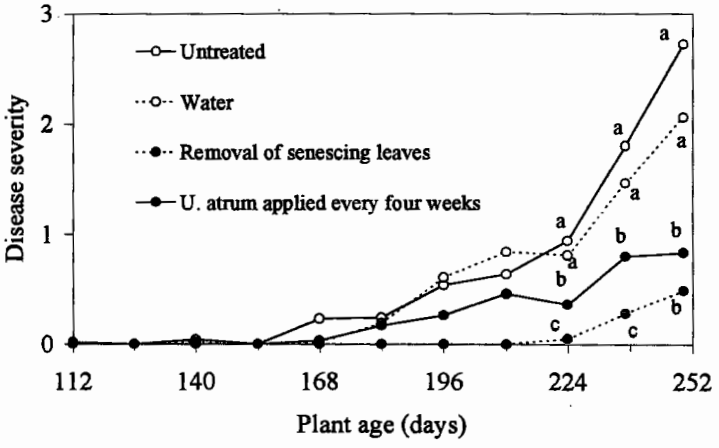

Figure 1. Effect of application of *U. atrum* ($1x10^6$ conidia per ml) and leaf sanitation on grey mould in cyclamen. Disease severity is estimated as number of diseased petioles per plant. Data points of the same plant age with the same letter do not differ significantly (LSD-test; $P \leq 0.05$).

Grey mould control in strawberry was achieved by weekly sprays of *U. atrum* during flowering (Table 1). There was no additional effect of foliar *U. atrum* sprays before flowering to prevent sporulation of *B. cinerea* on dead leaves. A manipulative treatment in which dead leaves were removed regularly, did not result in disease control. Field experiments in grapevine showed that *U. atrum* has a high potential in controlling grey mould of berries (data not presented; Schoene & Köhl, 1999). On the other hand, applications in lilies, targeting *B. elliptica* to prevent lily fire, a severe leaf disease in bulb production of lilies, did not control the disease (data not presented; Kessel, 1999).

From the overall results it can be concluded that three criteria determine the success of *U. atrum* during the interaction with *Botrytis* spp. on necrotic tissues:

(1) A mutual substrate for pathogen and antagonist must be present in the crop;

(2) *U. atrum* must be able to exclude *Botrytis* spp. from this specific mutual substrate;

(3) the mutual substrate must play an important role in the epidemiology of *Botrytis* spp. in the specific crop.

Table 1. Effect of applications of *U. atrum* ($2x10^6$ conidia per ml), leaf sanitation and a fungicide programme on grey mould in annual strawberries.

Treatment	Grey mould at harvest (%)
untreated	8.3 a*
U. atrum – weekly from transplanting	5.9 b
U. atrum – weekly from beginning of flowering	5.6 b
Leaf sanitation	8.3 a
Fungicides	3.6 c

* Values with the same letter are not significantly different (LSD-test; $P<0.05$).

The three criteria were evaluated for different crops in Table 2. In cyclamen, dead leaves are an essential bridge for *B. cinerea* to infect healthy petioles. *U. atrum* can out-compete *B. cinerea* on such dead leaves, so that the application of the antagonist has a high potential for biocontrol in this crop (Köhl *et al.*, 1998, 2000). In grapevine, dead flower parts in the berry cluster stimulate the infection of berries by *B. cinerea*. *U. atrum* may out-compete *B. cinerea* in these tissues, explaining the biocontrol achieved by spraying *U. atrum* in grapes during flowering (Schoene & Köhl, 1999). In annual strawberry systems, crop debris does not significantly contribute to the build-up of inoculum of *B. cinerea* (Boff, submitted). Therefore, an application of *U. atrum* onto dead leaves does not result in disease control.

Table 2. Competitive colonisation of cyclamen, grapevine, strawberry and lily tissues by *Botrytis* spp. and *U. atrum* and role of mutual substrates in *Botrytis* epidemiology.

Crop	Mutual substrate	Competitive abilities in mutual substrate	Importance of mutual substrate in epidemiology
cyclamen	dead leaves	Ua > Bc	Essential
grapevine *	dead flower parts	Ua > Bc	High
annual strawberry	dead leaves	Ua > Bc	Low
annual strawberry	dead flower parts	Ua > Bc	High
lily	Be lesion tissue	Be >> Ua	High
lily	dead tissue outside Be lesion	Ua > Be	Low

Bc: *B. cinerea*; Be: *B. elliptica*; Ua: *U. atrum;* * preliminary results.

However, applications to flowers, in which *U. atrum* can successfully compete with *B. cinerea*, reduce grey mould of strawberry fruit. *B. elliptica* is the superior competitor in lesions on lily leaves caused by the pathogen. Conidia of *B. elliptica* produced in these lesions are the driving force for lily fire. Although *U. atrum* can successfully compete with *B. elliptica* on dead leaf tissue outside *B. elliptica* lesions, no disease control was achieved by applications of the antagonist, since it reduces inoculum production of the pathogen only on a source of low epidemiological importance.

Based on the ecological analysis of the interactions between *U. atrum* and *Botrytis* spp.,

other crops, and possibly other pathogens can be identified in which applications of the antagonist may result in disease control. For example, it has been demonstrated that *U. atrum* can suppress *B. cinerea* development on dead leaves of *Pelargonium* stock plants and in pot roses under commercial growing conditions (Gerlagh *et al.*, unpublished; Köhl & Gerlagh, 1999). Since conidial inoculum in crops of *Pelargonium* and roses is an important driving force for grey mould epidemics (Hausbeck, 1996; Tatagiba, 1998), it can be expected that *U. atrum* may contribute to disease control in both crops.

Acknowledgements

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Biological Control of Fungal and Bacterial Plant Pathogens
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Mechanistic aspects of competitive substrate colonisation by *Botrytis cinerea* and *Ulocladium atrum*

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Abstract: *Ulocladium atrum* 385 is a successful saprophytic fungal biocontrol agent of *Botrytis cinerea* in cyclamen and in grapes. The biocontrol effect is based on competitive interactions between *B. cinerea* and *U. atrum* in necrotic plant tissue. Saprophytic colonisation of natural necrotic tissue is an important step for *B. cinerea* towards infection of healthy cyclamen tissue. The mechanistic principles behind this interaction were however not unequivocally established. Electron microscopy, immuno-histological quantification of mycelium, pairwise inoculations and replacement series confirm the hypothesis of competition for resources as the dominant mechanism of competition.

Key words: biological control, *Botrytis* spp., competition, cyclamen, *Ulocladium atrum*

Introduction

Ulocladium atrum is a saprophytic, fungal antagonist of *Botrytis* spp. *U. atrum* competes with *Botrytis* spp. during the colonisation of necrotic plant tissues. In specific pathosystems, necrotic tissue plays a key role in *Botrytis* epidemiology, facilitating mycelial contact infection of healthy plant parts originating from saprophytically colonised necrotic tissue (Kessel *et al.*, 2001). The mode-of-action of *U. atrum* during competitive substrate colonisation most probably is nutrient competition. This study aims to elucidate mechanistical principles behind the *U. atrum* - *B. cinerea* interaction. Substrate colonisation was studied at the ultrastructural level using electron microscopy and after spray and point inoculation. The resulting internal mycelium and/or sporulation was quantified. Additionally, the interaction was studied using a technique similar to a de Wit replacement series (De Wit, 1960).

Materials and methods

Sterilised, dead onion leaf segments were spray inoculated with conidial suspensions of: *B. aclada* alone, *U. atrum* alone and combinations of *B. aclada* plus *U. atrum* (Köhl *et al.*, 1997). Samples were taken after 6 days incubation at 18°C and prepared for electron microscopy. Additionally, the interaction between mycelia of the above fungi was also studied in samples taken at the meeting point of two colonies growing on malt extract agar plates.

Dynamics of the internal mycelial colonisation of sterilised, dead cyclamen leaf tissue were studied after spray inoculation with conidial suspensions of: *B. cinerea* alone, *U. atrum* alone and a combination of *B. cinerea* and *U. atrum*. Samples were taken in a time series. The mycelial colonisation was determined using a specific fluorescent label for *B. cinerea* and *U. atrum* in combination with image analysis and a stereological conversion as described by Kessel *et al.* (1999). The mycelial interaction occurring at the edge of colonies of *B. cinerea*

and *U. atrum*, growing radially in the same leaf, was studied after point inoculation of sterilised dead cyclamen leaf tissue with conidial suspensions.

Head-start experiments, by applying the biocontrol agent prior to pathogen inoculation, were started after spray inoculation of sterilised dead cyclamen leaf tissue with conidial suspensions of *B. cinerea* and *U. atrum*. A range of head-starts for *B. cinerea* and *U. atrum* were incorporated in the experiment. Sporulation was fully developed and quantified after two weeks incubation at 18°C. The results were analysed using a technique used for the analysis of de Wit replacement series (de Wit, 1960). A key concept is the relative yield [RY, yield (sporulation) in mixed culture divided by the yield in monoculture] for both competing species and the relative yield total (RYT, the sum of the relative yield for both species). The behaviour of the RY for both species and the RYT provides evidence for the nature and strength of interactions between species (Braakhekke, 1980).

Results and discussion

Electron microscopy determined that *U. atrum* mycelium was present and distributed throughout necrotic leaf tissues, completely excluding *B. aclada*. Although *B. aclada* could not be detected in leaf tissues in the presence of *U. atrum*, the interaction between both fungi in agar displayed no obvious signs of hyperparasitism or antibiosis as both fungi seemed unaltered. This would indicate that *U. atrum* relies exclusively on competition to antagonise *B. aclada* (Köhl *et al.*, 1997).

U. atrum and *B. cinerea* displayed similar temporal colonisation patterns in monoculture and in mixed culture. Maximum colonisation levels tended to be lower in mixed culture than in monoculture (Fig. 1). During the first 96 hours of incubation, the mycelial colonisation of the tissue steadily increased indicating that both fungal species grow in close proximity of each other. It is therefore unlikely that antibiosis is involved as the dominant competitive mechanism between *B. cinerea* and *U. atrum* in dead cyclamen leaf tissue.

Dual point inoculations resulted in radially growing colonies of *B. cinerea* and *U. atrum* in necrotic cyclamen leaf tissue. The colonies met without an inhibition zone being formed. At first sight, contact between the colonies resulted in a deadlock, effectively partitioning the

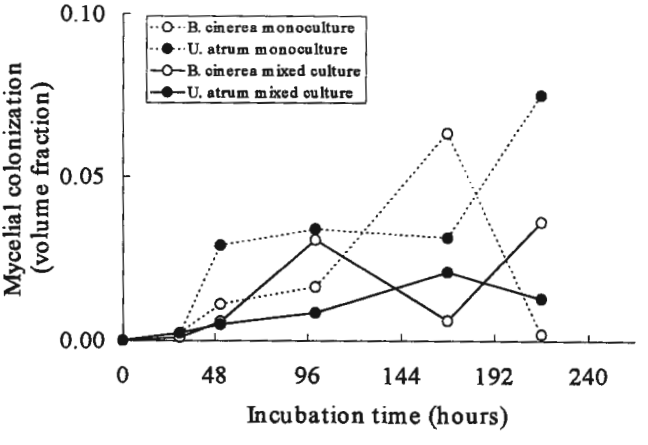
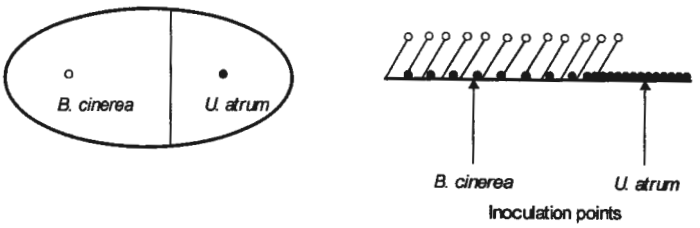


Figure 1. Dynamics of internal mycelial colonisation of sterilised necrotic cyclamen leaves by *B. cinerea* and *U. atrum* following spray inoculation.



A B

Figure 2. Schematic representation of a necrotic cyclamen leaf, point inoculated with *B. cinerea* and *U. atrum* (top view: A) and the resulting sporulation of *B. cinerea* (o) and *U. atrum* (•) from a lateral view point (B).

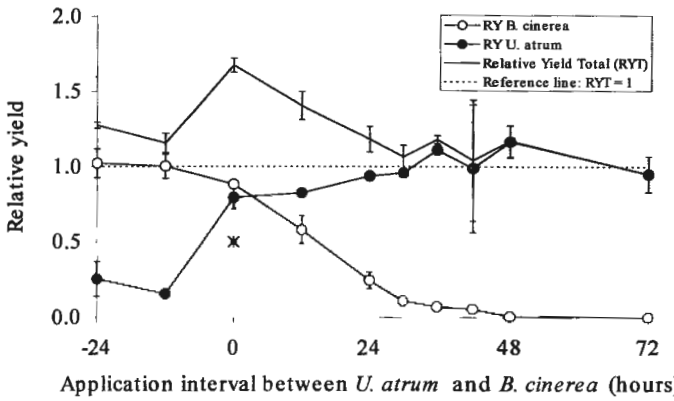


Figure 3. Relative yields (RY, sporulation in mixture / sporulation in monoculture) for *B. cinerea* and *U. atrum* growing in mixed culture in sterilised necrotic cyclamen leaf tissue with head starts for *B. cinerea* (-24 – 0 hours) or *U. atrum* (0 – 72 hours). The dotted line at RYT = 1 indicates a theoretical RYT in case of equal intra- and interspecific competition. The theoretical intersection between both RY lines in case of equal intra- and interspecific competition is indicated by (*).

leaf in a *B. cinerea* area and a *U. atrum* area. Closer observation, using a dissecting microscope at 50 – 100X magnification, showed that low level *U. atrum* sporulation were also found in the entire *B. cinerea* colony. *B. cinerea* sporulation was not found in the *U. atrum* colony (Fig. 2). *U. atrum* mycelium thus continued to expand into an area already colonised by *B. cinerea* mycelium. Based on the assumption of nutrient competition as the dominant competitive mechanism, this indicates that nutrients are available to *U. atrum* within the *B. cinerea* colony whereas no nutrients are available to *B. cinerea* within the *U. atrum* colony.

In the spray inoculated head-start experiments, *U. atrum* suppressed *B. cinerea* significantly when given a head-start of a few hours with complete suppression occurring at head starts of 48 hours or more. Relative yields (RY) of *B. cinerea* and *U. atrum* increased with increasing application advantages (Fig. 3). The relative yield total (RYT) was significantly higher than 1 for application intervals of 0 and 12 hours advantage for *U. atrum*. RYT values larger than 1 indicate some degree of niche differentiation between both fungi and absence of allelopathic effects. From these results it is highly unlikely that antibiosis fulfils a key role during competitive substrate colonisation. In case toxins would have been involved, the RYT line would be concave and fall below 1 (Braakhekke, 1980). The mycelia of *B. cinerea* and *U. atrum* exploit the resources available in necrotic plant tissue. The experimental data presented in this paper supports the hypothesis of nutrient competition as the dominant competitive mechanism. No evidence for involvement of antibiosis was found. Results of the head-start experiments and the point inoculations suggest that the resource spectrum available to *U. atrum* includes the resource spectrum available to *B. cinerea* which is a little wider since *U. atrum* is not excluded from established *B. cinerea* colonies and RYT values larger than one are found. The key nutrients themselves have however not been identified. Considering the short time for colonisation required by *U. atrum* to exclude *B. cinerea* (Fig. 3) it can be assumed that consumption of primary resources in necrotic tissue available to both fungi determines the outcome of the interaction. The outcome of interactions based on primary resource capture is determined on factors such as propagule dispersal, spore germination, mycelial growth possession of suitable enzymes and tolerance to adverse conditions associated with the resource. In a biocontrol setting, this type of interaction has the advantage that the biocontrol effect can be established well before the pathogens arrive. Thus, the ability of *U. atrum* to survive in the phyllosphere and rapidly colonise a substrate might be two of the key characteristics that make *U. atrum* a successful biocontrol agent.

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Biological Control of Fungal and Bacterial Plant Pathogens
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Selection of native yeasts for biological control of postharvest rots caused by *Botrytis allii* in onion and *Rhizopus stolonifer* in tomato

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Abstract: The biological control of phytopathogens during postharvest through the use of yeasts has shown consistent and promising results. The main objective of this work was to evaluate the biocontrol activity of native yeasts against the grey mould caused by *Botrytis allii* in onion and the soft rot of tomato caused by *Rhizopus stolonifer*. Out of 67 native yeast isolates obtained from vegetative material and soil, 10 isolates capable of growing both at 6°C and 22°C were selected. Yeast protection tests against *B. allii* in onion and *R. stolonifer* in tomato were carried out under controlled temperature conditions (6°C and 22°C). The results obtained showed that the 10 antagonistic microorganisms selected offered some protection against *B. allii* and *R. stolonifer*, which was evident by a lower development of disease symptoms of the infection caused by the two pathogens. Only three yeasts, Lv006, Lv027 and Lv031, identified as *Pichia onychis* in the case of bulb onion, resulted in protection higher than 85% at both storage temperatures (6°C and 22°C). In the case of tomato, the yeasts Lv050 (identified as *P. anomala)* and Lv031 and Lv027 (both identified as *P. onychis*) resulted in protection of more than 85% at the same temperature conditions.

Introduction

Harvested fruit and vegetables are highly perishable agricultural commodities. The major limiting factor in storage of fruits and vegetables is spoilage losses unstained during production, transportation, and storage (Wilson & Wisniewski, 1989). Post-harvest rots result in lowered quality and substantial economic losses to growers. Control of post-harvest diseases of fruits and vegetables relies heavily on chemical fungicides which pose a health risk when used on products that are consumed fresh, and are hazardous to the environment (Clifforf & Lester, 1988; National Research Council, 1987). Among the alternative methods to synthetic fungicides, naturally occurring antagonistic yeasts have been the most extensively studied (Janisiewicz, 1994). In Colombia, approximately 30% of harvested vegetables such as tomato and onion are discarded because of spoilage due to postharvest rots, which lowers quality and results in substantial economic losses to the growers. Therefore, the objective of this research was to investigate the biocontrol potential of native yeasts against *Rhizopus stolonifer* and *Botrytis allii* which are important postharvest pathogens of tomato and onion, respectively.

Materials and methods

Tomato (*Lycopersicon esculentum* var. milano) and onion fruits (*Allium cepa* var. Yellow granex) were purchased from commercial orchards. Strains of *R. stolonifer* and *B. allii* were isolated from decayed tomato and onion, respectively. Strains of the pathogens were cultivated in PDA at 22°C. Spore suspensions were prepared by scraping 15 days old colonies

with a spatula, suspending the propagules in 0.05% Tween 80, prepared with sterile water and diluting them to the required concentrations, as determined with a Neubauer cell.

Potential antagonistic yeasts isolated from flowers, fruits, rhizosphere and soil of tomato, onion and other fruits were cultivated in YM medium (yeast extract, malt) at 6°C and 22°C, and isolates capable of growing at both temperatures were selected for protection tests.

The protective effect of native yeasts was studied according to the methods described by Janisiwicz (1987) and Jijakli *et al.* (1993) by using the following method for their inoculation. Wounded sites were first inoculated with 25 µl of yeast suspension containing 10^7 cells per ml (measured for each isolate at 595 nm). Within 24 h of incubation, the wounds of the fruits treated with the potential antagonists were inoculated with 25 µl of the selected suspension of the pathogens ($1x10^4$ sporangiospores per ml of *R. stolonifer* and $1x10^6$ conidia per ml of *B. allii*). Inoculated fruits were incubated at 6°C and 22°C, on wet filter paper in closed foil containers (20cm x 14 cm x 4 cm).

For protection tests, symptom intensity was measured by the diameter of the lesion every two days during 6 days and 28 days for tomato incubated at 6°C and 22°C, respectively, and during 18 days for onion incubated at both temperatures. There were 15 replications of fruits (2 wound each) per treatment. Treatments were arranged in a completely randomized design. All experiments were repeated three times. Analysis of variance was performed on data from rot development with the General Lineal Model (GLM) procedure of the Statistical Analysis System (SAS). Means separation were calculated by Tukey method ($P \leq 0.05$). For evaluating protective effect of yeasts, results were expressed as protection percentages according to Jijakli (1996).

Results

Symptoms of the soft rot caused by *R. stolonifer* in tomato and grey mould caused by *B. allii* in onion were reproduced under the described experimental conditions.

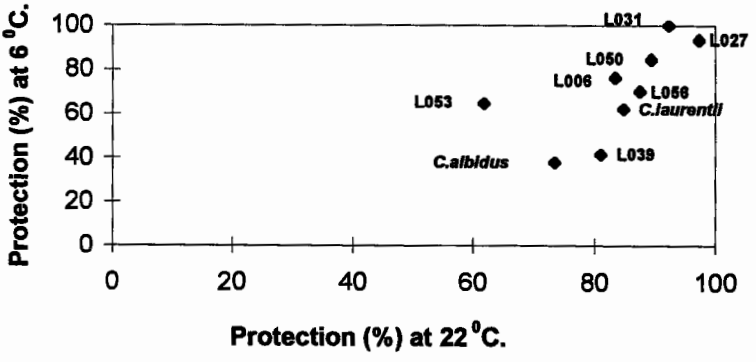

Figure 1. Protection obtained with different yeast strains against *Rhizopus stolonifer* in tomato. Strains Lv027 and Lv031, identified as *Pichia onychis* and strain Lv050, identified as *P. anomala*, were selected for exhibiting protection higher than 85%, at 6°C and 22°C.

Preliminary screening to determine development at 6°C and 22°C of a total of 67 yeasts, allowed to select 20 of them, that exhibited capacity for growing at both temperatures among these isolates, 11 were obtained from the surface of fruits, 9 were isolated from soil and

rhizosphere, and 2 were obtained from a yeast collection. According to the origin data, 9 and 10 yeasts, respectively, were selected and evaluated against *R. stolonifer* in tomato and *B. allii* in onion. The evaluated isolates exhibited their ability to control the pathogens at 6°C and 22°C, as compared with untreated controls. However, for *R. stolonifer* control, three isolates (Lv027 and Lv031 and Lv050) were finally selected for their protective level which ranged from 89.53% to 97.37% and from 84.72% to 100%, respectively, when fruits were incubated at 6°C and 22°C (Fig. 1). In addition, three isolates (Lv006, Lv027 and Lv031) were selected after evaluation of protective level against grey mould produced by *B. allii* in onion, ranging from 87.80% to 93.40% and from 86.20% to 93.44%, respectively, when fruits were incubated at 6°C and 22°C (Fig. 2). Among these, two yeasts Lv027 and Lv031, identified as *P. onychis*, exhibited enhanced protection (superior to 85%) against both pathogens at both incubation temperatures (Table 1).

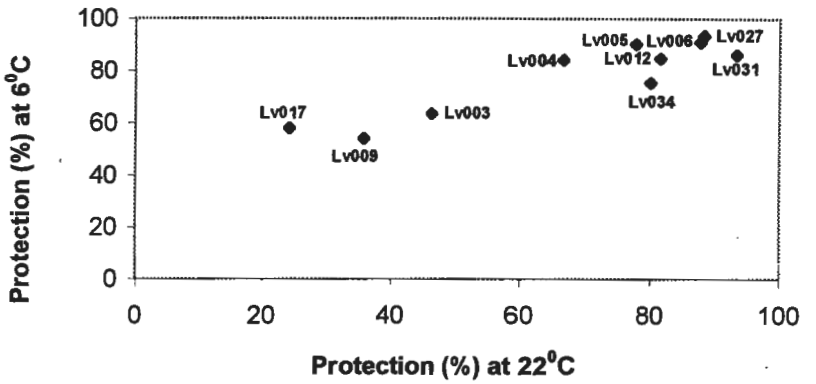

Figure 2. Protection obtained with different yeast strains against *Botrytis allii* in onion. Strains Lv027 and Lv031, identified as *Pichia onychis* and strain Lv050, identified as *P. anomala*, were selected for exhibiting protection higher than 85%, at 6°C and 22°C.

Table 1. Criteria used for selecting yeasts with potential biocontrol activity against *R. stolonifer* in tomato and *B. allii* in onion (Initial number of yeast strains: 67).

Selection criteria	Number of Strains
• Growth capacity at 22°C	67
• Growth capacity at 6°C	29
• Geographic distribution and host	14
• Yeasts exhibiting protection higher than 85%	Yeasts selected for *B. allii* control: Lv031 (*P.onychis*); Lv027 (*P.onychis*); Lv006 (*P.onychis*) Yeasts selected for *R. stolonifer* control: Lv031 (*P.onychis*); Lv027(*P.onychis*); Lv050 (*P. anomala*)

The ability of all yeast isolates investigated in the present work to reduce lesion diameter and, in most cases, the incidence of grey mould caused by *B. allii* in onion and the soft rot caused by *R. stolonifer* in tomato indicates that screening collection of yeasts from habitats other than fruits or bulbs may be helpful for the discovery of new biocontrol agents, which confirms the results found by Filonow *et al.*, (1996) on the biological control of *B. cinerea* in apple.

Two isolates of *P. onychis* reduced the severity of disease caused under postharvest conditions by *B. allii* in onion and *R. stolonifer* in tomato at 6^0C and 22^0C. In both cases, control was consistent in all replications. This is in contrast to earlier reports on control of plant pathogens, where the control was often impressive but not consistent or adequate enough for commercial development (Dik *et al.*, 1992).

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Biological control of *Botrytis cinerea* of pine seedlings in a forest nursery in Sweden

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The most common pathogen in Swedish forest nurseries is *Botrytis cinerea*. Most nurseries treat their seedlings with fungicides to prevent grey mould infections. Biological control has so far not been tested. However, biological control agents are used for example for treatments of grey mould infections in strawberries. In experiments on black spruce in Canada the fungus *Gliocladium roseum* reduced grey mould infections and influences of the pathogen on seedlings (*Can. J. Plant Pathol.* 18; 7 - 13, 1996).

In one forest nursery, pine (*Pinus sylvestris*) seedlings were sprayed with the commercial products Mycostop® (*Streptomyces griseoviridis*), Binab TF.WP® (*Trichoderma harzianum* and *T. polysporum*) and GlioMix® (*Gliocladium sp.*). Apart from these, a fungicide treatment was applied according to routine nursery practise. The experimental plot was subjected to spontaneous grey mould infections. Similar experiments were conducted in the laboratory where seedlings were kept in a growth chamber (20°C, 95% RH, 16h day-length). In those experiments seedlings were treated with spores of *Botrytis cinerea* as well as with the control agents.

The products inhibited grey mould infections, both when grey mould spores were sprayed on the seedlings in the laboratory experiment as well as on spontaneous grey mould infections in the forest nursery. However in all cases the variation within treatments was very high. Binab TF.WP® and GlioMix® performed better than Mycostop® and were able to suppress grey mould as effectively as the fungicide Euparen®.

Screening of epiphytic fungi from olive leaves for the biological control of *Spilocaea oleaginea*

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Olive scab caused by *Spilocaea oleaginea* (*Cycloconium oleaginum*) is widespread in many olive growing regions, including southern Spain. Similar to other scab diseases, the pathogen attacks mainly the olive leaf cuticle and foliar fungicides are the major disease control measures. No studies on biological control of this pathogen have been conducted. This work attempts to evaluate the antagonistic activity of some fungi obtained from healthy and scab-infected olive leaves. A total of 80 isolates were obtained from several olive orchards in southern Spain. Isolates were classified in the fungal genera *Aureobasidium*, *Cladosporium*, *Coniothyrium*, *Phoma*, *Alternaria* and *Aspergillus*.

An *in vitro* assay was used to determine the effect of the candidate antagonists on the germination of the conidia of *S. oleaginea*. Germination tests were performed in sterile well plates. Ten µl of a conidial suspension (10^5 conidia/ml) of *S. oleaginea*, plus 10 µl of a conidial suspension (10^6 conidia/ml) of the antagonist fungus, and 50 µl of sterile water were placed in each of at least three wells per candidate. Percentage of germination and germ tube length were evaluated after 48 hours in the dark at 15°C. Two *in vivo* assays were evaluated to determine the ability of the antagonist to reduce infection and development of the disease. The first assay was performed using individual olive leaves detached from plants, and the second one with open-pollinated olive seedlings. Detached leaves were inoculated by placing three droplets along the midrib, one droplet containing *S. oleaginea* alone (control), the second droplet with *S. oleaginea* plus the antagonist, and the third droplet with the antagonist alone. Olive seedlings were sprayed with a conidial suspension of the antagonist and, after 24 hours, they were sprayed with a conidial suspension of *S. oleaginea*. In both assays, antagonism was assessed by the relative ability of each fungal isolate to restrict incidence and size of the scab lesions respective to the control.

Six isolates inhibited the percentage of germination by greater than 50% compared to control. With the rest of the isolates, inhibition varied from 45% to 10%, and 19 isolates did not reduce germination or their inhibition was less than 10%. In general, isolates that inhibited the percentage of germination also reduced germ tube elongation. In the detached leaf assay, none of the isolates completely inhibited infection, and only two isolates of *Phoma* and *Alternaria* reduced lesion size by 75%, in respect to the control. Four isolates showed some antagonistic affect in inoculated seedlings. In these cases, plants treated with antagonists showed less than 25% scab lesions on leaf surfaces compared to 60% in the non-treated control seedlings. The large variation between *in vitro* and *in vivo* assays is in agreement with results from other experiments with similar pathosystems, such as *Venturia inaequalis* in apple.

Biological Control of Fungal and Bacterial Plant Pathogens
IOBC wprs Bulletin Vol. 24 (3) 2001
pp.189 - 192

Combination of fluorescent pseudomonads with acibenzolar-S-methyl induces synergistic disease resistance in tomato against bacterial and fungal pathogens

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Abstract: Application of selected isolates of *Pseudomonas fluorescens* (G309) and *Pseudomonas* spp. (CW2) in combination with acibenzolar-S-methyl (BTH) at 10-100µM showed synergistic effects against tomato diseases caused by *Fusarium oxysporum* f.sp. *lycopersici* and *Pseudomonas syringae* pv. *tomato* . In order to explore these synergistic effects, mechanisms of interaction between fluorescent pseudomonad isolates G309 and CW2 with BTH have been studied *in vitro* and *in vivo*. Activation of resistance in tomato and tobacco plants by combinations of fluorescent pseudomonad isolates (G309 and CW2) with BTH (10 and 20µM) was not salicylic acid dependant according to the HPLC analysis and activation of resistance against tobacco mosaic virus (TMV) in transgenic tobacco plants that constitutively express a salicylate hydroxylase gene (nahG). On the other hand, the content of shikimic acid among the 23 phenolic substances analysed was very high especially in the roots of tomato plants 4 and 8 days after treatment of BTH in combination with bacteria. Combinations of BTH (10 and 20µM) with fluorescent pseudomonad isolates G309 and CW2 activated the accumulation of the PR-1a protein in the transgenic tobacco plants (PR-1a;GUS) before and after inoculation with TMV. Reisolation of the transformed isolate G309-384 (containing the gfp-gene) from the tomato rhizosphere treated with BTH 10µM showed an increase in the fluorescent intensities.

Keywords: fluorescent pseudomonads, acibenzolar-S-methyl (BTH), salicylic acid (SA), shikimic acid (ShA), PR-1a;GUS and green fluorescent protein (gfp)

Introduction

In recent years, there has been an increased interest in improving the efficacy and reliability of the biological control agents by combining i.e. fluorescent pseudomonads, yeasts and/or antagonistic fungi (de Boer *et al.*, 1999; Fakhouri & Buchenauer, 1998; Raupach & Kloepper, 1998), with resistance inducers as DL-3-aminobutyric acid (Vogt & Buchenauer, 1997), or with a number of other compounds such as sugars and chitosan (Benhamou *et al.*, 1998; El-Ghaouth *et al.* 2000). Several studies indicated that combination of biocontrol agents resulted in improved biological control efficiency against various bacterial and fungal diseases (de Boer *et al.*, 1999; Raupach & Kloepper, 1998). Colbert *et al.* (1993) reported that amendments of sodium salicylate (1,0µg/ml) to agricultural ecosystems increased the population densities of *Pseudomonas putida* PpG7 in low-inoculum soils up to 26-and 29-fold in rhizosphere and nonrhizosphere soils.

The aim of this work was to study the possible benefits of the combined application of fluorescent pseudomonads with BTH with respect to improve the efficiency of controlling soil and air-borne plant pathogens on tomato. It was also of interest to gain an insight into the interactions and the mechanism of action *in vivo* of BTH and fluorescent pseudomonads.

Material and methods

Plants and pathogens

Tomato seeds (*Lycopersicum esculentum* L) cultivar TipTop were used in all experiments. Seeds of transgenic PR-1a:uidA tobacco plants were obtained from D. F. Klessig (Rutgers University, NJ) and seeds of transgenic NahG-10 tobacco were supplied by K. Lawton (Novartis Ltd., Research Triangle Park, USA). Non-transgenic tobacco (*Nicotiana tabacum* cv. Xanthi-nc), having the *N* gene derived from *Nicotiana glutinosa* for hypersensitive resistance to TMV, was used to study resistance induction. The wilt pathogen of tomato *Fusarium oxysporum* Schlecht. f.sp. *lycopersici* (DSM 62059) was obtained from German Collection of Microorganisms, Braunschweig and the isolate of *Pseudomonas syringae* pv. *tomato* was supplied by E. Moltmann (Landesanstalt für Pflanzenschutz, Stuttgart).

Greenhouse experiments

Three-week-old tomato seedlings were treated by soil drench with fluorescent pseudomonads ($2{,}0x10^8$cfu/ml; 50ml/pot), or BTH (100 and 200µM; 50ml/pot) and in combination. For Fusarium wilt, the plants were post-inoculated with the fungus ($5.2x10^7$ spores/ml) 6 days after treatment or at the same time as treatment the tomato plants with the bioagents and BTH. The wilt and browning index were evaluated one month after inoculation.

Quantification of salicylic acid and shikimic acid

The total contents (free plus conjugated) of salicylic acid (SA) and shikimic acid in tomato leaves were determined as described by Mölders *et al.* 1996 with some modifications.

Analysis of PR-1a gene expression

To analyse PR-1a gene activation after treatment, GUS activity was determined according to Conrath *et al.* 1997. The fluorescence of 4-methylumbelliferone (MU) formed after hydrolysis of 4-methylumbelliferoneglucoroinde (MUG) was determined by spectro-fluorometer (Hitachi F2000, Japan). GUS activity was calculated in nanomoles MU per mg fresh weight of leaf per hour at 37°C.

Population dynamics of the transformed Pseudomonas fluorescens G309-384 (gfp)

The fluorescent intensity was recorded by using the spectrofluorometer (Hitachi F2000, Japan) with the filter set including a bandpass excitation light source of 488nm via a fluorescence detector set with a longpass emission light of 520nm.

Results and discussion

Under greenhouse conditions, combinations of G309 or CW2 with BTH100 and 200µM completely inhibited the infection of tomato plants by *Fusarium oxysporum* f.sp. *lycopersici* (Fol) which were pretreated either 6 days before infection or at the same time of inoculation with Fol, while single treatments were less effective (Fig.1). In addition, soil application of G309 with BTH 5µM reduced disease incidence and severity of *Pseudomonas syringae* pv. *tomato* (Pst) in tomato plants to 17,35 and 5,66% while control plants showing corresponding disease values of 56,9 and 46,5%, respectively (data not shown). By repeating these experiments many times, the fluorescent pseudomonad isolates in combination with BTH showed very stabile and reliable antagonistic effect against Fol and Pst. Acibenzolar-S-methyl (BTH) is one of the non-toxic synthetic resistance inducer used against plant pathogens known under the commercial name Bion®. It has been shown that BTH induces disease resistance by expressing of genes coding for a wide spectrum of pathogenesis related proteins (PR1, 2, 3, 4 and 5). Furthermore, BTH action is independent on the SA accumulation (Friedrich *et al* 1996). According to our results, BTH in combination with fluorescent

pseudomonads mediates local accumulation of shikimic acid (Fig. 2) but not local or systemic accumulation of SA (Data not shown).

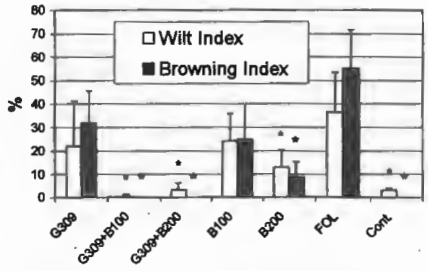

Figure 1. Effect of *Pseudomonas fluorescens* isolate G309 and acibenzolar-S- methyl (B100 and B200 μM) on wilt and browning index of Fusarium.

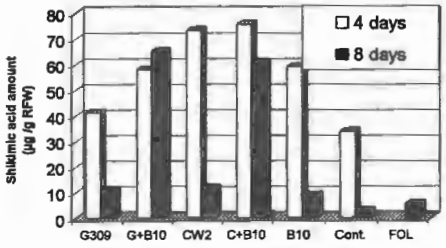

Figure 2. Local accumulation of free and conjugated shikimic acid (ShA) in tomato roots.

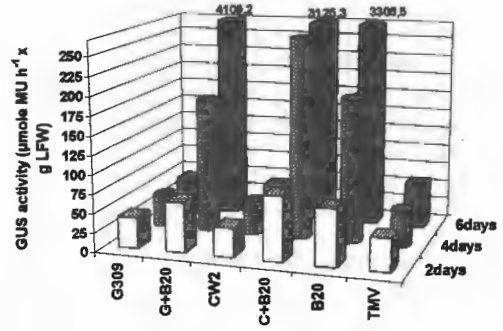

Figure 3. Expression of GUS activity in transgenic tobacco plants after inoculation with TMV.

BTH is one of the potent synthetic inducers activating the expression of the PR-1 protein group in tobacco (Friedrich *et al.* 1996). In our studies, the transgenic tobacco plants were challenge inoculated with TMV 4 days after treatment with BTH and pseudomonads. The highest expression of the GUS gene was reported 4 days after inoculation in the combined treatment of BTH 20µM with CW2. Furthermore, increased GUS activities were also more evident in the treatment G309 with BTH 20µM 6 days after challenge inoculation (Fig.3).
One of the important factors which may explain the synergistic effect of BTH in combination with fluorescent pseudomands is the positive effect of BTH on the population densities of fluorescent pseudomonad isolates G309 and CW2 not only *in vitro* but also *in vivo*. In addition, these positive effects of BTH on the transformed isolate G309-384 having the gfp-gene were also confirmed *in vivo* by determining the fluorescent intensities by using spectrofluorometer (Data not shown). In conclusion, the synergistic effect exerted by the combined treatment of fluorescent pseudomonads with BTH has the potential of a promising strategy to control diseases caused by soil and air-borne plant pathogens in agriculture, especially in integrated pest management programs.

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Biological Control of Fungal and Bacterial Plant Pathogens
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Establishment, survival and activity of biocontrol agents applied as a mixture in strawberry crops

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Abstract: Biocontrol of *Botrytis cinerea* by a mixture of a yeast and one of two bacterial isolates was followed during three years of commercial-like greenhouse experiments. Establishment and survival of the yeast and the two bacterial populations on strawberry leaflets and fruitlets were recorded in two experiments. Populations of the bacteria did not differ significantly in separate applications as compared to those in a mixture with the yeast. Similar results were obtained for the yeast. Moreover, in three out of 27 comparisons the yeast populations in the mixture were significantly higher than when applied alone. Application of the mixture resulted in significant disease reduction as compared to the control treatments. In one experiment, the biocontrol agents applied alone, reduced the number of diseased fruits by 50% whereas their mixture resulted in 75% reduction and additive disease control. In a second experiment, the mixture between the same yeast isolate and another bacterial isolate resulted in synergistic disease suppression as compared with their separate application.

Key words: *Botrytis cinerea*, phyllosphere, antagonism, population, establishment, survival

Introduction

Saprophytic bacteria, yeasts and filamentous fungi are common inhabitants of plant surfaces (Elad *et al.*, 1994; Guetsky *et al.*, 2001; Wilson *et al.*, 1992, 1993). Reports on the efficacy of yeasts and bacteria vary; while in some studies the isolates of yeasts and bacteria used result in low to moderate or high disease suppression, sometimes the biocontrol agents fail to reduce disease adequately (Elad *et al.*, 1994; Guetsky *et al.*, 2001). Occasionally, introduction of antagonists that were highly effective under controlled environments to the phyllosphere of commercially grown plants is only moderately effective and sometimes, totally ineffective. Introduction of two or more biocontrol agents, assuming that each one of them has different ecological requirements and different modes of action, may facilitate effectiveness under diverse conditions and result in increased control efficacy and consistency (Guetsky *et al.*, 2001). A basic requirement for successful mixture of microorganisms for biological control is the absence of antagonism between each other when applied in a mixture. It was shown that genotypically and hence ecologically similar strains would exhibit more competitive relationships than dissimilar strains (Janisiewicz, 1996; Janisiewicz & Marci, 1992). High levels of population establishment and survival in the phyllosphere may be important for the success of biocontrol.

Grey mould (*Botrytis cinerea*) inflicts serious losses in many crops. In strawberry the fungus attacks different organs. Chemical application is the major means by which grey mould is controlled. However, this may occasionally be ineffective due to the ability of the fungus to develop resistance to fungicides of several groups, if applied repeatedly (Hunter *et al.*, 1987). Thus, the development of nonchemical control measures is of great importance to

the strawberry industry worldwide. Since the ecological requirements of the bacterial and yeast isolates are markedly different, it was hypothesized that a mixture of the two agents would not result in antagonism as compared to applications of each organism alone. Moreover, it was hypothesized that the mixture would result in improved biological control of *B. cinerea* as compared to the separate application of these microorganisms. In the present work, we tested these hypotheses by application of the two biocontrol agents, separately or in combination for biocontrol of grey mould in strawberry.

Material and methods

Organisms

Three biocontrol agents, a yeast (Y2) and two bactera (B16 and B17) were used in all trials. Identification of the isolates is under progress. Y2 was grown on PDA for 24 to 48 h at 25°C before use. B16 and B17 were grown on nutrient agar medium (Difco) or on LB agar medium (Difco) for 24 h at 30°C. Cell concentrations were determined and adjusted to 10^7 cells/ml. Strawberry (*Fragaria ananassa* L.) plants (cv. Oso Grande) were used in all experiments. Transplants were planted at the beginning of September each year in 1-l plastic pots (containing a growth medium based on peat) and maintained in a greenhouse under temperatures of 20 to 30°C. Plants were irrigated and fertilized as needed. For the disease control experiments, strawberry plants were grown in vertical columns containing 32 potted plants (each column will be referred hereafter as an experimental plot).

Establishment and survival of the biocontrol agents on strawberry organs

Establishment and survival of Y2 and B16 were studied in two experiments on strawberry leaflets and fruitlets. The same yeast isolate was used in all experiments. Isolate B16 was used in 1996, while in 1997 isolate B17 was applied. The biocontrol agents were sprayed to runoff in 6 to 10-day intervals. Treatments consisted of control, yeast, bacterium and combination of the yeast and bacterium. There were 5 replicates (containing 8 pots each) per treatment. Leaflets and fruitlets were collected randomly from different plants and from different parts of the canopy (up and down, inside and outside) and were placed into 250 ml Erlenmeyer flasks containing 100 ml saline solutions (0.85% NaCl) and Tween-80. Flasks were shaken in a rotatory shaker for 60 minutes. The suspensions were serially diluted and 10 µl drops from the different dilutions were plated on the appropriate agar media. The quantitative results for cm^2 leaflet or for fruitlet were expressed as log of the colony forming units.

Disease control in greenhouse

Suppression of *B. cinerea* by sole and combined applications of the yeast and bacterium was examined in two experiments. The treatments were the same as those for the survival and establishment of populations of the biocontrol agents. Each treatment was repeated in five experimental plots. The biocontrol agents were sprayed alone or in combination at weekly intervals. Disease severity was assessed since the first appearance of disease in 3 to 7-day intervals and continued during the whole period of each experiment. In each assessment numbers and weights of diseased fruits and fruitlets per plot were recorded.

Data analysis

Statistical analyses of the data were performed using the JMP-in software, version 3 for Windows (SAS Institute Inc.). Survival of the biocontrol agents over time was quantified by means of regression analysis. Differences in population size between sole and combined application of the biocontrol agents were determined using the least significant difference (LSD) test.

Results and discussion

Survival and establishment of biocontrol agents on strawberry fruitlets and leaflets
Population size of the yeast Y2 and bacterium B17 applied alone or in combination was monitored on strawberry leaflets and fruitlets in two experiments. Populations of Y2 when applied alone did not differ significantly from its population when applied in the mixture with the bacterium on leaflets and on fruitlets (Fig. 1A). Moreover, in three out of 27 comparisons the yeast population was significantly higher in the combined treatment as compared to that of each organism alone. Similarly, population levels of the bacterium on leaflets and fruitlets when applied alone, did not differ significantly when applied in mixture with Y2 (Fig. 1B). This implies that there was no antagonism between the yeast and the bacterium applied in mixture to strawberry leaflet and fruitlets.

Control of* B. cinerea *by sole and combined applications
The efficacy of a mixture of biocontrol agents as compared to their sole use in suppression of *B. cinerea*, was examined in two experiments in a commercial-like greenhouse. In the first experiment sole application of B16 or Y2 reduced the number of infected fruits significantly, as compared to the untreated plots (Fig. 2A). Combined application of the two biocontrol agents further improved disease suppression and reduced the number of infected fruits significantly, relative to application of each biocontrol agent alone (Fig. 2B). In that case, control effect of the mixture was additive. In a second experiment, applications of B17 and Y2 alone did not result in significant disease suppression. However, application of a mixture of the two biocontrol agents resulted in a synergistic effect and significant disease suppression. Mixtures between the two biocontrol agents resulted in improved disease suppression as compared to control treatments, even when the yeast or the bacterium applied alone were not effective.

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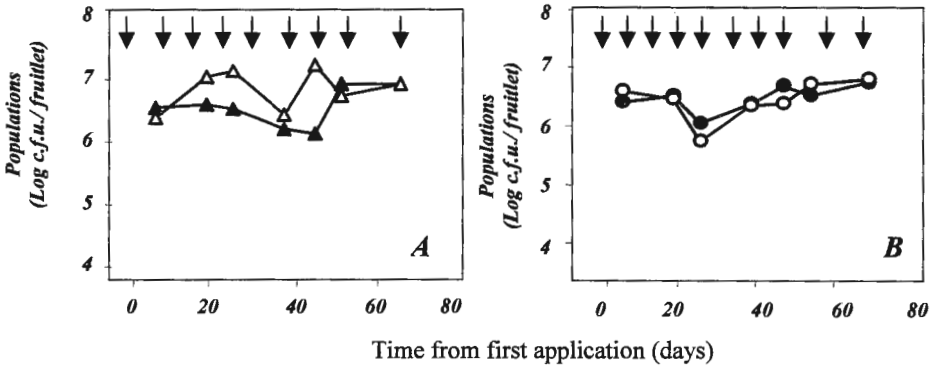

Figure 1. Survival and establishment of the yeast Y2 (A) and the bacterium B17 (B) on strawberry fruitlets at 1997. Filled symbols: populations of the microorganisms when applied alone. Open symbols: populations of the microorganisms when applied in mixture.

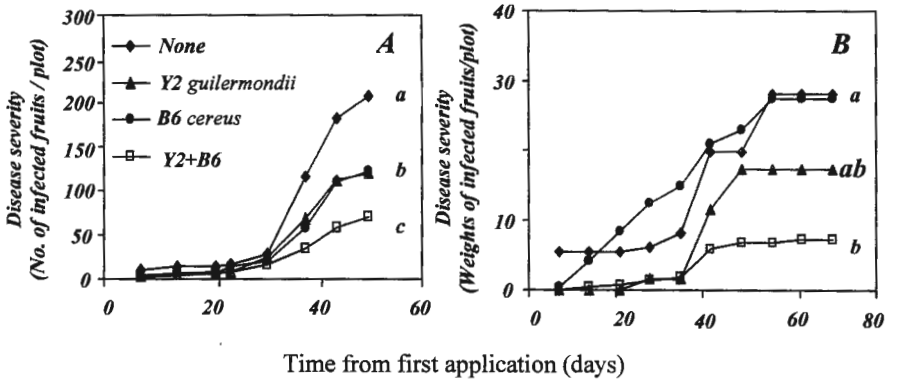

Figure 2. Control of *Botrytis cinerea* on strawberry by the bacterial isolates B16 (A) and B17 (B), the yeast Y2 or the mixture of the yeast and a bacterium, under conditions of a commercial-like greenhouse. Influence of the biocontrol agents on number of infected fruits per plot (A) and on the weights of infected fruits per plot (B).

Biological Control of Fungal and Bacterial Plant Pathogens
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Combining microbial seed treatment with priming of carrot seeds for control of seed borne *Alternaria* spp.

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Abstract: Carrot seed naturally infected with the pathogens *Alternaria radicina* and *A. dauci* were primed or bioprimed with the fungal antagonist *Clonostachys rosea* (isolate IK726) for 8 days at a constant seed moisture content of 40%. During priming incidences of *A. radicina* and *A. dauci* increased from about 32-35% to 59% and 86%, respectively. In contrast biopriming reduced the incidence of *A. radicina* to 4% and that of *A. dauci* to 2%. Effects of priming treatments and IK726-coating of unprimed seeds on seedling establishment were evaluated in sand tests. Primed seeds had a lower final seedling stand than untreated seeds which in turn had a significantly lower seedling stand than that obtained with biopriming or with IK726 coating. Thus, biopriming carrot seeds with IK726 almost eradicated the *Alternaria* pathogens on the seeds and improved the final seedling stand.

Key words: Seed priming, *Clonostachys rosea* IK726, *Alternaria radicina, A. dauci*, carrot

Introduction

Seed-priming is used commercially in many horticultural crops, including carrot, as a tool to increase speed, and uniformity of germination and final stand under environmental stress conditions. If seeds are infected or contaminated with pathogenic or saprophytic fungi, their growth can be enhanced during priming thus resulting in undesirable effects on plant health (Nascimento & West, 1998). Fungicides have been applied for elimination of microbial growth during priming processes (Maude *et al.*, 1992) but these fungicides are increasingly restricted in Europe. Therefore, application of antagonistic microorganisms during priming could be an environmentally friendly strategy for eradication of seedborne pathogens. Inoculation of seeds with biological control agents (BCAs) in combination with priming (biopriming), has in several cases been reported to enhance and stabilize the efficacy of BCAs. However, such approaches have mainly been used to control of soilborne seedling diseases (Welbaum *et al.*, 1998). An isolate of the antagonistic fungi *Clonostachys rosea* (isolate IK726) has a high potential for controlling cereal seedborne diseases under field conditions (Knudsen *et al.*, 1994, Jensen *et al.*, 2000). In recent experiments with carrot (unpublished), IK726 controlled pre- and post-emergence death caused by seedborne *A. dauci* and *A. radicina* as effectively as the fungicide iprodione. The objectives of the present work were to (1) to evaluate the effect of priming and IK726 biopriming on the incidence of *A. dauci, A. radicina* and *A. alternata* on naturally infected carrot seeds, and (2) to test the effect of priming and biopriming on seedling emergence and post emergence infection in sand tests.

Materials and methods

Plant material and antagonist

Carrot seeds of the variety Royal Chantenay Rola (germination 55%) naturally infected with *A. radicina* (29%) and *A. dauci* (11%) and contaminated with *A. alternata* (30%) were used throughout the experiments. Isolate IK726 of the fungus *C. rosea*, (Knudsen *et al.*, 1995) was used for coating and priming carrot seeds.

Treatments

Priming: seeds were imbibed in aerated water (50 g seed/500 ml H_2O) for 16 hours. Seeds were then surface dried for about 1 h and the water content determined whereupon seeds were dried back to a final water content of 40%. These seeds were incubated in plastic containers with two 1 mm holes in the lid at 15°C for a further 7 days. For priming with IK726, a clay preparation of the fungus (2 x 10^9 cfu/g) was applied during imbibing of seeds (2.5 g clay inoculum/500 ml water). One set of samples for blotter-tests and bioassays was taken immediately after drying to 40% water content (day 1) and a second set were assayed after a further 7 days incubation (day 8), when seeds were dried overnight. *Coating of unprimed seeds:* 2 g of seeds were immersed for 5 minutes in either 8 ml water or in 8 ml of a water suspension of the IK726 clay preparation (0.01 g/ml) used above. Seeds were air dried for 1 h in a laminar hood before sowing.

Assessments of pathogens and disease symptoms

Blottertest: Freeze-blotter tests were conducted according to ISTA rules for detection of *Alternaria* spp. After 7 and 10 days incubation under near-UV light (NUV), presence of *A. radicina, A. dauci* and *A. alternata* on 4 x 100 seeds was recorded under a stereomicroscope for primed and bioprimed seeds. *Bioassays:* 4 x 50 seeds per treatment were sown in water-moistened sand (3:1 v/v) and incubated in a growth chamber (12 h light at 20°C/12 h darkness at 15°C). Plant emergence was recorded regularly from 6 to 28 days after sowing. Dead and wilted plants were removed and checked for *Alternaria* infection after incubation under NUV. Bioassay results are given as number of healthy plants per pot (infected plants subtracted). Data for each assessment time were analyzed separately.

Results

Priming and biopriming of carrot seeds naturally infected with *A. radicina, A. dauci* and *A. alternata* significantly affected incidence of the fungi as seen in Table 1.

Table 1. Presence of *Alternaria radicina, A. dauci* and *A. alternata* on carrot seeds primed, with or without *Clonostachys rosea* (IK726), one or eight days at 15°C.

Seed treatment	Pathogens identified in blotter tests	Incidence of pathogens (%)	
		Primed for 1 day	Primed for 8 days
Primed	*A. radicina*	34.5±7.2	59.0±6.1
	A. dauci	32.0±5.0	86.3±6.4
	A. alternata	33.0±3.7	74.3±8.7
Primed with IK726	*A. radicina*	6.0±1.4	4.3±1.3
	A. dauci	5.3±1.9	2.0±1.4
	A. alternata	2.5±1.0	2.5±1.7

After priming for one day the incidence of *A. radicina*, *A. dauci* and *A. altanata* was between 32-35% while the incidence of the three species was between 2.5%-6% on bioprimed seeds. Thus biopriming reduced both pathogens by 83% in comparison to the priming treatment. After an additional 7 days of priming the incidence of *Alternaria* species significantly increased to 59, 86 and 74% for *A. radicina*, *A. dauci* and *A. alternata*, respectively. In contrast, biopriming still strongly suppressed *Alternaria* spp. as the incidence of all three species was below 4.3% corresponding to a control efficacy of above 90%.

Priming, biopriming and coating seeds with IK726 affected establishment of healthy carrot seedlings as shown in figure 1. When seeds were primed for one day, the initial emergence of primed seeds was as high as for unprimed seeds. However, in the priming treatment 37% of the emerged seedlings subsequently died (post emergence infection), resulting in a final seedling stand of 30%, which was significantly lower than the seedling stand (42%) of the unprimed seeds. Biopriming and coating with IK726 were equally effective in increasing the final seedling stand to approximately 60%, which was significantly higher than the seedling stand from unprimed seeds. Priming of carrot seeds for 8 days affected the establishment of carrot seedlings even more (Fig. 2). Thus, although the total emergence was about 30%, post emergence death (71 %) reduced the final stand to 10%.

Biopriming still successfully controlled disease caused by *Alternaria* spp. compared to unprimed seeds as the final seedling stand was 60%, which was not significantly different from that obtained with unprimed IK726-coated seeds.

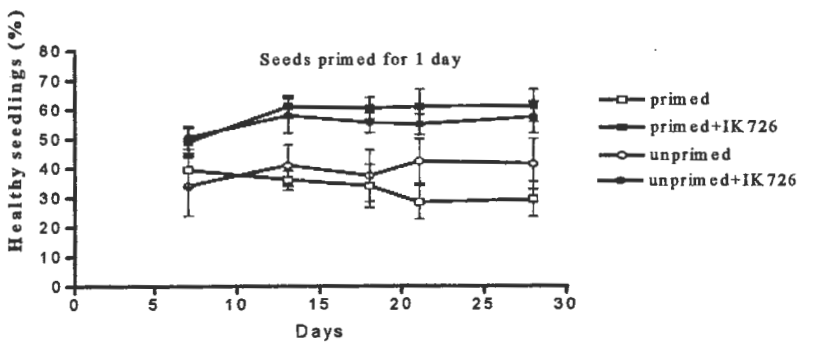

Figure 1. Effect of priming and of biopriming with *Clonostachys rosea* (isolate IK 726) on establishment of healthy stands of carrot seedlings in sand. Priming treatments lasted one day. Treated seeds were naturally infected with *Alternaria radicina* and *A. dauci*.

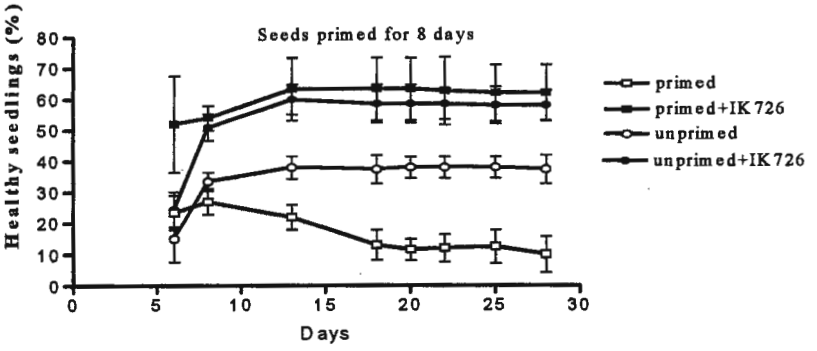

Figure 2. Effect of priming and of biopriming with *Clonostachys rosea* (isolate IK 726) on establishment of healthy stands of carrot seedlings in sand. Priming treatments lasted eight day. Treated seeds were naturally infected with *Alternaria radicina* and *A. dauci*.

Discussion

Carrot seeds naturally infected with *Alternaria* spp. were primed or bioprimed with *C. rosea* (isolate IK726) at 15°C for 8 days at a constant seed moisture content of 40%. Priming alone approximately doubled the incidence of *A. radicina* to 59% and that of *A. dauci* to 86%. In agreement with our results, Biniek & Tylkowska (1987) had previously shown that osmo-priming of carrot seeds increased the general microflora, although *A. radicina* decreased or remained unaffected. On the other hand Nascimento & West (1998) recently reported that osmo-priming increased *Alternaria* spp. on melon seeds from 2% to 50%. Maude *et al.* (1992), however, concluded that osmo-priming did not affect the rate of transmission of *A. dauci*. In the present study the enhanced growth of *A. dauci* and *A. radicina* during priming could mainly explain the large reduction in final stand of healthy seedling compared to unprimed seeds. In contrast, biopriming almost eradicated the two pathogens and incidences of *A. radicina* and *A. dauci* was reduced from 35% to 4% and from 32% to 2 %, respectively. This lead to a highly improved seedling stand, which was significantly higher than that of unprimed seeds. Thus biopriming with IK726 has shown a high potential for suppression of *Alternaria* spp. and bio-priming of carrot seed with IK726 will be tested by the Danish seed company Dæhnfeldt using a commercialized priming technique.

Acknowledgements

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Biological Control of Fungal and Bacterial Plant Pathogens
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Evaluation of antagonistic bacteria and fungi for biological control of sugar beet and cucumber damping-off caused by *Pythium ultimum*

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Abstract: Five bacterial (*Pseudomonas fluorescens*, *P. corrugata*, *Bacillus subtilis*) and two fungal antagonists (*Gliocladium virens*, *Trichoderma viride*) were evaluated for damping-off biocontrol of sugar beet and cucumber *in vitro* and *in vivo*. *Pseudomonas* seed treatments efficiently controlled sugar beet damping-off, but not cucumber. *Bacillus* seed treatments (sugar beet and cucumber) and fungal compost treatments (cucumber) were ineffective. Biocontrol efficacy of cucumber damping-off was improved with bacterial compost treatments and with use of *Pseudomonas* antagonists in a peat carrier based inoculum. This formulation had a good shelf life of 2 years. Integrated control was achieved when *Pseudomonas* antagonists were combined with thiram at 1/4 of the commercial rate.

Key words: Damping-off, biocontrol, sugar beet, cucumber, integrated control, peat-based inocula

Introduction

Damping-off is a common problem in many field and greenhouse crops. A number of soilborne pathogenic fungi have been associated with damping-off, such as various species of *Pythium*, *Phytophthora*, *Fusarium*, *Aphanomyces* and *Rhizoctonia solani*. Chemical fungicides and soil disinfestation are today the main commercially available options for plant protection. These fungicides usually provide good control against pre-emergence damping-off but are less effective in protecting from post-emergence damping-off. Soil disinfestation is common practice in greenhouses, with methyl-bromide being the most effective chemical compound. However, it is now being phased-out from agriculture, making urgent the search for alternative control methods.

Materials and methods

Seed and microorganisms

Cucumber seed cultivar "Knossos" (Agris S.A., Athens, Greece) and sugar beet seed cv. "Turbo" (Hellenic Sugar Industry, Thessaloniki, Greece) were used in this study, along with the following bacterial and fungal antagonists: *Pseudomonas fluorescens* strains B5 and X, *P. corrugata* strain R117, *Bacillus subtilis* strains B2 and B6, *Trichoderma viride* strain K2 and *Gliocladium virens* strain K3. Various *Pythium* spp. strains were used in experiments *in vitro* and *P. ultimum* strain ZPT8 (isolated from cotton, Benaki Phytopathological Institute, Kifissia, Greece) was used in experiments *in vivo*.

Dual culture inhibition assays
Standard tests were performed in various media. Bacterial antagonists were inoculated at the center of a plate and pre-incubated at 21°C. *Pythium* isolates were then inoculated at either side of the bacterial growth in three replicate plates. Inhibition of fungal growth was assessed two days later.

Evaluation of antagonist biocontrol activity
The following experiments were performed: (1) Three experiments for control of sugar beet damping-off with individual bacterial antagonists applied by soaking seed in bacterial suspensions in 10mM phosphate buffer. Inoculum level was 10^5 (*Bacillus*) to 10^6 (*Pseudomonas*) cfu/seed. (2) Four experiments for control of sugar beet damping-off with four selected dual combinations (1:1) of bacterial antagonists [based on *in vitro* compatibility assays (results not shown)] applied on seed. (3) Three experiments for control of cucumber damping-off with individual bacterial antagonists applied on seed. (4) Two experiments for biocontrol of cucumber damping-off by mixing bacterial suspensions into infested compost at a level of 32 OD^{600} units/kg. (5) Three experiments for control of cucumber damping-off by mixing spore suspensions of *G. virens* K2, *T. viride* K3 and a 1:1 combination of K2 and K3 into compost at a level of 10^6 conidia/g. Compost was artificially infested with *P. ultimum* grown on PDA for 3 days at 21°C. Experiments were conducted either in a growth chamber (21°C for sugar beet and 26°C for cucumber, 12h photoperiod) or a glasshouse. Sugar beet seed was planted in four replicate 15cm pots of twenty seeds each. Cucumber was planted in seedling trays in four replicates of 18 seeds each. Treated and untreated seed in *Pythium*-free compost were used as controls.

Biocontrol of cucumber damping-off and integrated control with* Pseudomonas *seed inocula and thiram in a peat carrier
All bacterial antagonists were grown in broth and incorporated into sterilized peat, according to a proprietary procedure developed by MicroBio Ltd. *Bacillus* inoculants were made with spores. The shelf life of these inocula at room temperature was determined up to twenty-four months from production. Cucumber seed was coated with a slurry of peat inocula made with an adhesive polymer to achieve a population of around 10^5 cfu/seed. Experiments were performed as described previously. Seed adhesion and colonization was assessed in a pilot experiment during the first forty-eight hours after planting. Integrated control experiments included low rates of thiram (1/2, 1/4 and 1/8 of a commercial rate of 4 mg a.i./seed). Antagonist viability on seed during integration of thiram into the coating process was also assessed.

Statistical analysis
One-way ANOVA and Fisher's Least Significance Test at the 5% confidence level were performed with the MSTAT statistical program (Michigan State University).

Results

Dual culture inhibition assays
All assays generally showed that *Pseudomonas* antagonists were more efficient in inhibiting various *Pythium* isolates *in vitro* than *B. subtilis* antagonists (results not shown).

Evaluation of antagonist biocontrol activity
Results of three experiments with individual antagonists (Table 1) and four experiments with antagonist combinations (results not shown) showed that *Pseudomonas* seed treatments generally controlled sugar beet damping-off better than *Bacillus* treatments, which were effective in only one experiment. Only the combination of strains B5 and R117 was found to

be a better seed treatment than individual components in three out of four experiments, although this combination was assessed as a negative one *in vitro*. Cucumber damping-off was not efficiently controlled with bacterial seed treatments or fungal compost treatments (results not shown). Bacterial antagonists, however, adequately controlled cucumber damping-off when applied as compost treatments (Table 1). *P. fluorescens* X was particularly consistent in most experiments and provided the best control, compared to other treatments.

Table 1. Biocontrol of sugar beet damping-off with bacterial seed treatments and cucumber damping-off with bacterial compost treatments. Numbers represent percentage of healthy seedlings.

	Sugar beet seed treatments			Cucumber compost treatments	
Treatments	1st Exper.	2nd Exper.	3rd Exper.	1st Exper.	2nd Exper.
Pythium	52,5 f	48,8 d	55 bcd	18 d	32 d
Pythium/R117	70 de	73,4 bc	48,8 cd	76 bc	68 c
Pythium/B5	73,8 cd	83,8 abc	65 abcd	88 ab	79 bc
Pythium/X	80 bcd	87,8 abc	67,5 abcd	95 a	89 ab
Pythium/B2	45 f	71,3 c	48,8 cd	67 c	46 d
Pythium/B6	55 ef	77,5 bc	46,3 d	67 c	not done
Control	88,8 abc	90 ab	88,8 a	94 a	97 a

Table 2. Biocontrol of cucumber damping-off with antagonists in a peat-based carrier material (seed treatments). Numbers represent percentage of healthy seedlings.

Treatments	1st Exper.	2nd Exper.	3rd Exper.	4th Exper.
Pythium	11,8 d	33 c	2,8 c	31,8 c
Pythium/R117	56,5 bc	68 b	8,5 bc	50 bc
Pythium/B5	62,3 bc	57 bc	14 bc	58,5 b
Pythium/X	80,3 ab	69,8 b	24,8 b	56,8 b
Pythium/B2	44,3 c	41,8 c	16,8 bc	41,5 bc
Pythium/B6	44,3 c	44,5 bc	7 bc	36 bc
Control	95,5 a	97 a	88,8 a	94 a

Biocontrol of cucumber damping-off with bacterial antagonists applied in a peat carrier
All bacterial antagonists adequately colonized cucumber spermosphere during the first forty-eight hours from planting (results not shown) and controlled damping-off, with *Pseudomonas* antagonists being more efficient than *Bacillus* antagonists (Table 2).

Bacterial peat inoculants had good shelf-life at room temperature (results not shown). *B. subtilis* B6 was particularly stable, with a drop of only log 0,1 cfu/g peat after storage for 2 years. Populations of *Pseudomonas* inoculants decreased consistently after production, but this decrease was only log 1 cfu/g peat after two years. Viability tests for strain X are still in progress.

Integrated control of cucumber damping-off with* Pseudomonas *peat inocula and low rates of thiram

Thiram was not found to be toxic to *Pseudomonas* antagonists when integrated into the seed coating process (results not shown). Moreover, combinations of low thiram rates and antagonists provided good control of the disease at rates as low as 1/4 of the commercially applied (Table 3). These experiments also reconfirmed the biocontrol potential of *Pseudomonas* antagonists.

Table 3. Integrated control of cucumber damping-off with *Pseudomonas* spp. seed treatments in a peat-based carrier material and low rates of thiram. Numbers represent percentage of healthy seedlings. Only results with ¼ rate of thiram are presented.

Treatments	Thiram	1st Exper.	2nd Exper.	3rd Exper.
Pythium	no thiram	7 e	48,5 e	51,5 e
Pythium/R117		30,5 d	72,3 bcd	50,8 e
Pythium/B5		37,5 d	66,5 cde	56,5 de
Pythium/X		62,8 c	66,8 cde	72,5 bcde
Pythium	¼ rate	27,8 d	56,8 de	66,3 cde
Pythium/R117		81,8 ab	72 bcd	73,8 bcde
Pythium/B5		87,5 ab	73,8 abcd	82 abc
Pythium/X		91,8 ab	77,5 abcd	86 abc
Control	no thiram	88,8 ab	94,5 a	98,5 a

Discussion

Among the tested antagonists, *Pseudomonas* strains were effective in damping-off biocontrol with remarkable consistency. Although antagonist combinations have been successfully used, this did not happen with the microorganisms used in this study. Generally, biocontrol potential of a microorganism depends on its mode of action (Whipps & Lumsden, 1991) as well as timing of antagonistic activity (Georgakopoulos *et al.*, 1994) in relation to infection (Osburn *et al.*, 1989). This may explain the failure of *Bacillus* and fungal antagonists in controlling damping-off. Formulation of *Pseudomonas* antagonists in a peat carrier with good shelf life of at least 2 years from production is a breakthrough in viability of gram-negative microbial inocula. Peat-based inocula are easy to apply on seeds and compatible with low rates of thiram for an integrated control approach.

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Biological Control of Fungal and Bacterial Plant Pathogens
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Isolation of nonpathogenic mutants of *Fusarium oxysporum* for biocontrol of Fusarium wilt in cucurbits

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Abstract: A nonpathogenic mutant (isolate 4/4) of *Fusarium oxysporum* f. sp. *melonis* was isolated following UV-mutagenesis. Isolate 4/4 colonized a variety of melon cultivars without causing any disease symptoms. In Ein Dor melon cultivar, the optimal concentration of $5X10^6$ conidia/ml was required for colonization of between 30 to 50% of the lower stem tissue following 7 days continuous dip inoculation. In addition, isolate 4/4 colonized watermelon seedlings at varying degrees, either applied as a continuous dip or mixed into soil. In cross-protection experiments with melon cultivars, no significant differences were observed between 4/4 colonized wildtype-challenged plants compared with wildtype-challenged plants alone. However, isolate 4/4 was able to reduce mortality of watermelon seedlings (cv. Odem and Malali) caused by *F. o.* f. sp. *niveum* race 2. Colonization percent corresponded to percent of protection. This was observed following optimal colonization at 4 days, when percent mortality of protected seedlings was most reduced as opposed to lower percent colonization resulting in higher seedling mortality. This novel approach of "creating" nonpathogenic mutants in *Fusarium* and other fungal pathogens from the wildtype parent may be used to improve biocontrol strategies which have previously utilized hypovirulent strains or non-pathogenic saprophytes by allowing the mutants to compete more efficiently for infection sites and nutrient sources.

Keywords: *Fusarium*, melon, nonpathogenic mutant, watermelon

Introduction

Fusarium wilt of melon and watermelon caused by *F. o.* f. sp. *melonis* and *F. o.* f. sp. *niveum*, respectively, inflict considerable yield loss worldwide (Biles & Martyn, 1989; Katan *et al.*, 1994). Due to the persistent nature of these pathogens in soil, once the disease is established in the field the pathogen is likely to remain indefinitely because subsequent crops of susceptible melon and watermelon cultivars increase the pathogen population. *Fusarium* of melon and watermelon cause plant wilting by colonizing the hosts vascular system and eventually cause seedling or mature plant death. Because of the persistent nature of the wilt pathogens, without adequate control measures, the diseases are best managed using wilt-resistant cultivars. However, as resistant cultivars are utilized, new virulent populations (physiological races) are continuously overcoming resistance. In FOM (*F. o.* f. sp. *melonis*), the three common races are designated 0, 1 and 2. Reports on a fourth race (1,2) indicate its prevalence in Israel and the US (Cohen *et al.*, 1989). Race 1,2 is virulent to muskmelon cultivars possessing resistance genes FOM-1, FOM-2 and FOM-3 (Katan *et al.*, 1994) and to date no known resistant cultivars are commercially available.

With FON (*F. o.* f. sp. *niveum*) a similar phenomenon of resistance is found. Resistant watermelon cultivars have been developed to races 0 and 1, whereas no resistant cultivars are currently available to race 2 (Biles & Martyn, 1989). In an effort to develop alternative control strategies, biological control methods relying on induced resistance and activation of

host defenses have been attempted (Alabouvette & Couteaudier, 1992; Biles & Martyn, 1989; Mandeel & Baker, 1991).

Fusarium pathogens penetrate the vasculature via root tips. Vascular colonization by *F. o.* f. sp. *lycopersici* (FOL) (specific to tomato) is extensive in wilt-susceptible plants but remains limited to the basal part of resistant plants (Beckman, 1987). It is presumed that resistant tomato plants are able to "wall-off" potential FOL isolates by trapping spores, depositing callose, tylose occlusion, infusing phenolic compounds and synthesizing phytoalexins (Beckman, 1987). The pathogen may cause disease by avoiding or overcoming any of these defense mechanisms, or by vascular occlusion. The cause of wilting by the pathogen may be due to either vascular occlusion resulting in the failure of water transport and/or toxin production. With FOM (specific to melon) however, wilt-resistant plants also harbor the pathogen extensively within the vascular elements (Cohen *et al.* 1987; Netzer *et al.* 1979). This phenomenon may be due to detoxification of toxins and/or degradation of fungal cell wall degrading enzymes by the resistant host as a defense response. Alternatively the fungus may not be expressing these factors in resistant plants.

It has been previously demonstrated that UV-mutagenesis of a wildtype virulent pathogen can result in genetic conversion to a nonpathogenic, endophytic mutualist (Freeman and Rodriguez, 1993a). The nonpathogenic mutant isolate of *Colletotrichum magna* (path-1) retained the wildtype phenotype, colonized curcurbit and other host tissues and protected host plants against the wildtype and *F. o.* f. sp. *niveum* pathogens in watermelon, by priming the host defense response. Therefore, the main objectives of this study were to isolate non-pathogenic mutants of *F. o. melonis* and test feasibility for protecting different cucurbit plants against the respective Fusarium wilt pathogens.

Materials and methods

UV mutagenesis for isolation of nonpathogenic mutants

UV-mutagenesis was conducted on a wildtype isolate of *F. o.* f. sp. *melonis* (race 1,2) and on wildtype isolate FON1 of *F. o.* f. sp. *niveum* (race 2) using the previously described method which resulted in the isolation of path-1 of *C. magna* (Freeman & Rodriguez, 1992). Approximately 800 survivers of UV-mutagenesis were screened for pathogenicity on a set of differential cucurbit cultivars. Of these, isolate 4/4 of *F. o.* f. sp. *melonis* was completely nonpathogenic and did not cause any disease symptoms using the dip-inoculation screening method (Freeman & Rodriguez, 1993b). Four nonpathogenic isolates of *F. o.* f. sp. *niveum* were also isolated but were not characterized in this study.

Plant colonization and inoculation assays

Four physiological races of *F. o.* f. sp. *melonis* exist worldwide with race 1,2 being the most virulent race without a resistant cultivar available. Isolate 4/4 was assessed for the control of the wildtype *F. o.* f. sp. *melonis* isolate of race 1,2 and *F. o.* f. sp. *niveum* isolate of race 2. Seedlings of Ein-Dor melon cultivar and Odem watermelon cultivar were colonized by nonpathogenic mutant isolate 4/4 after planting in a peat/vermiculite mixture (1:2/v:v) containing $5X10^6$ conidia/g. Plants were grown for 7 days before inoculation with a 20 ml conidial suspension of 10^6 conidia/ml of the respective wildtype *F. o.* f. sp. *melonis* and *F. o.* f. sp. *niveum* isolates. Colonization of the plants were assessed by plating on a *Fusarium*-selective medium and disease incidence was evaluated periodically.

Results and discussion

Colonization and control of F. o. *f. sp.* melonis *and* F. o. *f. sp.* niveum *by mutant isolate 4/4 of* F. o. *f. sp.* melonis

Nonpathogenic mutant isolate 4/4 of *F. o.* f. sp. *melonis* colonized a variety of melon and watermelon cultivars without causing any disease symptoms. In Ein Dor melon cultivar, the optimal concentration of $5X10^6$ conidia/ml was required for 56% colonization of lower stem following 20 days continuous dip inoculation. Likewise, in Odem watermelon cultivar, 36% colonization of lower stem was measured following 20 days continuous dip inoculation.

In cross-protection experiments with melon (cv. Ein Dor) no significant differences were observed between 4/4 colonized wildtype-challenged plants compared with wildtype-challenged plants alone. However, in watermelon (cv. Odem) cultivars, isolate 4/4 (originally from melon) was able to reduce wildtype mortality caused by *F. o.* f. sp. *niveum*. Disease reduction in Odem cultivar after 11 and 20 days challenge was 25 and 53%, respectively. No disease was observed in either water- or isolate 4/4-treated plants of both cucurbit cultivars.

In a related study, it was recently demonstrated that the nonpathogenic mutant path-1 of *C. magna* colonized watermelon plants resulting in protection of 90 - 100% against disease caused by *C. orbiculare* (Redman *et al.*, 1999). Collectively, these data indicated that the path-1 mutation converted a virulent pathogen to a nonpathogenic endophytic mutualist. Furthermore, biochemical studies indicated that path-1 avoids activation of host defenses and that path-1 colonized plants, challenged with virulent isolates, activate host defenses more rapidly than control plants (Redman *et al.*, 1999). These results were determined by monitoring the activity of peroxidase and the deposition of lignin in path-1 colonized and control plants.

The nonpathogenic *Fusarium* and *Colletotrichum* mutants isolated are potential novel biocontrol agents. To date no biocontrol agents of *Fusarium* have been used that were derived from wildtype pathogenic isolates of *Fusarium*. For example, in *Fusarium*, only saprophytes and specific pathogenic isolates have been utilized for biocontrol. The nonpathogenic mutants therefore may compete better than the wildtype isolates when applied in the correct manner. This research has provided information toward a better understanding of host-parasite interactions; specifically, endophytes, pathogens and their hosts. It will also allow us to assess the potential for utilizing nonpathogenic mutants as biological control agents against fungal pathogens.

Besides the potential use as biocontrol agents, the non-pathogenic mutants will enable a better understanding of factors involved in pathogenicity. Complementation of the nonpathpogenic isolates, isolates path-1 of *C. magna* (Freeman & Rodriguez, 1993a) and 4/4 of *F. o.* f. sp. *melonis* with wildtype genomic libraries, will also enable characterization of genes involved in pathogenicity which may allow development of improved control measures.

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Biological Control of Fungal and Bacterial Plant Pathogens
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Integrated control of rhizomania disease by *Trichoderma* and cultural management

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Abstract: Potential biocontrol strains of *Trichoderma* spp., including a combination of *T. asperellum*, *T. atroviride* and *T. inhamatum*, were selected for control of the sugar beet pathogenic complex of rhizomania virus (BNYVV) and its vector (*Polymyxa betae*). *Trichoderma* irrigation in transplanting (paper-pot) systems and *Trichoderma* seed coating were demonstrated as more effective in both number of roots and sugar production than chemical control with 8 g thiram and 18 g hymexazol/unit (1 sugar beet unit is equivalent to 100,000 seeds).

Key words: *Trichoderma*, *Polymyxa*, rhizomania, BNYVV, biocontrol, sugar beet

Introduction

Rhizomania causes the most economic damage among sugar beet viral diseases. Initially described by Canova (1959) in Italy, who first attributed this disease to the association of the beet necrotic yellow vein furovirus (BNYVV) with a vector fungus (*Polymyxa betae* Keskin), rhizomania has been detected around the world in most of geographical areas where sugar beet is cultivated. The term "rhizomania" refers to the root bearding where most of the virus concentrates. Biocontrol efforts have been conducted to avoid the development of *P. betae* and transmission of the virus.

Protection of sugar beet against rhizomania disease was tested by antagonism using a combination of selected strains of *Trichoderma* from the species *T. asperellum*, *T. atroviride* and *T. inhamatum* (Hermosa *et al.*, 2000). These species belong to the former *T. harzianum* aggregate that has been demonstrated as an important source of fungal antagonists (Hjeljord & Tronsmo, 1998). We have studied the efficacy of *Trichoderma* irrigations and seed coating and expressed the results according to yield in the trials in terms of the number of seedlings and amount of sugar produced per surface unit (Tm of sugar/Ha) on both normally-sown and transplanted sugar beets.

Materials and methods

Six different trials were performed, during the seasons 1996, 1997 and 1998, in fields in three locations of Castilla y León region: Nava de Arévalo, Iscar and Ataquines, infected with rhizomania where BNYVV was detected and identified using molecular methods (Suárez *et al.*, 1999). Depending on the experimental conditions, between 60,000 to 95,000 seeds/Ha were sown. Transplanted sugar beet plants were grown in paper-pots maintained in greenhouses for 60 days before the transplanting date and protected with Tusal®, a mixture of

five strains of the genus *Trichoderma*, corresponding to the species *T. inhamatum* (1 strain), *T. atroviride* (2 strains) and *T. asperellum* (2 strains). In all cases Tusal® applications were carried out by means of four pulverisations of 6 l/Ha and 20 million conidia of each strain/ml, performed every fifteen days during the two months following the transplanting date. A comparative study was performed with a standard fungicidal treatment of 8 g thiram and 18 g hymexazol/unit (1 sugar beet unit is equivalent to 100,000 seeds).

Normal-sowing was performed with sugar beet seeds treated with the insecticide gaucho at a concentration of 90 g/unit and two different treatments: Standard and Piltusal®, a mixture of at least 500.000 conidia/seed of a combination of five strains of the genus *Trichoderma* corresponding to the species *T. inhamatum* (1 strain), *T. atroviride* (2 strains) and *T. asperellum* (2 strains). Alternatively, four Tusal® irrigations were also carried out during the two months following the sowing date.

Field 1: tested with seeds of the Riposte variety (SES Ibérica, Zaragoza, Spain), tolerant to BNYVV, in Nava de Arévalo (Avila) between the 19th of April 1996 (transplanting date) and the 17th of October of 1996 (date at which the beets were uprooted).

Fields 2 and 3: tested with Riposte seeds in two farms of Nava de Arévalo between 19th of March 1997 (transplanting date) and the 27th of August 1997, under conditions of low sugar beet production.

Field 4: transplanted in Iscar under conditions of high sugar beet production between the 9th of April 1997 (transplanting date) and 1st of September 1997. Two treatments were carried out in these four fields: Treatment 1: Standard, and Treatment 2: Standard + Tusal®.

Field 5: performed with Rima (SES Ibérica, tolerant to BNYVV) variety in Ataquines between 26th of February 1998 (normal-sowing date) and 26th of October 1998, treated with Tusal®, Piltusal® and standard treatments.

Field 6: carried out in Nava de Arévalo between the 5th of April 1998 (normal-sowing date) and the 14th of October 1998, with seeds of Rima and Oryx (SES Ibérica, sensitive to BNYVV) varieties, with identical treatments applied as those in field 5.

Results

Field 1

Trichoderma irrigations on transplanted sugar beet (Nava de Arévalo, 1996). Treatment 1 (standard) presented 63,700 roots/Ha, 58.6 Tm of roots/Ha, 16.4% polarization and 9,658 Kg of sugar/Ha; whereas Treatment 2 (standard + Tusal®) resulted in 69,630 roots/Ha, 73.4 Tm of roots/Ha, 16.9% polarization and 12,406 Kg of sugar/Ha. The variation in production of Treatment 2 with respect to Treatment 1 was: Roots/Ha: +9.3%, Tm of roots/Ha: +25.3%, Percentage polarization: +3% and Kg sugar/Ha: +28.5%; and the economic yield increased 29% in favour of Treatment 2.

Field 2

Trichoderma irrigations on transplanted sugar beet (Nava de Arévalo, 1997). Treatment 1 (standard) resulted in 55,200 roots/Ha, 59.5 Tm of roots/Ha, 16.2% polarization and 9,674 Kg of sugar/Ha; and Treatment 2 (standard + Tusal®) resulted in 59,200 roots/Ha, 66.5 Tm of roots/Ha, 16% polarization and 10,655 Kg of sugar/Ha. The variation in production of Treatment 2 with respect to Treatment 1 was: Roots/Ha: +7.2%, Tm of roots/Ha: +11.8%, Percentage polarization: -1.2% and Kg sugar/Ha: +10.1%. The economic yield increased 10% in favour of Treatment 2.

Field 3

Trichoderma irrigations on transplanted sugar beet (Nava de Arévalo, 1997). Treatment 1 (standard) resulted in 55,200 roots/Ha, 59.5 Tm of roots/Ha, 16.2% polarization and 9,674 Kg

of sugar/Ha and Treatment 2 (standard + Tusal®) resulted in 59,200 roots/Ha, 60 Tm of roots/Ha, 16.4% polarization and 9,830 Kg of sugar/Ha. The variation in production of Treatment 2 with respect to Treatment 1 was: Roots/Ha: +7.2%, Tm of roots/Ha: +0.8%, Percentage polarization: +1.2% and Kg sugar/Ha: +1.6%; the economic yield increased 1.6% in favour of Treatment 2.

Field 4

Trichoderma irrigations on transplanted sugar beet (Iscar, 1997). Treatment 1 (standard) resulted in 73,500 roots/Ha, 75.7 Tm of roots/Ha, 14.8% polarization and 11,152 Kg of sugar/Ha; and Treatment 2 (standard + Tusal®) resulted in 76,500 roots/Ha, 82.2 Tm of roots/Ha, 14.9% polarization and 12,232 Kg of sugar/Ha. The variation in production of Treatment 2 with respect to Treatment 1 was: Roots/Ha: +4.1%, Tm of roots/Ha: +8.6%, Percentage polarization: +0.7% and Kg sugar/Ha: +9.7%; and the economic yield increased 9.9% in favour of Treatment 2.

The mean values (%) of 1997 rhizomania trials did not give statistically significant variation in polarization. However, production between treatments, expressed in roots/Ha (+6.0%), Tm/Ha (+7.1%) and Kg sugar/Ha (+7%), was always statistically significant ($P \leq 0.05$) in favour of Tusal® in the three trials.

During the 1998 season, two more biocontrol tests were performed (fields 5 and 6). Three treatments were applied in these trials: Tusal®, Piltusal® and standard (Tables 1 and 2).

Table 1. Field 5. Ataquines (1998). Biocontrol of rhizomania disease by *Trichoderma* irrigations (Tusal®) and seed coating (Piltusal®) on normally-sown sugar beet (cultivar Rima: tolerant to BNYVV).

	Roots/Ha	Tm/Ha	Polarization (%)	Sugar Kg/Ha
Tusal®	73.010 (-2.1%)	52.8 (-6%)	16.8 (+2.4%)	8.901 (-3.6%)
Piltusal®	80.000 (+7.2%)	57.5 (+2.4%)	16.4 (-0.2%)	9.479 (+2.6%)
Standard	75.240	56.6	16.4	9.281

Table 2. Field 6. Nava de Arévalo (1998). Biocontrol of rhizomania disease using *Trichoderma* irrigations (Tusal®) and seed coating (Piltusal®) on normally-sown sugar beet (cultivar Rima: tolerant to BNYVV; and cultivar Oryx: sensitive to BNYVV).

Rima	Roots/Ha	Tm/Ha	Polarization (%)	Sugar Kg/Ha
Tusal®	90.833 (+13.5%)	45.3 (+9.9%)	14.9 (+2.0%)	6.728 (+12.5%)
Piltusal®	77.917 (-2.6%)	43.8 (+6.3%)	15.0 (+2.7%)	6.560 (+9.6%)
Standard	80.000	41.2	14.6	5.981
Oryx				
Tusal®	80.000 (+0.5%)	48.3 (-5.9%)	15.0 (+4.9%)	7.212 (-1.8%)
Piltusal®	74.166 (-6.8%)	48.9 (-4.7%)	15.3 (+7.0%)	7.501 (+2.2%)
Standard	79.583	51.3	14.3	7.340

Conclusions

1. *Trichoderma* irrigation in transplanting systems resulted in increased number of roots and sugar production, under low and high production of sugar beet plants of a cultivar tolerant to BNYVV (Fields 1-4).
2. *Trichoderma* seed coating was demonstrated as more effective increasing number of roots and sugar production compared to chemical control in normally-sown sugar beet systems (Fields 5-6).
3 Polarization is independent of both biocontrol and chemical treatments.

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Integrated control of *Sclerotinia sclerotiorum* in glasshouse lettuce using the mycoparasite *Coniothyrium minitans*

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Abstract: Six fungicides, four insecticides and one herbicide were incorporated into agar at a range of concentrations up to twice the recommended dosage and the effects on radial growth and spore germination on *Coniothyrium minitans* was determined. Only the fungicides had any significant effect on *C. minitans*. *C. minitans* was highly sensitive to iprodione (EC_{50} 7-18 µg a.i. ml^{-1}), moderately sensitive to thiram (EC_{50} 52-106 µg a.i. ml^{-1}) but less sensitive to the other tested pesticides. The same pesticides were also applied weekly, at twice the recommended dosage, to soil trays containing *C. minitans* and sclerotia of *S. sclerotiorum*. *C. minitans* survived in soil and infected sclerotia equally well in all pesticide-treated and untreated control soils. Two glasshouse trials each of three sequential crops of lettuce, were carried out in a glasshouse infested with *S. sclerotiorum* to determine the potential for *C. minitans* to provide integrated control with a single application of iprodione. In the first glasshouse trial, disease caused by *S. sclerotiorum* was significantly reduced by both iprodione tolerant and standard strains of *C. minitans* and was enhanced when combined with a single application of iprodione. This enhanced control was irrespective of whether the *C. minitans* was iprodione tolerant or not. In the second experiment, the enhanced control achieved by the combination of *C. minitans* and a single application of iprodione was shown to be equivalent to the level of control achieved by fortnightly prophylactic sprays with iprodione. These results indicate that integrated control of *S. sclerotiorum* using soil applications of *C. minitans* and reduced foliar iprodione applications is feasible and does not require an iprodione tolerant isolate.

Key words: *Coniothyrium minitans, Sclerotinia sclerotiorum*, integrated control, lettuce, glasshouse

Introduction

Coniothyrium minitans is a sclerotial mycoparasite of the plant pathogen *Sclerotinia sclerotiorum* and provides reproducible control of Sclerotinia disease of lettuce under glasshouse conditions (Budge & Whipps, 1991; Budge *et al.*, 1995; McQuilken & Whipps, 1995; McQuilken *et al.*, 1997). However, integration with chemicals used routinely in the glasshouse environment has not been examined. This paper describes the effect of all pesticides used in protected lettuce in the UK on the mycelial growth and spore germination of *C. minitans*, and on the ability of *C. minitans* to infect sclerotia in soil. The relative effectiveness of an iprodione tolerant strain of *C. minitans* and an iprodione susceptible strain in combination with a reduced application of iprodione was also determined in lettuce trials.

Materials and methods

Fungi and inoculum production

An isolate of *S. sclerotiorum* was originally obtained from diseased glasshouse lettuce and was maintained on potato dextrose agar (PDA). Sclerotia used for the soil tray experiments were produced on sterilised wheat grain following inoculation with agar plugs of *S. sclerotiorum*.

After incubation for three weeks at 20°C, sclerotia were removed and graded, to use those between 2 - 4 mm diameter.

Coniothyrium minitans (Conio; IMI 134523) was originally isolated from a sclerotium of *S. sclerotiorum* (Turner & Tribe, 1976). An iprodione tolerant strain of *C. minitans* used in the first glasshouse trial was obtained from fast-growing sectors on PDA containing 500 mg l^{-1} iprodione. Stability of fungicide resistance was tested by subculturing eight times on PDA and then transferring onto iprodione containing media. Maizemeal perlite inoculum of *C. minitans* used in the tray tests and glasshouse trials was prepared following the method of McQuilken & Whipps (1995).

In vitro *and soil tray pesticide tests*

The effect of pesticides on radial growth rate and conidial germination of the three isolates of *C. minitans*, was tested on PDA amended with a range of seven concentrations determined from preliminary experiments up to a maximum of twice the manufacturers application dosage. To evaluate the toxicity of pesticides to *C. minitans* and the effects of pesticides on colonization of sclerotia by the biocontrol agent, soil tray experiments were carried out. Plastic trays, 53 x 38 x 7.5 cm, with small holes in the bottom for drainage, were filled with a 2 cm layer of gravel, and a 5 cm layer of soil (brickearth; silt loam, Hamble Series). Maizemeal perlite inoculum of *C. minitans* (120 ml per tray = 0.6 litres m^{-2}) was added to appropriate trays and lightly worked into the top 3 cm. Fifty sclerotia of *S. sclerotiorum* were lightly pressed into the soil surface of each tray so they were evenly spaced and remained just visible. For each pesticide, there were four treatments: i) sclerotia only; ii) sclerotia plus pesticide; iii) sclerotia plus *C. minitans* and iv) sclerotia plus *C. minitans* plus pesticide. Pesticides were applied at double the manufacturers recommended application rate immediately after setting up the trays and three times thereafter at weekly intervals. All trays were covered using a second empty tray placed upside down on top of the filled tray and incubated at 18 °C for four weeks. Soil moisture was maintained at 75% field capacity by spraying water on to the soil surface using a hand sprayer. The sclerotia were then recovered, surface sterilised, disected and placed onto PDA amended with chlortetracycline (McQuilken & Whipps, 1995). Numbers of viable sclerotia and those infected by *C. minitans* were recorded. The pesticides used in all tests were: fungicides (iprodione, mancozeb, metalaxyl + thiram, thiram, tolclofos-methyl, zineb), insecticides (cypermethrin, dimethoate, malathion), and a herbicide (chlorpropham with cetrimide). In addition, for the soil tray tests only, the fumigant fungicide, dicloran and the fumigant insecticide, pirimiphos methyl were also used.

Glasshouse trials

Two trials each of three sequential crops of lettuce, were carried out in a glasshouse infested with *S. sclerotiorum* to determine the potential for *C. minitans* to provide integrated control with a single application of iprodione (Rovral WP) following the protocol of McQuilken & Whipps (1995). In the first trial there were five treatments: i) control, no treatment; ii) standard *C. minitans* only; iii) standard *C. minitans* plus single Rovral spray; iv) iprodione tolerant *C. minitans* only and v) iprodione tolerant *C. minitans* plus single Rovral spray. In the second trial the treatments comprised: i) control, no treatment; ii) single Rovral spray; iii) fortnightly Rovral spray applications; iv) standard *C. minitans* only and v) standard *C. minitans* plus single Rovral spray. Immediately before planting each crop, 1.7 l (0.6 litres m^{-2}) of *C. minitans* maizemeal perlite inoculum was evenly applied to each appropriate plot and raked into the soil surface. The single application of Rovral WP was applied during each lettuce crop to the appropriate plots until run-off as disease symptoms first began to proliferate. At harvest plant disease, sclerotial numbers, sclerotial viability and infection, and survival of *C. minitans* were assessed.

Results and discussion

In vitro *and soil tray pesticide tests*

In agar tests, only the fungicides had any significant effect on *C. minitans*. The pathogen was highly sensitive to iprodione (concentration to inhibit growth or spore germination by 50% (EC_{50}) 7-18 μ g a.i. ml^{-1}), moderately sensitive to thiram (52-106 μ g a.i. ml^{-1}) but less sensitive to the other tested pesticides. However, in soil tests, despite weekly applications of pesticides at twice their recommended concentrations, *C. minitans* survived in soil and infected sclerotia equally well in all pesticide-treated and untreated control soils. The number of colony forming units (CFU) remained constant over the four-week period of the tests between 0.2 – 5.6 x 10^6 CFU per cm^3 soil. The level of infection of sclerotia by all isolates of *C. minitans* was always greater than 80% and was normally over 95%. Sclerotial viability in *C. minitans* treatments was reduced to less than 20% in comparison with control treatments lacking *C. minitans*, where sclerotial viability was always above 88%. This demonstrates the importance of assessing pesticide compatibility in environmentally relevant tests.

Glasshouse trials

In the first glasshouse trial, disease caused by *S. sclerotiorum* was significantly reduced by both iprodione-tolerant and standard strains of *C. minitans* and was enhanced when combined with a single application of iprodione. This enhanced control was irrespective of whether the *C. minitans* was iprodione tolerant or not (Table 1). Populations of *C. minitans* in the soil at harvest were the same in all *C. minitans* treatments between 3 – 12 x 10^6 CFU per cm^3 soil. The iprodione-tolerant isolate may have had no advantage over the standard *C. minitans* isolate in these trials because the soil prevented exposure of the mycoparasite to an inhibitory dosage of fungicide.

Table 1. Effect of standard and iprodione tolerant *C. minitans* soil incorporation and Rovral sprays on control of *S. sclerotiorum* in three sequential lettuce crops.

Treatment	Diseased plants (%)		
	1st Crop	2nd Crop	3rd Crop
i) Control (nil)	3	63a	55a
ii) Standard *C. minitans*	1	24b	31b
iii) Iprodione tolerant *C. minitans*	0	30b	37ab
iv) Standard *C. minitans* + Rovral	1	17b	22c
v) Iprodione tolerant *C. minitans* + Rovral	2	19b	24c

Numbers in the same columns followed by the same letters are not significantly different according to L.S.D. ($P \leq 0.05$) based on the F test in ANOVA.

In the second experiment, the enhanced control achieved by the combination of *C. minitans* and a single application of iprodione was equivalent to the level of control achieved by fortnightly prophylactic sprays with iprodione (Table 2). These results indicate that integrated control of *S. sclerotiorum* using soil applications of *C. minitans* and reduced foliar iprodione applications is feasible and does not require an iprodione tolerant isolate.

Table 2. Effect of *C. minitans* soil incorporation and Rovral sprays on control of *S. sclerotiorum* in three sequential lettuce crops.

Treatment	Diseased plants (%)		
	1st Crop	2nd Crop	3rd Crop
i) Control (nil)	12a	32a	84a
ii) Single Rovral spray	7b	20b	72a
iii)Fortnightly Rovral spray	5b	5b	21c
iv) *C. minitans*	7b	19b	44b
v) *C. minitans* + Single Rovral spray	1b	15b	29c

Numbers in the same columns followed by the same letters are not significantly different according to L.S.D. ($P \leq 0.05$) based on the F test in ANOVA.

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Biological Control of Fungal and Bacterial Plant Pathogens
IOBC wprs Bulletin Vol. 24 (3) 2001
pp.221 - 224

Efficacy of a new liquid formulation from *Fallopia sachalinensis* (Friedrich Schmidt Petrop.) Ronse Decraene as an inducer of resistance against powdery mildew in cucumber and grape

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Abstract: In trials performed in The Netherlands, Germany and Greece in greenhouse-grown cucumber and/or field-grown grape, weekly applications of a new liquid formulation prepared from *Fallopia sachalinensis* (formerly *Reynoutria sachalinensis*) called Milsana® (VP99), resulted in significant reduction of infection by *Sphaerotheca fuliginea* and *Uncinula necator*, respectively. Increase in yield (fruit weight) after induction of resistance amounted up to 29.5 % in cucumber (Milsana® 0.5 %) and up to 50.5 % in raisin grapes (Milsana® 1 %). No difference in the effectiveness on grape cultivars differing in their susceptibility to the pathogen was observed.

Key words: induced resistance, plant extract, *Sphaerotheca fuliginea, Uncinula necator*

Introduction

The giant knotweed, *Fallopia sachalinensis* (syn. *Reynoutria sachalinensis*) has been reported earlier for its resistance-inducing properties in several crops, especially against powdery mildew fungi (Herger & Klingauf, 1990; Daayf *et al.*, 1995). Previously, water extracts were prepared freshly from the plant powder each time before application. Since this procedure is too time-consuming for use in practice, and not always reproducible, a liquid extract formulation is under development. This formulation (Milsana® VP99) was tested in Germany, Greece and The Netherlands in small scale trials and/or large scale greenhouse and field trials in grape and/or cucumber for its efficacy against powdery mildew, influence on yield, and performance in cultivars differing in their susceptibility towards the pathogens.

Material and methods

Greenhouse trials in cucumber

In The Netherlands and in Germany the cv. Jessica, and in Greece cv. Sandra were used. Trials were arranged as randomised blocks with 4 or 6 replicates and 4 or 6 treatments. The fungicide controls were: in Greece alternating application of pyrazophos (Afugan EC; 0.3 ml/l), sulphur (Netzschwefel WP; 2.0 g/l), myclobutanil (Systhane 12E EC; 0.5 ml/l) and penconazol (Topas 10EC; 0.17 ml/l) as needed; in The Netherlands fenarimol (Rubigan; 0.2 ml/l) and tolylfluanid (Eupareen M; 1.5 g/l) as needed; in Germany, lecithin (Bioblatt

Mehltaumittel; 1.5 ml/l) applied weekly. Milsana® (VP99) was applied at a rate of 2.0 and/or 5.0 ml/l, starting when first disease symptoms appeared on cotyledons. Artificial inoculation was conducted in Germany. Milsana® and water were applied weekly, 8 to 12 times. Percentage diseased leaf area (EPPO-guidelines) was recorded weekly in Germany and The Netherlands and 3 times in Greece. Number and weight of fruit was recorded after harvesting 2 to 3 times per week.

Field trials in grape

In Germany, the cv. Müller-Thurgau and Samtrot and in Greece the cv. Black Corinth were used. Trials were arranged as randomised blocks with 4 replicates. Milsana® (VP99) was applied at a rate of 10 and/or 5ml/l. As a standard, wettable sulphur (Netzschwefel WP) was sprayed (2.0 g/l in Greece applied 5 times, on 14 day intervals; 2.0 or 6.0 g/l in Germany as needed). In Greece, applications started at ES 15 (BBA-scale). Milsana® was applied 10 times at weekly intervals. In Germany, applications in the vineyard of Korb started at ES 17, amounting to a total of 8 sprays at 8 to 13 day intervals. In the vineyard of Bad Kreuznach, Milsana® was applied 10 times, starting at ES 09 and continuing in intervals of 9 to 13 days. Disease severity on fruit was recorded after BBA-guidelines in classes, and transformed into percentage after the Townsend-Heuberger formula.

Tests on different cultivars of grape

The cv. Soultanina, Black Corinth and Cabernet Sauvignon were treated with Milsana® 10 ml/l, dinocap (Karathane EC; 0.175 g/l) and water at 7-day intervals. First application took place at the 1-leaf stage with 5 sprays in total. Artificial inoculation was performed every 7 days. Percentage diseased leaf area was assessed 18 and 40 days after the first artificial inoculation.

Results

Large scale greenhouse trials in cucumber against powdery mildew* (Sphaerotheca fuliginea) *in Germany, Greece and The Netherlands

In all three greenhouse trials, final disease severity on the upper surface of cucumber leaves reached between 89.1 and 100 % in the control plots. Weekly applications of Milsana® (0.5 %) resulted in final disease levels of 68.1 % in The Netherlands, 14.2 % in Germany and 1.4 % in Greece. The corresponding efficacy was 28.5, 85.8 and 98.3 % respectively. Even though the level of disease in the trial in The Netherlands was relatively high in the plots treated with Milsana®, the disease development was significantly slower than in the control plots. This was reflected in the Area Under the Disease Progress Curve (AUDPC), that was reduced by 43 % compared to the control. Extract-treated cucumber leaves showed a dark green colour in all trials. In Greece and Germany however, browning on leaf edges, due to the treatments were recorded.

Application of Milsana® at a concentration of 0.2 % (trials in Germany and Greece) also reduced the infection with powdery mildew to a high degree, resulting in an efficacy of 52.3 and 79.9 % (upper leaf surface), respectively. The increase in yield (weight of fruit) for plants treated with 0.5 % Milsana® reached between 21.6 and 29.5 % in all trials, whereas after application of Milsana® 0.2 % an increase of 11.6 and 25.7 % was achieved. In all cases, yield of cucumber after treatment with the plant extract was comparable with or higher than yield after fungicide application (Table 1). The number of fruit per plant was increased by 13 and 14.5 % (Milsana® 0.2 %) and by 16.7 and 22 % (Milsana® 0.5 %) in Germany and Greece, respectively (data not shown). The increase in yield after Milsana® treatment was in all trials significant at $P \leq 0.05$.

Table 1. Increase [%] in total weight of harvested cucumber fruit over yield in water-treated control plants in greenhouse trials against powdery mildew.

Treatments	Increase [%] in total weight of harvested cucumber fruit		
	Germany	Greece	The Netherlands
Milsana® (VP99) 0.2 %	25.7	11.6	/
Milsana® (VP99) 0.5 %	24.7	21.6	29.5
Fungicides	11.4	14.2	4.8

Large scale field trials in grapevine against powdery mildew* (Uncinula necator) *in Germany and Greece

In Germany, two trials were performed in wine grapes (vineyards of Korb and Bad Kreuznach). Disease severity on berries reached 93.9 and 89.8 % in the water-treated control plants, after weekly treatments with Milsana® (0.5 %) 2.6 and 16.7 %, and after sulphur treatment 2.5 and 32 % in Korb and Bad Kreuznach, respectively. In Greece, (vineyard Aigion; organic culture), disease severity on untreated raisin grapes was 82.1 %, while weekly treatments with Milsana® (0.5 and 1 %) resulted in 72.1 and 62.8 %, respectively. In all trials, leaves of extract-treated plants showed a dark green colour. However, some necrotic browning on leaf edges was visible.

Table 2. Weight of harvested grape berries in field trials against powdery mildew.

Treatments	Weight of grape berries in the vineyards	
	Korb (wine grape) [kg/ha]	Aigion (raisin grape) [kg/grapevine]
Milsana® (VP99) 0.5 %	8000	2,84 b*
Milsana® (VP99) 1 %	/	3,10 b
Sulphur	9050	2,58 ab
Water control	0	2,06 a

*Numbers followed by different letters are significantly different at $P \leq 0.05$.

Due to the high infection of wine grapes with powdery mildew, no berries could be harvested in the control plots in Korb. From plots treated with the plant extract, 8 t/ha were harvested, while sulphur- treated plots yielded 9.05 t/ha. In Greece, the yield of raisin grapes amounted to 2.06 kg/grapevine in the control plots, while treatment with 0.5 and 1 % Milsana® increased in yield by 37.9 and 50.5 %, respectively (Table 2).

Effectiveness of Milsana® in cultivars of grape with different susceptibility to powdery mildew

In three grape cultivars, Cabernet Sauvignon (less susceptible), Soultanina and Black Corinth (more susceptible to powdery mildew), weekly application of 1 % Milsana® resulted in a complete control of powdery mildew on the leaves independently of disease pressure, which reached between 44.9 and 64.29 % in the control plants.

Discussion

In all greenhouse trials in cucumber, weekly application of Milsana® (VP99) reduced disease severity significantly compared to control plants. Treatment with 0.5 % Milsana® reduced infection with powdery mildew more effectively than 0.2 % extract concentration. In most trials, yield increase occurred also to a higher extend when 0.5 % Milsana® was applied, reaching levels between 21 and 30 %, which was always higher than after treatment with fungicides. Furthermore, the results from the trial in Germany indicated that Milsana® had a direct positive effect on yield despite the prevailing infection level.

In all grape trials, infection of berries in control plots was high, however, percentage reduction in powdery mildew severity by Milsana® was more prominent in the German (wine grape) than in the Greek trial (raisin grape). The reasons for this are not yet clear. For infection on leaves it was shown that an extract concentration of 1 % resulted in equally low disease levels independent of the grape cultivars used. Other studies pointed to the fact that cultivars differing in their susceptibility would respond differently to induction of resistance (Dik & Van der Staay, 1995). Further investigations will be necessary to determine inducibility in grape berries with respect to different cultivars.

Similar to the cucumber trials, the higher extract concentration (1 %) tended to give better results than the lower ones. Taking into account that raisin grapes could be harvested even though berries are infected with powdery mildew, the 50 % increase in yield achieved after treatment with Milsana® in the Greek trial would be highly valuable for organic growers.

The experiments showed that regular applications of the new liquid formulation Milsana® (VP 99) resulted in significant reduction of infection with powdery mildew and in an enhancement of yield. Browning of leaves appeared to a certain extend while the overall appearance was dark green. Further development of the formulation is in progress.

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Biological Control of Fungal and Bacterial Plant Pathogens
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Influence of organic matter on the interaction between the biocontrol agents *Glomus intraradices* and *Burkholderia cepacia*

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Abstract: Several biocontrol agents (BCAs) acting against root pathogens have been identified. Combining antagonists from different trophic levels might enhance biocontrol efficacy compared to the use of single BCAs. In the present experiment, we wanted to examine the compatibility of two potential BCAs, the biotrophic arbuscular mycorrhizal fungus *Glomus intraradices* and the saprophytic bacterium *Burkholderia cepacia,* under different nutritional conditions. Cucumber (*Cucumis sativus* L.) was grown in a compartmented growth unit, which enabled us to study interactions between *G. intraradices* and *B. cepacia* in root-free sand, with and without organic matter. Fatty acid signatures, from a whole-cell fatty acid analysis, were used to quantify the individual microorganisms. The fatty acid 16:1ω5 was used as a biomass marker of *G. intraradices*, and the cyclic fatty acids, cy17:0 and cy19:0, as biomass markers of *B. cepacia.* In addition, hyphal length density of *G. intraradices* was also quantified. Organic matter had a stimulatory effect on the biomass of *G. intraradices* and *B. cepacia* as indicated by the different fatty acid signatures and hyphal length density of *G. intraradices*. Generally, *G. intraradices* was stimulated by *B. cepacia,* whereas *B. cepacia* was unaffected by *G. intraradices*, suggesting that these two BCAs are compatible.

Key words: *Glomus*, *Burkholderia*, fatty acid, biological control, organic matter, interaction

Introduction

Several biocontrol agents (BCAs) acting against root pathogens have been identified, such as arbuscular mycorrhizal fungi (Linderman, 1994) and bacteria (Gaskins *et al.*, 1985; Bevivino *et al.*, 2000). A biocontrol strategy where antagonists from different trophic levels are combined might enhance biocontrol efficacy compared to the use of single BCAs. The objective of the present experiment was to examine whether the biotrophic arbuscular mycorrhizal fungus *Glomus intraradices* (Schenck and Smith, BEG 87) and the saprophytic bacterium *Burkholderia cepacia* (Bc2, kindly provided by Daniel Roberts, USDA), are compatible under different nutritional conditions. Furthermore, we wanted to study whether fatty acid signatures, assayed by a whole cell fatty acid analysis, could be used to monitor *G. intraradices* and *B. cepacia* specifically.

Materials and methods

Experimental design

Mycorrhizal and non-mycorrhizal *Cucumis sativus* L. cultivar Aminex were grown in a growth unit with root-free sand compartments (Fig. 1) as described by Larsen *et al.* (1998). The soil in the root chamber (RC), consisting of a mixture of sandy loam and quartz sand 1:3

(w/w) with a low phosphor content (8 mg kg^{-1} soil), and a pH of 6.1, was irradiated, (10 kGy, 10 MeV electron beam) to eliminate intrinsic propagules of mycorrhiza. Basal nutrients where added to the soil in the following amounts (mg kg^{-1}): NH_4NO_3 (86), KH_2PO_4 (132), K_2SO_4 (70), $CaCl_2$ (70), $CuSO_4,5H_2O$ (2.2), $ZnSO_4,7H_2O$ (5), $MgSO_4,H_20$ (10), $CoSO_4,7H_2O$ (0.33), $NaMoO_4,2H_2O$ (0.2), and $MgSO_4,H_2O$ (20). The inoculum of *G. intraradices* was obtained from *Cucumis sativus* L. pot culture and consisted of soil, minor roots, and spores. This inoculum replaced 50 g of the 740 g soil used in each RC in treatments with *G. intraradices*. To establish a uniform microbial community 10-ml soil filtrate (20 µm-nylon mesh) was added to each RC. The growth units were placed in a greenhouse and rearranged 3 times a week when watered. The RCs were watered to approximately 60% (w/w) of the water holding capacity, and once a week, 20 mg N was added to the RC in the form of NH_4NO_3. Four weeks after sowing, when the cucumber-*G. intraradices* symbiosis was established, each root-free chamber (RFC) was filled with 104 g semi-sterile quartz sand. Buffer zones were made at both ends of each RFC, thereby enclosing the interaction zone. The sand in the interaction zone (62 g) was mixed with fine ground barley leaves (cultivar Anne-Sofie, harvested 6 weeks after sowing) to obtain 0.5% (w/w) content of organic matter, whereas the sand in the second RFC received no organic matter. At the same time, *B. cepacia* was mixed into the sand in both RFCs (10^9 cfu g^{-1} sand). The batch culture of *B. cepacia* was harvested after 24 hours growth in nutrient broth at 37°C on a rotary shaker at 150 rpm. After centrifugation for 10 minutes at 20°C and 6000 rpm, the pellet was resuspended in 0.9% NaCl and washed twice in sterile distilled water. After plating on trypticase soy broth agar (TSBA), the number of colony forming units (cfu) was counted after 24 and 48 hours incubation at 22°C. The experiment had a full factorial design with 8 treatments (± *G. intraradices* × ± *B. cepacia* × ± organic matter), each with 4 replicates. The control was without *G. intraradices* and *B. cepacia*.

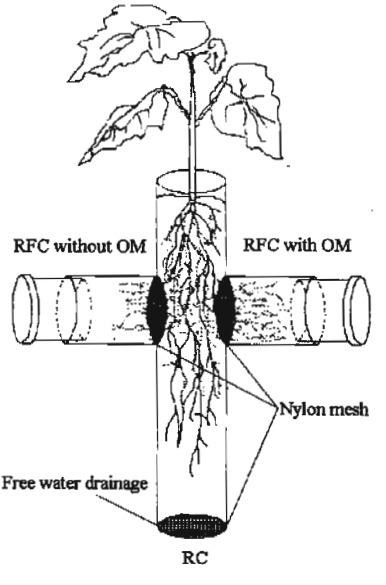

Figure 1. Cucumber plant grown in a compartmented growth unit with a central root chamber (RC), and two lateral root-free sand chambers (RFCs) with and without organic matter (OM).

Harvest and analysis

Plants were harvested four weeks after adding sand to the RFCs. Freeze-dried sand samples (2.5 g) from the RFCs were subjected to lipid extraction and analysed for content of whole cell fatty acids (WCFA), as described by Larsen *et al.* (2000). To enable quantification of the amount of the individual WCFA, a known amount of nonadecanoate (19:0) solution was used as an internal standard and was added to each tube containing the sand sample. The fatty acid 16:1ω5 was used as a biomass marker for *G. intraradices*, and the cyclic fatty acids cy17:0 and cy19:0 as markers for *B. cepacia.* Hyphal length density of *G. intraradices* was measured as described by Jakobsen *et al.* (1992).

Statistical analysis

The treatments in the two RFCs, amended or unamended with organic matter, belonging to the same growth unit were assumed to be independent. The fatty acid contents and the hyphal length density data were transformed logarithmically before analysis to obtain variance homogeneity based on Bartlett's test. Levels of significance of main treatments and their interaction were analysed using multifactor ANOVA (STATGRAPHICS Plus, Version 4.0, Copyright Manugistics Inc., Rockville, USA). Means were compared by Fisher's protected least significant difference (LSD) test at $P \leq 0.05$.

Results and discussion

The experiment confirmed the specificity of the fatty acids signature used to quantify *G. intraradices* and *B. cepacia.* The fatty acid signature 16:1ω5c was exclusively found in treatments with *G. intraradices* (Table 1). The fatty acid cy19:0 was useful as a marker for *B. cepacia.* However, in this system, the presence of *B. cepacia* could not be distinguished from the background of other gram negative bacteria when using the fatty acid cy17:0. The amount of specific fatty acids for *G. intraradices* and *B. cepacia* increased considerably in the presence of organic matter ($P \leq 0.001$). Similarly, the hyphal length density of *G. intraradices* increased in the presence of organic matter ($P \leq 0.001$). Also the presence of *B. cepacia* stimulated the hyphal length density of *G. intraradices*, but this was only significant ($P \leq 0.001$) in treatments without organic matter. Interestingly, the amount of the biomass marker 16:1ω5c in the same treatments showed that the presence of *B. cepacia* had a negative effect on *G. intraradices* without organic matter and a stimulating effect with organic matter. Since the WCFA 16:1ω5 originates from both mycelium and spores of *G. intraradices,* the results mentioned above can be explained by a reduced sporulation of *G. intraradices* in the presence of *B. cepacia* without organic matter compared to when *B. cepacia* was present with organic matter. The additive effect of *B. cepacia* and organic matter on *G. intraracides*, as indicated by the increased amounts of 16:1ω5c, might be caused by the release of unidentified compounds from *B. cepacia*, which may have a stimulating effect on the growth of *G. intraradices*. The mycelium of *G. intraradices* seemed to have no effect on the biomass of *B. cepacia.*

This experiment indicates that *G. intraradices* (BEG 82) and *B. cepacia* (Bc2) are compatible. The use of specific fatty acid markers to study the interaction between *G. intraradices* and *B. cepacia* were confirmed and WCFA analysis was shown to be a reliable, fast, and useful method for specific quantification of *G. intraradices* and *B. cepacia.* However, as *B. cepacia* is a rhizosphere coloniser, a complementary study should be carried out to examine its interaction with *G. intraradices* in the rhizosphere. Also, future investigations will be carried out to study the biocontrol efficacy of the combined use of *G. intraradices* and *B. cepacia* against the root pathogen *Pythium ultimum.*

Table 1. Whole cell fatty acid (WCFA) content and hyphal length density in freeze-dried sand from root-free compartments. Different letters indicate significant differences according to Fisher's least significant difference procedure ($LSD_{0,05}$). The mean values were obtained from non-transformed data whereas the LSD-values of 16:1ω5c, cy19:0, and hyphal length density were obtained from log transformed data.

Treatment			WCFA (nmol g^{-1} dry sand)			Hyphal length
G. intraradices	*B. cepacia*	Organic matter	16:1ω5c[$]	cy17:0[£]	cy19:0[£]	(m g^{-1} dry sand)
-	-	-	0^{a}	0^{a}	0^{a}	0.79^{a}
-	-	+	0^{a}	2.00^{c}	0.67^{bc}	6.38^{d}
-	+	-	0^{a}	0.41^{ab}	0.44^{b}	1.02^{a}
-	+	+	0^{a}	1.75^{c}	1.39^{d}	3.70^{c}
+	-	-	2.74^{c}	0.18^{a}	0^{a}	1.95^{b}
+	-	+	30.32^{d}	1.46^{bc}	0.98^{cd}	28.76^{e}
+	+	-	1.82^{b}	1.75^{c}	0.42^{b}	6.84^{d}
+	+	+	45.16^{c}	2.08^{c}	1.65^{d}	34.27^{e}

Ad[$] Used as specific marker for *G. intraradices*.
As[£] Used as specific marker for *B. cepacia*.

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Biological Control of Fungal and Bacterial Plant Pathogens
IOBC wprs Bulletin Vol. 24 (3) 2001
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Interaction of the biocontrol agent *Brevibacillus brevis* with other disease control methods

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Abstract: Biocontrol agents, under certain circumstances, have been shown to be as effective as chemical fungicides in controlling plant disease. For an integrated approach to the control of foliar disease, however, it is likely that microbial biocontrol agents, biologicals such as plant extracts, and chemical treatments (fungicides and insecticides) will be used in combination. To determine how successful such an integrated approach might be, the effect on the behaviour of the biocontrol agent in terms of microbial activity and growth must be studied in combination with these other treatments to highlight the compatibility between them. This research is part of an EU initiative (BIOCOMBI) to look at the effect of combinations of biological treatments on two important plant diseases; grey mould and powdery mildew on tomato, cucumber and grape. *Brevibacillus brevis* (formerly *Bacillus brevis*) has been shown to control grey mould (*Botrytis cinerea*) *in vitro* and *in vivo* on tomato and lettuce (Seddon *et al.*, 2000). The compatibility between a range of fungicides, insecticides and a plant extract from *Fallopia sachalinensis* (formerly *Reynoutria sachalinensis*) and, *B. brevis* was tested using plate inhibition diffusion assays and growth in liquid culture. In addition, for the plant extract, population dynamics of *B. brevis* on young tomato plants were determined. Of the 14 fungicides and 7 insecticides/acaricides tested (at 1 mg ml^{-1}), six fungicides: chlorothalonil, dichlorofluanid, dinocap, penconazol, thiram and tolylfluanid, but none of the insecticides, inhibited the growth of *B. brevis* in liquid culture. Agar diffusion assays were ineffective at detecting growth inhibition due to the insolubility in water of these compounds. The plant extract was found to inhibit the growth of *B. brevis*, but not spore viability in liquid culture. On tomato plants, there was a significant decease in cell numbers after 2 days, which did not appreciably change until after 10 days. Considering these observations it is now possible to formulate compatible combinations for an integrated disease control programme.

Key Words: *Brevibacillus brevis*, plant extracts, pesticides, biological combinations

Introduction

Microbial control of plant pests and diseases has to a large extent focussed upon the use of single inoculants which are unlikely to offer a broad spectrum solution. An integrated approach of both biological and chemical applications may therefore be necessary. Knowledge of the combinations of biological control and chemical treatments that are compatible are important for efficient use of biocontrol agents in practice.

The plant extract Milsana®, extracted from *Fallopia sachalinensis*, (formerly *Reynoutria sachalinensis*) and the biological control agent *Brevibacillus brevis* (formerly *Bacillus brevis*) are being investigated as part of an EU initiative (FAIR-CT98-4413: BIOCOMBI) to look at the effectiveness of new combinations of biological treatments to control two important plant diseases; grey mould and powdery mildew on tomato, cucumber and grape. The plant extract

from *F. sachalinensis* and *B. brevis* have been shown to control both *Sphaerotheca fuliginea* and *Botrytis cinerea* by using different mechanisms. Seddon and Schmitt (1999) have suggested that combinations of the two biological treatments may also work synergistically leading to a reduction in the application levels. This research was conducted to determine the compatibility between *B. brevis*, Milsana® and several pesticides used in glasshouse grown crops.

Materials and Methods

***Effect of Milsana® VP2000 on growth and, vegetative and spore viability of* B. brevis**

The effect on cell growth was determined by culturing cells of *B. brevis* in 100 ml Tryptone Soy Broth (TSB) containing Milsana® VP2000 at 0.2 - 2.0 % (v/v) at 37°C in a rotary incubator and samples taken over 7 d. The effect on vegetative cells and spores were determined by initially heat treating (80°C, 10 min) young (6h) (containing vegetative cells) and old (7d) (containing both vegetative cells and spores) cell suspensions and exposing both heat treated and non-heat treated cell suspensions to Milsana® for 30 min. Cells were enumerated by 1:10 serial dilutions and plating (5 x 20 µl) onto Tyrosine agar (containing (g l^{-1}); nutrient broth (6.5), tyrosine (5) and agar (15)). Plates were incubated for 2 d at 37°C.

Population dynamics of* B. brevis *on young tomato plants

Milsana® VP2000 was added at 0.2% to a 7 day old culture of *B. brevis* grown at 37°C in TSB. The suspension was sprayed onto the leaves and stem of 4 week old tomato plants (cv. Moneymaker) grown at 25°C with 16h light. Plants were destructively sampled by removing 3 leaves and 3 stem sections (approx. 3-4 cm in length) from each replicate (n=5 plants). The population of *B. brevis* was determined from the tissue sections by placing each section in 5 ml ¼ strength Ringers solution, sonicating for 10 min. and vortexing for 30 seconds. Cell numbers were estimated as above.

***Effect of pesticides on growth of* B. brevis**

The effect of a range of fungicides and insecticides (see tables 1 a, b) on the growth of *B. brevis* in TSB (1 ml) was measured by monitoring the change of culture OD_{595}. Pesticides were initially prepared as a 100 mg ml^{-1} stock solution in either methanol, ethanol or acetone and 1:10 serially diluted in $d.H_2O$ to give a final pesticide concentration of 1 mg ml^{-1} and solvent concentration of 1%. Growth rates from 6-24 h, representing the exponential growth period, were compared (Tukey HSD, n=3). No significant difference ($P \leq 0.05$) was found in the growth of cells exposed to 1% solvent. Plate inhibition assays were performed using *B.brevis* seeded TSB agar plates (20 ml per plate) with pesticide (1 mg ml^{-1}) added into previously cut wells (approx. 100 µl per well, wells 6 mm dia.) (n=5). Plates were incubated for 24h at 37°C.

Results

Compatibility between* B. brevis *and Milsana®

In vitro, no growth of *B. brevis* was observed at Milsana® concentrations at 0.5% and above (data not shown). At 0.2%, there was a decrease in viable cell numbers from the starting inoculum value (taken after 3 h) to the levels which were undetectable after 1 d and which did not recover during the experiment. Exposing spores, vegetative cells or both spores and vegetative cells to Milsana® indicated that spores were more resistant to Milsana® than vegetative cells (data not shown).

In vivo, when combined with Milsana® at 0.2%, there was a significant difference in the populations of *B. brevis* on both leaves and stem (Fig. 1) 2 days after spraying. The levels of *B. brevis* maintained on these plants did not however decrease appreciably until after 10 days.

Compatibility between* B. brevis *and pesticides

Agar diffusion assays indicated that at 1 mg ml^{-1} that the fungicides dinocap and thiram inhibited the growth of *B. brevis* (data not shown). These assays depend on the mobility of the substance under test to diffuse through the agar. All of the pesticides tested have either very low solubilities or are insoluble in water, which may account for the low number of inhibitory substances found. A much more sensitive assay was found with the pure culture studies. To reduce the amount and volume of pesticide used in the experiment, growth of *B. brevis* was followed by monitoring changes in culture optical density using small (1 ml) culture volumes. In this assay, of the 21 pesticides tested, 6 fungicides but none of the insecticides (Table 1a, b) inhibited growth.

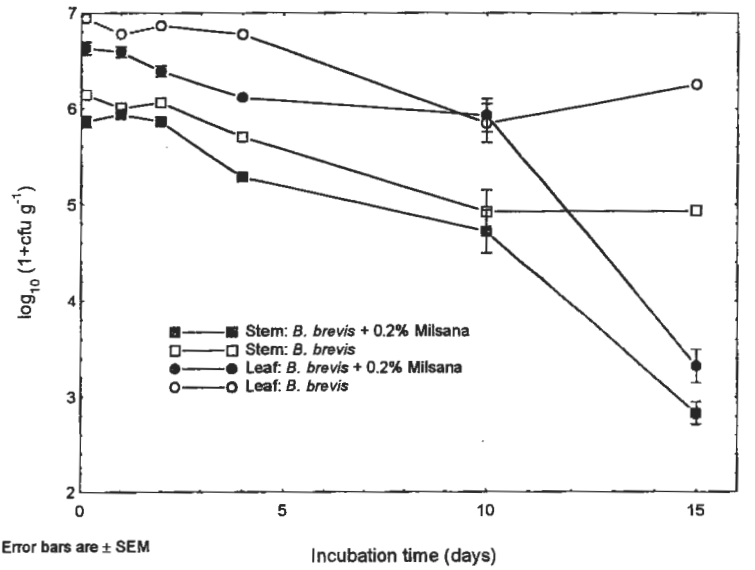

Figure 1. Effect of Milsana® (0.2%) on population dynamics of *B. brevis* on young tomato plants.

Discussion

The combination of two or more disease control methods such as biological inocula, plant extracts and certain agrochemical treatments can offer a tremendous advantage over one treatment. Determining their compatibility is an initial step in assessing their effectiveness *in situ*. From this research, it is evident that some combinations of pesticides are possible and may lead to their combined use in some IPM programmes. The effect on the viability of vegetative cells but not on spores of *B. brevis* by Milsana® may not be a problem when used in the field. Gramicidin S production by *B. brevis*, required for antifungal activity, occurs after spore formation and current formulations of *Bacillus* sp. will normally use spores due to their longevity and ease of handling.

Of the 6 fungicides that inhibited the growth of *B. brevis*, tolylfluanid and dichlorofluanid have been recommended for IPM programmes. It is therefore evident that combinations of *B. brevis* and possibly other microbial biocontrol agents with these fungicides may not be possible depending on the application site, for example thiram is often used as a seed coat formulation for the control of *Pythium* damping-off. As there may also be spatial and concentration effects *in vivo*, experiments are planned to determine their compatibility on plants. None of the insecticides/ acaricides tested inhibited the growth *B. brevis* which may indicate either, the biocontrol agent is resistant perhaps due to an incompatible metabolism or will tolerate higher concentrations of these compounds.

Table 1a. Effect of fungicides (1 mg ml^{-1}) on growth of *B. brevis*.

Fungicide	*B. brevis* Growth rate $x10^2$ (OD/h)	Fungicide	*B. brevis* Growth rate $x10^2$ (OD/h)
carbendazim	4.00	penconazol	0.01*
chlorothalonil	0.52*	pyrazophos	2.00
Cu oxychloride	3.39	pyrimethanil	2.54
dichloran	4.80	quintozen	2.15
dichlorofluanid	0.05*	thiram	0.05*
dinocap	0.00*	tolylfluanid	0.09*
iprodione	1.78	triflorin	4.69

Table 1b. Effect of insecticides and acaricides (1 mg ml^{-1}) on growth of *B. brevis*.

Insecticide/ acaricide	*B. brevis* Growth rate $x10^2$ (OD/h)	Insecticide/controls	*B. brevis* Growth rate $x10^2$ (OD/h)
buprofezin	3.90	Deltamethrin	1.58
dimethoate	1.98	Permethrin	2.27
fenpyroximate	2.94	d.H_2O	2.90
malathion	2.46	Acetone	2.30
pirimiphos-methyl	2.44	Ethanol	2.46
		Methanol	2.28

*Rates which are significantly different from the control (at $P \leq 0.05$), n=3.

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Biological Control of Fungal and Bacterial Plant Pathogens
IOBC wprs Bulletin Vol. 24 (3) 2001
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Isolation and characterization of heavy metal resistant mutants from mycoparasitic *Trichoderma* strains

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Abstract: The effect of 10 heavy metals on the *in vitro* activities of β-glucosidase, cellobiohydrolase, β-xylosidase and endoxylanase enzymes, all of them important for effective competition, was investigated in the case of six *Trichoderma* strains. At a concentration of 1 mM, only mercury showed significant inhibitory effects. In the case of other heavy metals, the enzymes of *Trichoderma* remained active, suggesting that screening for heavy metal resistant *Trichoderma* strains could result in biocontrol agents effective against phytopathogenic fungi, even under heavy metal stress. A total number of 177 heavy metal resistant mutants were isolated and tested for possible cross-resistance to each of the heavy metals. Some of the mutants were effective antagonists of *Fusarium*, *Pythium* and *Rhizoctonia* strains, even on media containing the corresponding heavy metal. Our results suggest that such mutants might be the preferred choice for combined application with heavy metal-containing pesticides in the frame of complex integrated plant protection.

Key words: *Trichoderma*, biocontrol, extracellular enzymes, heavy metals

Introduction

The mycoparasitic ability of *Trichoderma* species against phytopathogenic fungi, e.g. *Pythium*, *Fusarium* and *Rhizoctonia* species, allows for the development of biocontrol strategies (Papavizas, 1985). When planning the application of biocontrol strains, it is important to consider the environmental stresses affecting microbial activities. The presence of heavy metals in the soil is among the most important stress factors. Heavy metal sorption (Morley & Gadd, 1995), and accumulation (Ledin *et al.*, 1996) by *Trichoderma* strains, and the influence of some heavy metals on the growth, sporulation (Babich *et al.*, 1982), and differentiation (Frank *et al.*, 1993) of these fungi was examined. The effect of heavy metals on the *in vitro* activities of extracellular enzymes involved in mycoparasitism (β-1,4-*N*-acetyl-glucosaminidase, β-1,3-glucanase, trypsin-like and chymotrypsin-like proteases) was also studied in some *Trichoderma* strains (Kredics *et al.*, 2000). This study was designed to investigate the effect of heavy metals on the *in vitro* activities of extracellular enzymes important for the competititive abilities of *Trichoderma* strains, and to isolate and characterize heavy metal resistant mutants.

Materials and methods

Microorganisms and culture conditions

Strains of *T. aureoviride* T122, *T. harzianum* T66 and T334, and *T. viride* T124 and T228 were isolated by Manczinger *et al.* from forest soils in southern Hungary. *T. viride* T114

originated from the strain collection of the Budapest Technical University. These are cold tolerant strains antagonistic against *Pythium, Fusarium* and *Rhizoctonia* species (Antal *et al.*, 2000). The *P. debaryanum, R. solani, F. culmorum* and *F. oxysporum* strains were obtained from the strain collection of the Cereal Research Institute, Szeged.

One per cent of cellulose MN300 or xylan (Sigma), depending on the enzyme activities to be examined, was incorporated as an inducer in 20 ml liquid media (0.5% KH_2PO_4, 0.1% $NaNO_3$, 0.1% $MgSO_4 \cdot 7H_2O$). Fifty ml Erlenmeyer flasks were inoculated with conidial suspensions to a final concentration of 10^5 conidia/ml, and incubated at 25°C on a shaker at 200 rpm. Mycelial pellets were removed by centrifugation after 4 days of incubation, and enzyme activities were measured in the supernatants.

Enzyme assays

β-glucosidase, cellobiohydrolase and β-xylosidase activities were assayed using p-nitrophenyl-β-D-glucopyranoside, p-nitrophenyl-β-D-cellobioside and p-nitrophenyl-β-D-xylopiranoside (Sigma) substrates, respectively. From the supernatants, 100 µl was incubated with 50 µl substrate (2 mg/ml) and 50 µl heavy metal solution (4 mM) at 25°C for 1 hour. The measurements were carried out as described earlier (Kredics *et al.*, 2001). Endoxylanase activities were assayed using dinitrosalicylic acid reagent (Miller, 1959).

Isolation and characterization of mutants

Heavy metal resistant mutants were isolated by UV-mutagenesis using the following heavy metal concentrations for direct selection of the mutants: $AlCl_3 \cdot 6H_2O$: 25 mmol, $CuSO_4 \cdot 5H_2O$: 1.6 mM, $NiSO_4 \cdot 7H_2O$: 3 mM, $CoCl_2 \cdot 6H_2O$: 4 mM, $CdNO_3 \cdot 4H_2O$: 0.3 mM, $ZnSO_4 \cdot 7H_2O$: 2 mM, $MnSO_4 \cdot 4H_2O$: 12 mM, $PbNO_3$: 1 mM, $HgCl_2$: 0.4 mM, $FeSO_4 \cdot 7H_2O$: 1.5 mM. These concentrations resulted in 90% growth inhibition of the wild type strains. All mutants were tested on each of the heavy metals for cross resistance. The *in vitro* antagonistic properties of the wildtype and mutant *Trichoderma* strains were investigated against *Pythium, Rhizoctonia* and *Fusarium* strains by coinoculation of 4 mm diameter agar discs of the *Trichoderma* strains and the phytopathogens, 3 cm apart onto Petri plates containing 0.5% glucose, 0.1% yeast extract and the corresponding heavy metal in its IC_{50} concentration.

Results and discussion

Effect of heavy metals on* in vitro *activities of extracellular enzymes

The effect of 1 mM concentration of the heavy metals on the *in vitro* activities of the enzymes β-glucosidase, cellobiohydrolase, β-xylosidase and endoxylanase was investigated. β-Glucosidase activities (Fig. 1A) increased in the presence of 1 mM aluminium, copper, cadmium, zinc and lead, and decreased in the case of mercury, the other ions did not influence the *in vitro* activities of this enzyme significantly. Similar results were obtained for cellobiohydrolase (Fig. 1B), with the difference that manganese and iron seemed to activate this enzyme. The β-xylosidase activities (Fig. 1C) were also inhibited by mercury and activated by aluminium, lead and iron. There was a variation between the β-xylosidase activities of the strains in the response to nickel, cobalt, cadmium and zinc. Endoxylanase activities (Fig. 1D) were inhibited by mercury and activated by cobalt, manganese and iron.

The concentration of 1 mM mercury showed the strongest inhibition on the *in vitro* activities of all enzymes. The other heavy metals did not inhibit the investigated enzymes to such an extent, in some cases even increased enzymatic activities were measured. These results suggest, that screening for heavy metal resistant *Trichoderma* strains could reveal biocontrol agents effective against phytopathogenic fungi even in soils with heavy metal contamination.

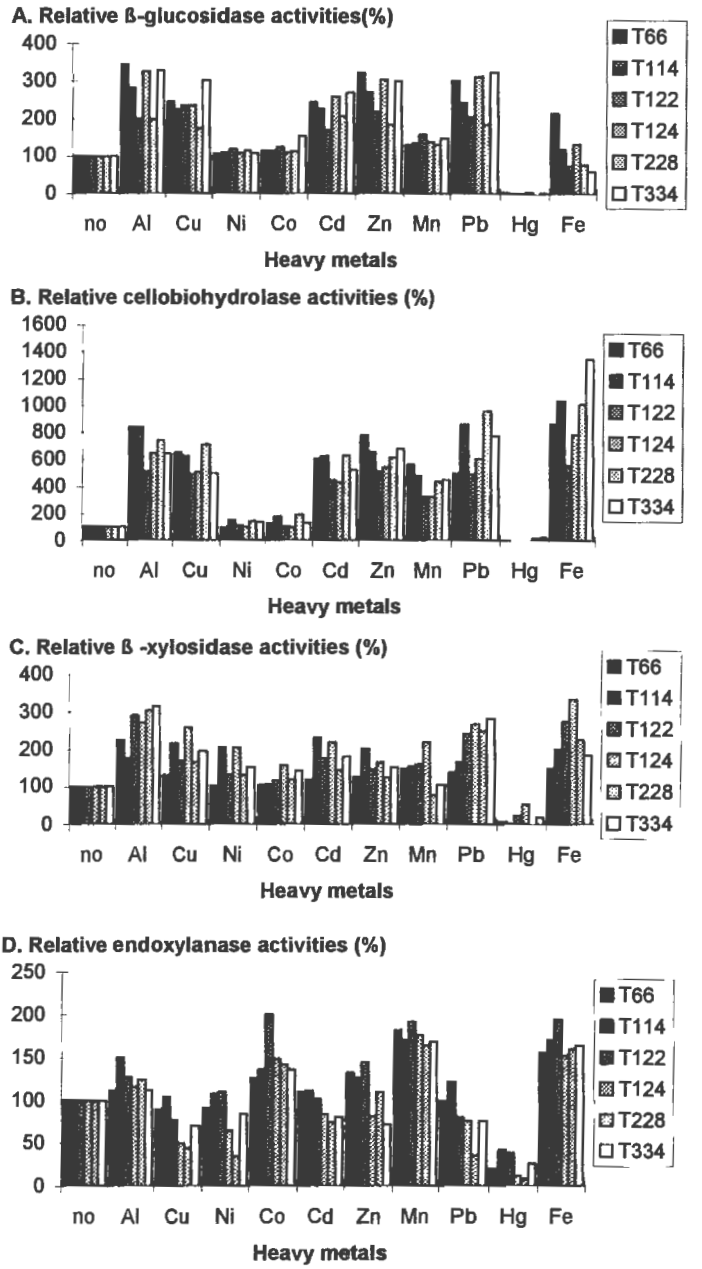

Figure 1. Effect of heavy metals on extracellular enzyme activities of *Trichoderma* strains.
no: control without heavy metals.
A.β-glucosidase, B.cellobiohydrolase, C.β-xylosidase, D.endoxylanase

Isolation and characterization of heavy metal resistant mutants

A total number of 177 heavy metal resistant mutants were isolated by direct UV-mutagenesis. Sixty-three % of the aluminium and 68% of the nickel-resistant mutants proved to be resistant to copper. Significant cross resistance was found also in the case of the lead-resistant mutants to cadmium and in the case of those resitant to copper and nickel.

Table 2. shows the *in vitro* antagonism of *T. harzianum* strain T66 and its copper-resistant mutant Cu_2 against *P. debaryanum*, *R. solani*, *F. culmorum* and *F. oxysporum* on yeast extract medium containing $CuSO_4 \cdot 5H_2O$. The effectiveness of antagonism in this cases and that of some other mutants was significantly higher, when compared with the wild-type strains. Such mutants may be preferred when applying biocontrol agents combined with heavy metal-containing pesticides for integrated plant protection.

Table 1. *In vitro* antagonism of *T. harzianum* T66 and T66Cu2 against phytopathogenic fungi on yeast extract medium containing 0.15 mM $CuSO_4 \cdot 5H_2O$ (colony diameters of phytopathogens in mm after 7 days of incubation).

Strains	*P. debaryanum*	*R. solani*	*F. oxysporum*	*F. culmorum*
T66	69.2	69.4	55.4	81.3
T66Cu2	24.2	55.4	36.3	69.0

Acknowledgements

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Biological Control of Fungal and Bacterial Plant Pathogens
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Fusarium wilt in tobacco burley: cultural and biological management in Tucumán (República Argentina)

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Abstract: Due to a request from the tobacco company Nobleza Piccardo SAICyF (BAT – British &American Tobacco Group) we monitored tobacco diseases in the Northwest of Argentina (Salta, Jujuy, Tucumán provinces). Fusarium wilt (*Fusarium oxysporum* f. sp. *nicotianae*) was the main fungus disease in Tucumán. The aim of this work was to emphasize improved cultural management practices and to start biological control tests for reducing incidence of disease. Tests were conducted during the 1999/00 season in 4 very affected farm. Burley tobacco var Ky 17 was used, in macro-plots with 5 rows (100 m each) with 2 repetitions. Treatments were: A) cultural management, B) biological control, C) biological control plus cultural management; D) control or conventional management. Cultural management consisted of constructing high and wide hills and minimizing machinery access into crop for avoiding root damage. *Trichoderma harzianum* was used as the biocontrol agent, applied 3 times: pre-transplant (root immersion), and 15 and 30 days after transplant by stem drip. Tobacco plants in 3 central macro-plot rows were evaluated at 15, 30,60 days from transplant and at harvest. At the last evaluation, percentage affected plants varied between: A) 3,6 - 6% (38,7%), B) 3,3 - 5,7% (38,7%); C) 3,7 - 10,7% (30%) and D) 14,7 - 26,7% (45,7%). Values between parentheses were from a field test where cultural practices were not done properly. Affected plants in *Trichoderma* treated plots, generally, did not show intense wilt, but their leaves were more yellow and smaller than healthy plants. In conclusion, for certain cases, executing proper cultural practices may improve tobacco plant stand at harvest time even without application of a fungal biocontrol addition.

Key words: tobacco, wilt, biocontrol, management

Introduction

Tobacco is a very important crop in Northwest of Argentina (Salta, Jujuy and Tucumán provinces). Due to a request from the tobacco company Nobleza Piccardo SAICyF (BAT – British & American Tobacco Group) for reducing chemical pa./ha, it was decided to monitor tobacco crops for determining major diseases and to work on cultural control practices. Monitoring, carried out during 1997-98, indicated that Fusarium wilt (*Fusarium oxysporum* Schlectend ex Fr. f. sp. *nicotianae* J. Johnson W.C. Snyder & H.N. Hans) was the main fungal disease in Tucumán. The aim of this work was to emphasize improved cultural management practices and to start biological control tests for reducing incidence of disease.

Materials and methods

Tests were conducted during the 1999/00 season in 4 very affected farms: a) Rodolfo Antonio Plaza (loc. La Florida); b) José Lascano (loc. Los Arroyos); c) Juan Nicanor Bustos (loc.0o Yánima); d) Carlos Esteban Ruiz (loc. El Sacrificio). The treatments were: A) cultural management, B) biological control, C) biological control plus cultural management; D) control or conventional management. Cultural management consisted of constructing high

and wide hills and minimising machinery access into crops to avoid root damage. The biocontrol agent *Trichoderma harzianum* was used. It was applied 3 times: pre-transplant (root immersion), and 15 and 30 days after transplant by stem drip. Burley tobacco var Ky 17 was planted in macro-plots with 5 rows (100 m each), with 2 repetitions, located in the middle of the crop. Tobacco plants in 3 central macro-plot rows were evaluated at 15, 30,60 days from transplant and at harvest time. Tobacco plant showing incipient symptoms or death due to Fusarium wilt, were considered "diseased plants".

Results and discussion

Results of field monitoring are described in Table 1.

Table 1: Incidence of Fusarium wilt (%) registered at 30 and 60 days from treatments and at harvest time.

Treatment	*Trichoderma*			control			cult. pract			trich+cult. pract		
Date	30d	60d	harv	30d	60d	harv	30d	60d	harv	30d	60d	harv
Plaza, R.	1,07	1,6	2,8	3,87	8,57	12,27	1,07	3,5	5,6	1,27	1,43	2,47
Lascano,J.	1,61	2,47	3,17	2,83	6,63	14,03	2,27	3,7	5,43	1,6	2,47	3
Bustos, J.	5,97	11,43	20,33	8,77	16,3	24,03	8,27	14,57	14,03	7,37	12,8	20,3
Ruiz, C	0,9	1,27	1,43	1,07	1,43	7,7	0,87	1,43	1,93	0,9	1,27	1,76

At the last evaluation, percentage affected plants varied between: A) 3,6 - 6% (38,7%), B) 3,3 - 5,7% (38,7%); C) 3,7 - 10,7% (30%) and D) 14,7 - 26,7% (45,7%). Values between parentheses were from a field test where cultural practices were not done in a proper manner.

Figures 1-4 present results of Fusarium wilt incidence obtained in 3 rows monitored at each treatment and at each studied farm. In Plaza, Lascano and Ruiz fields, treatments of *Trichoderma*, cultural practices and *Trichoderma* + cultural practices were very similar. In Bustos farm, diseased plant numbers were high for all treatments; probably since cultural practices were not properly conducted. Affected plants in *Trichoderma* treated plots, generally, did not show intense wilt, but their leaves were more chlorotic and smaller than healthy plants.

It can be concluded that in the cases demonstrated here, executing proper cultural ractices resulted in improved tobacco plant stand at harvest time even without fungal biocontrol addition.

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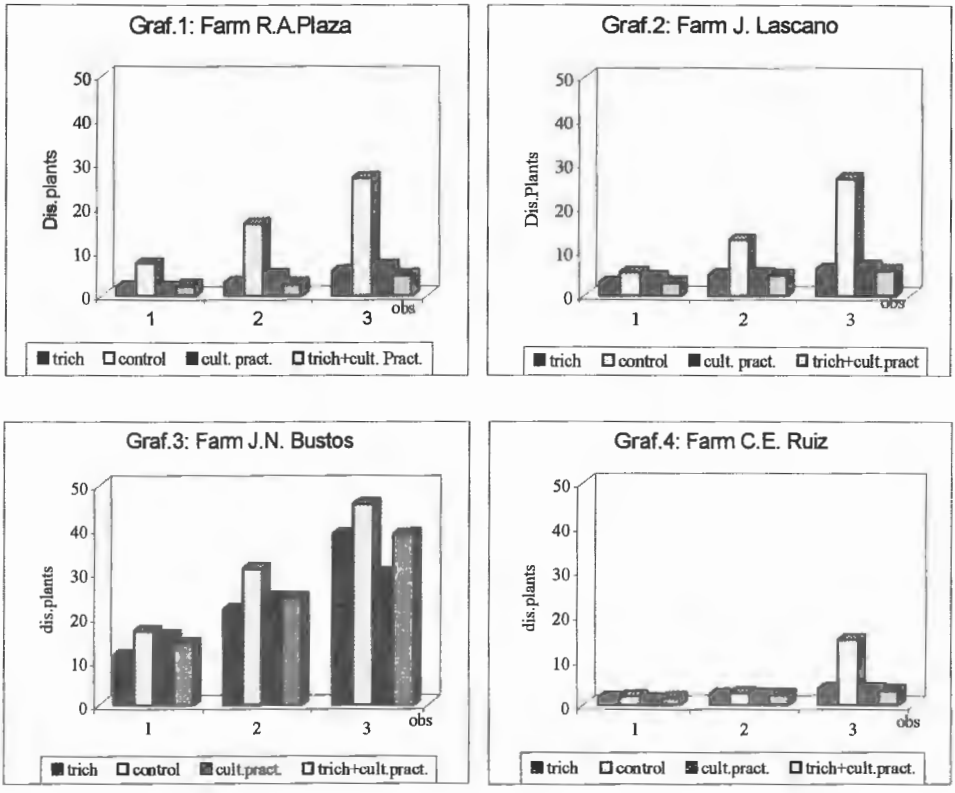

Figure 1. Incidence of Fusarium wilt in tobacco plants in four farms.

Biological Control of Fungal and Bacterial Plant Pathogens
IOBC wprs Bulletin Vol. 24 (3) 2001
pp.241 - 242

Engineering disease resistance in crop plants through the expression of fungal and bacterial genes

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Some of the current strategies for plant protection involves the expression of antifungal genes in transgenic plants. As an example, genes coding plant chitinases and β-1,3-glucanases have been transferred into several plant species. Strategies based on the use of plant genes to genetically improve plant disease resistance, however, had shown limited success, mainly due to the narrow spectrum of antifungal activity. As a complemantary approach to the use of plant antifungal genes to control pathogens, Lorito *et al.* (1998) demonstrated for the first time the feasibility of using genes from sources other than plants (namely, *Trichoderma harzianum*) to increase plant resistance against phytopathogens. There is therefore an evident interest in identifying antifungal genes that upon transfer may render target plants resistant to fungal pathogens, particularly in crop plants.

Clearly, the genome of mycoparasitic and antagonistic fungi, which has evolved specifically to attack other fungi but not plants, represents a potential source of antifungal genes. *Trichoderma* spp., is known to produce fungal cell wall degrading enzymes, such as chitinases and glucanases. These enzymes exhibit more antifungal activity than plant chitinases and ß-1,3-glucanases. Most importantly, they are effective against a much wider range of pathogens, and are not toxic to plants. The production of antifungal proteins, however, is not restricted to these fungi. Thus, antimicrobial peptides/proteins have been found in many different kinds of organisms, such as bacteria, insects, amphibians, mammals and plants.

We are interested in studying the antifungal properties of compounds that are produced as part of the defense response of different organisms against phytopathogens, as well as their application for the development of fungal resistant plants through gene transfer. Towards this goal, we previously reported the ability of cecropin A-derived peptides, cecropins being a key component of the immune response in insects, to inhibit growth of several fungal plant pathogens (Cavallarin *et al.*, 1998). On the other hand, the mould *Aspergillus giganteus*, isolated from the soil of a farm in Michigan (USA), has been reported to produce a small protein (51 amino acids) showing antifungal properties, the AFP (antifungal protein). Results will be presented on the antifungal properties of *Aspergillus* AFP against three economically important fungal pathogens, *Magnaporthe grisea, Fusarium moniliforme* and *Phytophthora infestans*, as well as on the protection afforded by direct application of AFP on rice plants. Work in progress in our group involving expression of the *afp* gene and the *cecropin A* gene, as well as the *ChiA* gene from *S. marcescens* (kindly provided by Dr. I. Chet, Israel), will be presented. This information will be useful to design strategies for engineering fungal disease resistance into important crop species.

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Biological Control of Fungal and Bacterial Plant Pathogens
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Compatibility with seed treatment chemicals of rhizobacteria antagonistic to the sugar-beet damping-off pathogen *Aphanomyces cochlioides*

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Abstract: Rhizobacteria showing antagonism against *Aphanomyces cochlioides* were tested for compatibility with the routinely used seed treatment fungicides, thiram and hymexazol. Exposure in liquid culture to a concentration of hymexazol equivalent to the standard rate used in pelleted seed resulted in loss of viability of all bacterial isolates tested, except for *Arthrobacter histidinolovorans*. In survival studies on fungicide-treated seed, the *Arthrobacter* showed potential for integration into the standard commercial seed pellet. Integration of the Pseudomonads with thiram in a pellet without hymexazol is also feasible. This study highlights the importance of screening BCAs individually for compatibility with single chemical components of a seed-treatment mixture.

Key words: *Aphanomyces*, sugar-beet, seed treatment, rhizobacteria, thiram, hymexazol, compatibility

Introduction

Sugar beet seed pelleted in the UK has been treated with a combination of the fungicides thiram and hymexazol since 1983. Thiram is used as a general protectant against seed- and soilborne diseases whilst hymexazol is used more specifically for the control of *Aphanomyces cochlioides* (Payne & Williams, 1990). Hymexazol-treated sugar-beet seed is currently sown on over 2 million hectares per annum in Europe (Asher & Dewar, 1994). This constitutes an enormous selective pressure for the development of hymexazol-resistant strains of the pathogen. No alternative form of *Aphanomyces* control is currently available. One possible alternative is the use of bacteria with antagonism to *Aphanomyces* in combination with either thiram alone or thiram plus reduced rates of hymexazol. Rhizobacteria selected in this laboratory have shown activity against *Aphanomyces* when applied to pelleted sugar-beet seed in controlled environment tests. This study was undertaken to test the compatibility of these isolates with hymexazol and thiram with a view to possible future integration of bacteria with seed treatment chemicals. Studies have concentrated on the survival of bacterial isolates marked with resistance to the antibiotic rirampicin (*Rif*$^{+}$) on stored seed treated with thiram plus hymexazol and thiram alone and the effect of reduced rates of hymexazol on these isolates in liquid culture.

Materials and methods

Bacterial wild-type and antibiotic-resistant marked strains

The bacterial isolates used throughout this study had been previously isolated from roots of sugar-beet seedlings and selected on the basis of their antagonism *in planta* to *A. cochlioides* (Williams & Asher, 1996). The Gram positive isolates were identified as strains of *Bacillus*

megaterium and *Arthrobacter histidinolovorans* whilst the Gram negatives were *Pseudomonas fluorescens* and *P. syringae*. Rifampicin-resistant strains of the isolates were generated by spread plating wildtype liquid cultures in log growth phase in Tryptone Soya Broth (TSB) onto Nutrient Agar (NA) containing 25 μg ml^{-1} rifampicin. The resulting rifampicin-resistant colonies were then streak- plated onto NA with 250 μg ml^{-1} rifampicin (NA$_R$) and the resulting *Rif*$^+$ strains were subsequently maintained on this medium.

Effect of hymexazol on bacterial isolates in liquid culture

Flasks containing 100 ml of TSB were amended with different rates of hymexazol (Tachigaren 70 WP) with the highest rate being equivalent to that applied to standard commercial seed during pelleting. Controls consisted of TSB without hymexazol. All liquid cultures were inoculated with 100 μl *Rif*$^+$ bacterial suspension from 24 h cultures in Nutrient Broth (NB) and incubated at 30°C and 100 rpm in rotary culture (Gallenkamp, UK). The numbers of cfus ml^{-1} were assessed over time by spiral plating onto NA$_R$. The influence of different rates of hymexazol on the growth curve of each isolate was determined.

Survival of bacterial isolates on stored seed pelleted with fungicides

Flasks containing TSB were cultured as described previously until each *Rif*$^+$ isolate had reached the mid-log phase of growth. Resulting bacterial cells were spun down at 8000*g* and 22°C for 30 min (4K10 Centrifuge, Sigma, Dorset, UK). The supernatant was discarded and the pellet re-suspended in 100 ml sterile distilled water (SDW). This washing procedure was repeated. The concentration of each bacterial isolate was determined by total cell counts in an Improved Neubauer haemocytometer (Weber, Lancing, Sussex, UK) at x 400 magnification on a Reichert Diastar with phase contrast. The concentration was adjusted to 9 $\log_{10}$ cells ml^{-1}. The *Rif*$^+$ isolates were applied to seed as described by Williams and Asher (1996). Standardisation was achieved by applying 500 μl aliquots of the cell suspension to 10 seeds in a single compartment of a 100 mm, 25 compartment repli-dish (Bibby Sterilin, Stone, Staffordshire, UK) to give a final application rate of 7.5 $\log_{10}$ cells per seed. The treatments were allowed to soak into the seed pellet for 6 h at 22.5°C. The seed was dried for 16 h at room temperature in a laminar air-flow cabinet and stored in glass bijous at 5°C. Survival curves on stored seed were determined for each *Rif*$^+$-marked isolate over a time course by spiral plating seed homogenised in Maximum Recovery Diluent (MRD) onto NA$_R$.

The sugar-beet seed used was var Gash pelleted with EB3 commercial seed coating with and without thiram (4.8 g ai/kg) plus the standard commercial seed containing both thiram (4.8 g ai/kg) and hymexazol (10.5 g ai/kg) [Germain's (UK) Limited, King's Lynn, Norfolk, UK].

Results and discussion

Effect of hymexazol on bacterial isolates in liquid culture

Exposure in liquid culture to the highest concentration of hymexazol resulted in loss of viability of all bacterial isolates tested except for *A. histidinolovorans* that survived at a reduced growth rate (Fig. 1). A ten-fold reduction in the fungicide rate resulted in survival of all the isolates tested although growth rates and population levels of the Gram - were reduced.

Survival of bacterial isolates on stored seed pelleted with fungicides

All four isolates remained viable on both types of fungicide-treated seed for the duration of the study. It may be that the intermediate porosity of the EB3 pellet afforded protection to the bacterial cells at the pesticide-inoculum interface compared with liquid culture. Despite this, the survival of the Gram negative bacteria was significantly reduced by hymexazol although these isolates showed more tolerance to thiram. The Gram positive bacteria were tolerant to hymexazol at the commercial rate of application to seed but were more sensitive to thiram but this sensitivity was less significant than the effect of hymexazol on the Pseudomonads.

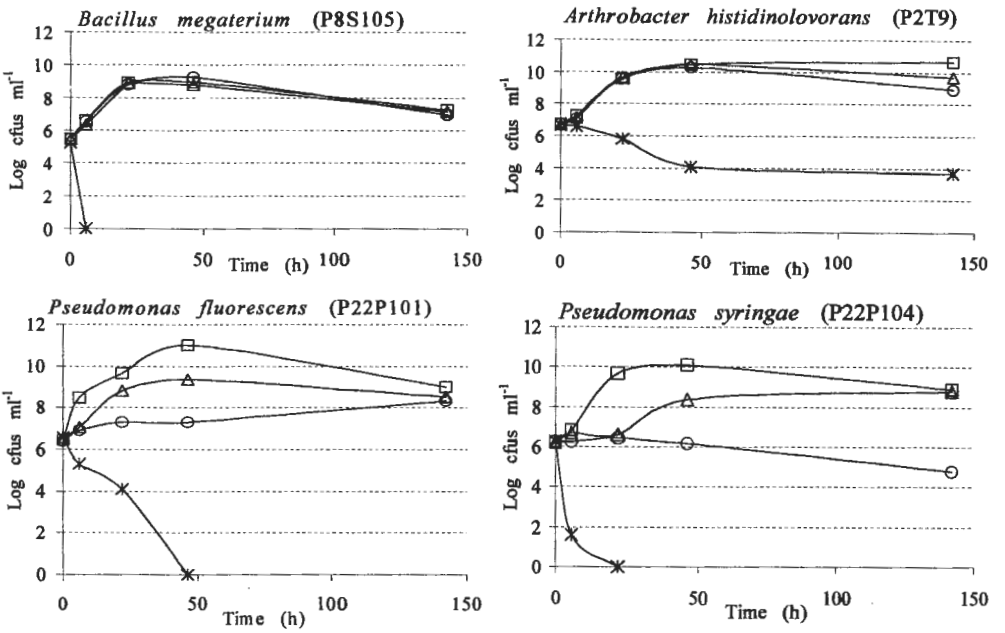

Figure 1. Effect of hymexazol on bacterial isolates in liquid culture. Concentration of hymexazol in TSB (mg l^{-1}): 0 (-□-), 5 (-Δ-), 50 (-○-), 500 (Standard Rate) (-*-).

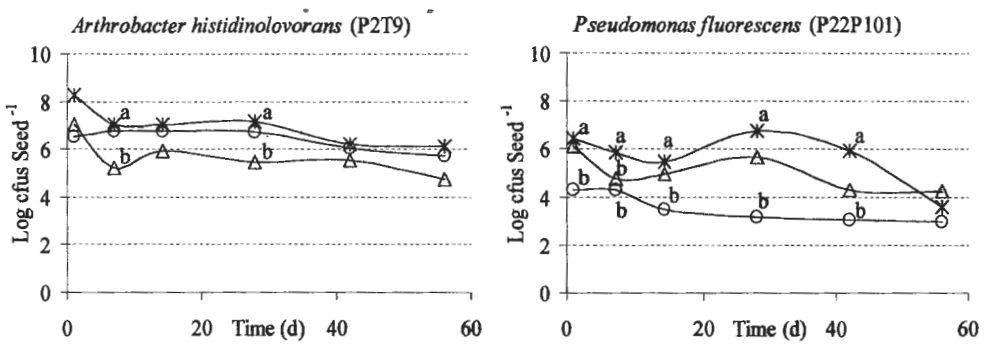

Figure 2. Effect of fungicides on survival of bacterial isolates on pelleted seed. Control (pelleted without fungicides) (-*-), thiram-treated (-Δ-), hymexazol and thiram-treated (-○-). Significant differences between treatments at each sample time [Fisher's protected LSD ($P \leq 0.05$)] represented by 'a-b'.

In fact, the *Arthrobacter* survived at levels efficacious for biocontrol (*c.* 5 $\log_{10}$ cfus g^{-1} seed) on thiram-treated seed. This isolate has potential for integration into the standard commercial seed pellet. Integration of the Pseudomonads with thiram in a pellet without

hymexazol is also feasible. This study highlights the importance of screening BCAs separately for compatibility with single chemical components of a seed treatment mixture.

Previous investigations have shown that integration of either fungal or bacterial BCAs with fungicides in seed treatments for the control of soilborne damping-off pathogens is a viable proposition. Hwang & Chakravarty (1991) and Adams & Wong (1993) proposed the integration of thiram with *Gliocladium virens* (for the control of *Rhizoctonia* on pea) *and Sporodesmium sclerotivorum* (for the control of *Sclerotinia minor*), respectively. Other workers have shown that a combination of metalaxyl with *Rhizoctonia* spp. (Harris & Nelson, 1999), *Bacillus* spp. (Hwang *et al.*, 1996), *P. aureofaciens* AB254 (Mathre *et al.*, 1995) and *Chaetomium globosum* (Dipietro *et al.*, 1991) successfully controlled seedling damping-off in tomato, pea, sweet corn and sugar-beet, respectively. Some workers have stated that combinations of BCAs with fungicides can improve the disease control of either strategy used alone. For example, combined application of pencycuron with *P. fluorescens* 2-79 was more effective than either treatment alone for controlling root rot of spring wheat (Duffy, 2000).

Acknowledgements

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Biological Control of Fungal and Bacterial Plant Pathogens
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Control of *Phytophthora* spp. with grapefruit extract

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Abstract: Addition of grapefruit extract to peat leachate at 1,6 µg/cm³ strongly inhibited zoosporangia production of *Phytophthora cryptogea*. At 200 µg of the product/cm³ sporulation was not observed. Amendment of peat with grapefruit extract at dose 165 µg/cm³ resulted in a drastic decrease of colony forming units of the pathogen and suppression of its development within at least one month. Treatment of peat with grapefruit extract at a dose of 165 µg/cm³ immediately after gerbera and cypress planting, suppressed *Phytophthora* rot development for at least 7-14 weeks.

Key words: *Phytophthora*, zoosporangia, formation, population, control, gerbera, cypress

Introduction

Phytophthora rot, incited by *Phytophthora* species are some of the most widespread and destructive pathogens of many major ornamental plants, growing under covering and in open fields. *P. cryptogea* is the main threat causing *Phytophthora* foot rot of gerbera and many pot plants. The fungus is also the casual agent of root and stem rot of some coniferous plants (Orlikowski *et al.*, 1995). *P. cinnamomi* Rands is know as a pathogen of at least 1000 host plants among them Lawson cypress, rhododendron, heather's (Orlikowski *et al.*, 1995). Some plant products have been reported to posses pathogen control properties (Orlikowski & Saniewska 1995). Grapefruit extract exhibited significant antifungal activity against *Penicillium* spp., *Colletotrichum* spp., and *Botrytis cinerea* (Angioni *et al.*, 1998; Caccini *et al.*, 1998, Caceres *et al.*, 1992; Esterio *et al.*, 1992). In those reports, however, a mode of grapefruit extract activity against plant pathogens was not studied. In this work, the commercially formulated product, on the base of grapefruit extract, was evaluated *in vitro* and *in vivo* in reducing growth and development of *P. cryptogea* and its activity toward *Phytophthora* spp. in substratum.

Materials and methods

Grapefruit extract
Biosept 33 SL (containing 33% of grapefruit extract) was supplied by Cintamani Poland. In *in vitro* trials the extract was applied at a concentration from 0 (control) to 200 µg/cm³ whereas in greenhouse experiments at doses of 165 and 660 µg/cm³.
Fungi
Phytophthora cryptogea Peth. et Laff. and *P. cinnamomi* Rands were used. Stock cultures were maintained on PDA at 25°C. For peat infestation, both species were grown on Quick oats for 2 weeks at 24°C. Population density of fungi was estimated using gallic acid selective medium (Flowers & Hendrix, 1969) and a procedure described by Orlikowski (1999).

Plants

Gebera (*G. jamesonii*) and Lawson cypress (*Chamaecyparis lawsoniana*) were used. Immediately after planting, plants were drenched with grapefruit extract and standard fungicides (furalaxyl and phosethyl-Al) using 50 cm^3 of solution/m^3 of peat.

The experimental design was completely randomised with 4 replications and 5 Petri dishes or 10 plants in each rep. Trials were repeated at least twice.

Results and discussion

Addition of grapefruit extract to peat leachate at a concentration of 1,6 µg /cm^3 resulted in inhibition of 65% of zoosporangia formation of *P. cryptogea*. At a concentration of 40 µg/cm^3 zoosporangia were found only sporadically. Increase of GE concentration to 200 µg/cm^3 caused complete inhibition of the pathogen sporulation (Table 1). On mycelial disks of *P. cryptogea* transferred from solution containing 1,6 µg of GE/cm^3 into peat leachate, inhibition of zoosporangia formation was not observed. A 5-fold increase in initial concentration of GE resulted in the inhibition of zoosporangia production of approximately 85% (Table 1).

Table 1. Influence of grapefruit extract on zoosporangia formation of *Phytophthora cryptogea*; reduction of zoosporangia number (%) after 6 days of incubation.

µg of a.i./cm^3	Soil leachate with grapefruit extract	Transferring of mycelial mats from grapefruit extract into soil leachate
1,6	65 a	0 a
8	81 b	85 b
40	97 c	97 bc
200	100 c	100 bc

Note: Means in columns, followed by the same letter, do not differ with 5% of significance (Duncan's multiple range test).

Complete inhibition of mycelial growth of the pathogen (Orlikowski, unpubl.) and strong suppression of its zoosporangia production in this study indicate direct action of grapefruit extract against *P. cryptogea*. Microscopic observation of the pathogen, incubated in soil leachate, containing 8 µg of GE/cm^3 showed abundant production of swellings and deformation of zoosporangia. It is possible that such activity of grapefruit extract toward *P. cryptogea* occurs also in peat. Amendment of substratum, artificially infested by the pathogen, with grapefruit extract at a concentration of 165 µg/cm^3 caused a drastic decrease in colony forming units within 5 days after application. During the next 25 days population density of *P. cryptogea* in treated peat was very low (Fig.1). Application of grapefruit extract as a peat drench, immediately after gerbera planting, resulted in strong suppression of *Phytophthora* foot rot development within 7 weeks of growth (Table 2). There were no significant differences between dose of grapefruit extract and number of diseased gerberas (Table 2).

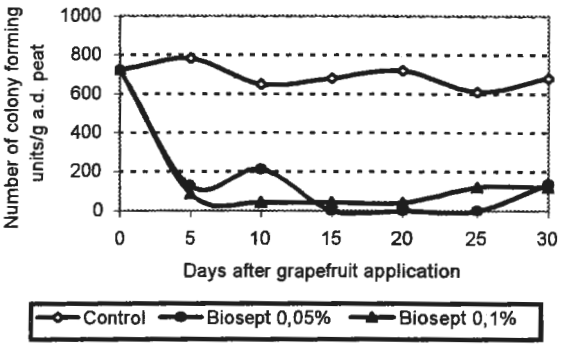

Figure 1. Biological activity of grapefruit extract (a.i. of Biosept) in the reduction of *Phytophthora cryptogea* population density in peat.

Table 2. Biological activity of grapefruit extract in the control of *Phytophthora* foot rot of gerbera; % of diseased plants. Initial population of *P. cryptogea*: 330 cfu/g of air dry substratum.

Treatment	µg of a.i./cm³	Weeks after planting		
		3	5	7
Control	-	5 a	65 b	80 d
Grapefruit extract	165	5 a	10 a	20 b
- " -	300	10 a	15 a	30 bc
- " -	660	0 a	20 a	35 bc
Furalaxyl	80	0 a	0 a	5 a

Note: See Table 1

Application of grapefruit extract in the control of *Phytophthora* root and stem rot of cypress had a similar effect on pathogen control as that of phosethyl-Al (Table 3). After 14 weeks growth of cypress, in peat amended with grapefruit extract at a dose of 165 µg/cm³, significantly less plants showed disease symptoms than in other treatments (Table 3).

The suppression of *Phytophthora* rot in greenhouse trials corresponds with the ability of grapefruit extract to reduce sporulation of *P. cryptogea* and fungal populations in peat. This product has been used for foliar application (Caceres *et al.*, 1998; Esterio *et al.*, 1992) and no data exists for its activity toward *Phytophthora* spp. in soil or substratum.

Additional studies will be directed towards understanding the interaction between grapefruit extract and *Phytophthora* spp. It has been shown that the product is capable of pathogen growth and sporulation inhibition. Effect of the product on *P. cryptogea* population over time and development of disease symptoms are issues that need to be considered when determining whether repeated peat treatment is necessary throughout the cultivation period. The data obtained here suggests that grapefruit extract protected most of plants for at least 7-14 weeks.

Further research in this area is necessary to determine the potential of the tested product in crop production.

Table 3. Biological activity of grapefruit extract in the control of *Phytophthora* root and stem rot of Lawson cypress; % of diseased plants. Initial population of *P. cinnamomi*: 250 cfu/g of air dry substratum.

Treatment	µg of a.i./cm³	Weeks after planting		
		7	10	14
Control	-	10 a	35 b	60 c
Grapefruit extract	165	0 a	5 a	5 a
- " -	330	5 a	15 a	30 b
Phosethyl-Al	1600	5 a	15 a	25 b

Note: See Table 1

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Biological Control of Fungal and Bacterial Plant Pathogens
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Microscopic studies on the mode of action of fluorescent pseudomonads alone and in combination with acibenzolar-S-methyl effective against *Fusarium oxysporum* f.sp. *lycopersici* in tomato plants

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Abstract: The antagonistic effect of fluorescent pseudomonads and acibenzolar-S-methyl (BTH) against *Fusarium oxysporum* f.sp. *lycopersici* (Fol), the causal agent of tomato wilt, was studied by scanning and transmission electron microscopy. The fluorescent pseudomonad isolates G309 and CW2 colonise the outer surface and the xylem of tomato roots of plants treated alone or in combination with BTH (100µM) 4 d after application. In control plants, Fusarium hyphae penetrated the root cells immediately after spores germination. In plants pretreated with the pseudomonad isolates alone or with BTH, no penetration into root tissues was noticed after germination of Fol microspores. This inhibition could be explained by formation of thick layers of lipopolysaccharides and other substances, and/or by production of antifungal substances as pyoluteorin and phenazine derivatives causing a collapse of Fusarium hyphae according to the scanning electron microscopic studies. In the plants treated only with BTH (20µM), Fol spores produced long germ tubes after germination but no penetration sites were recognised. In this case, it may be elucidated that Fol could not recognise its host and lost its orientation. The bacterial cells of fluorescent pseudomonad isolates G309 and CW2 colonised large areas of the xylem of tomato roots and formed a thick layer of lipopolysaccharides and other substances almost similar to the layer formed on the surface of tomato roots and thereby inhibited the expansion and growth of Fusarium hyphae to the upper parts of tomato plants.

Introduction

Fluorescent pseudomonads are well known as bioagents for controlling many soilborne and few foliar plant pathogens (Baker & Cook, 1982; Howell & Stipanovic, 1979). The control mechanism may be divided into direct and indirect effects. The direct effect includes production of certain substances such as antibiotics, cyanide, siderophores and extra-cellular enzymes that inhibit the growth or cause mortality of the pathogens. The indirect effect relies on induction of resistance in host cells against plant pathogens (Buchenauer, 1998, Chen *et al.*, 2000; Voisard *et al.*, 1989). According to our previous studies, application of selected isolates of *Pseudomonas fluorescens* (G309) or *Pseudomonas* spp. (CW2) alone or in combination with acibenzolar-S-methyl (BTH) at 100µM was antagonistic to *Fusarium oxysporum* f. sp. *lycopersici* in tomato plants. The aim of this work was to study the mode of action of selected antagonistic fluorescent pseudomonades alone and in combination with BTH against *F. o.* f.sp. *lycopersici* using scanning and transmission electron microscopy.

Material and methods

Plants and Pathogens

Tomato plants (*Lycopersicum esculentum* L) cultivar TipTop were used in all experiments. The wilt pathogen of tomato *Fusarium oxysporum* Schlecht. f.sp. *lycopersici* (DSM 62059) was obtained from the German Collection of Microorganisms.

Greenhouse experiments

Three-week-old tomato seedlings were treated by soil drench with fluorescent pseudomonads ($2{,}0x10^8$cfu/ml; 50ml/pot), or BTH (100µM; 50ml/pot) and in combination. The plants were inoculated with Fol ($5.2x10^7$ spores/ml) by two methods; firstly 6 days after application of fluorescent pseudomonad isolates and BTH and secondly by co-inoculation at the same time.

Preparation of samples for scanning and transmission electron microscopy

Samples of control and treated tomato roots were cleaned and fixed in 3% glutaraldehyde in phosphate buffer (v/v, pH 6.8). Four hours after incubation, the samples were post-fixed using 1% osmium tetroxide (pH 6.8) for 2h at 4°C. After that, the specimens were further prepared for transmission electron microscopy (TEM) and scanning electron microscopy (SEM).

Results

Fluorescent pseudomonad isolates G309 and CW2 were sufficiently able to colonise and to form a thick layer of lipopolysaccharide and other substances on the outer surface (Fig. 4 and 5) and in the xylem cavity of tomato roots (Fig. 11), 4 days after application. The thick layers of lipopolysaccherides formed by bacterial cells (G309 and CW2) prevented penetration of Fol hyphae into the root tissues of tomato plants after spore germination (Figs. 4 and 5) compared to control plants infected only with Fol spores (Figs. 2 and 3). The pseudomonad isolates G309 and CW2 were also able to inhibit the infection of tomato plants by Fol through other mechanisms of action like production of antifungal substances and extracellular enzymes. The presence of collapsed Fusarial hyphae (Fig. 8) on the surface of tomato roots is an indication of antibiotic activity in the plants pretreated with isolate CW2 that produces the antibiotics PCA and 2OH-Phz *in vitro,* compared to positive control plants (Fig. 7). In plants treated with G309 that produces extracellular enzymes, lysis Fol spores and germ tubes were used (Fig. 5). Also, the bacterial cells of fluorescent pseudomonad isolates G309 and CW2 colonised large areas of the xylem of tomato roots and inhibited the expansion and growth of *Fusarium* hyphae to the upper parts of tomato plants (Figs. 11, 12 and 13) compared to control plants (Figs. 9 and 10). The same results were obtained in TEM studies (Figs. 14, 15 and 16). In tomato plants treated only with BTH 100µM, the Fol spores produced long germ tubes after germination, but no penetration into root tissues was observed which could be explained by a loss of orientation of the hyphae (Fig. 6).

Figure 1. Outer surface of tomato root of the control.

Figures 2 and 3. Germinated microconidia of Fol and direct penetration of Fol hyphae into tomato roots control plants, 4 days after inoculation.

Figure 4. G309 and Fol, the bacterial isolate G309 efficiently colonised the root surface and prevented penetration of germinated Fol conidia inside the root tissue.

Figure 5. Combined treatment of bacteria with BTH, no penetration of the pathogen was observed.

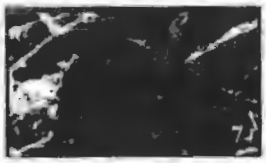


Figure 7. Two weeks after inoculation with Fol, the outer surface of control roots was massively colonised with hyphae.
Figure 8. In contrast, collapsed Fusarial hyphae were observed in plants pretreated either with the isolate CW2 alone or in combination with BTH, 6 days before inoculation.

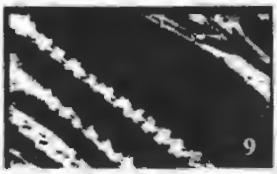


Figure 9. Longitudinal sections in the xylem of tomato roots of a healthy control plant.
Figure 10. Fol hyphae colonise the xylem that led to wilt symptoms.
Figure 11. A large area of the xylem was colonised with the bacterial cells of G309.

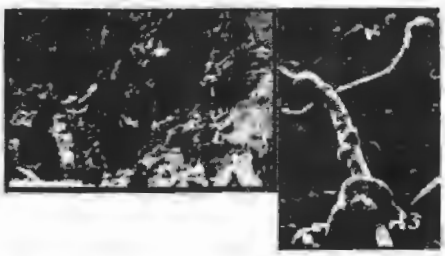
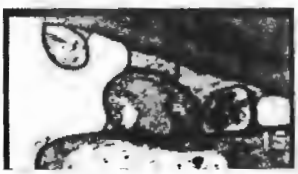


Figure 12. Fol hyphae growing upward through xylem pits colonised by G309.
Figure 13. The bacterial cells established themselves in the xylem, formed a thick lipopolysaccharide layer and inhibited the growth of Fol hyphae to the upper parts of tomato plants.



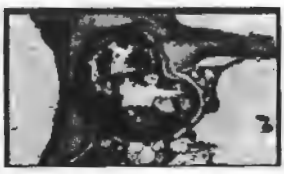


Figure 14. TEM studies 3 weeks after Fol inoculation, colonization of xylem and wilting due to Fol.
Figure 15. Pretreatment with G309 caused the Fol hyphae to stick close to xylem cells due to extracellular lipopolysaccharides produced by the bacteria.
Figure 16. Later, Fol hyphae destroyed in pretreated plants with G309.

Discussion

The antagonistic fluorescent pseudomonad isolates G309 and CW2 were able to colonise effectively the outer surface of tomato roots and the root xylem by formation of thick layers

of lipopolysaccharides or other substances. Our results agreed with the results of Troxler *et al.* (1997) using immunofluorescent microscopy showing that *P. fluorescens* isolate CHA0 was found between and inside cells in the epidermis and cortex, as well as in the xylem vessels, 4-7 days after planting tobacco seedlings. However, CHA0, was seldomly found in contact with the mycelium of *Thielaviopsis basicola*, the causal agent of black root rot in tobacco. Our SEM and TEM studies, however, showed a direct inhibition of Fol hyphae in the xylem of tomato roots by the fluorescent pseudomonad isolates G309 and CW2. Mechanism of actions of fluorescent pseudomonads against plant pathogens have been already described in several articles (Howell and Stipanovis, 1980; Voisard *et al.*, 1989). The two isolates G309 and CW2 inhibited Fol in tomato plants by production of antifungal substances and extracelluar enzymes. In our studies, a new mode of action of BTH against Fol was illustrated. In tomato plants treated with BTH (100 µM) alone, the Fol spores produced long germ tubes after germination, but no penetration into root tissues was noticed which could be explained by a loss of orientation of the hyphae. Finally, in order to protect the plant against a pathogen, a biocontrol agent should not only produce a protective factor, but it should also be able to colonise at the right site and at the right time at optimal level (Lugtenberg, *et al.* 1994).

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Biological Control of Fungal and Bacterial Plant Pathogens
IOBC wprs Bulletin Vol. 24 (3) 2001
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Soil colonization by *Bacillus subtilis* M51 pre-conditioned in organic matrix and its survival in soil

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Abstract: The effectiveness of a physiological pre-adaptation of the biocontrol agent *Bacillus subtilis* M51 to an organic matrix has been assessed. The strain showed a greater affinity to mature compost from exhaust olive husk in respect to potting or agricultural soil. The release of the strain in 3 type of agricultural soils amended with olive husk compost bacterized with M51/rs, showed the ability of the matrix to enhance the growth and diffusion of the bacteria. Moreover, *in planta* test, performed with soil naturally infected by *Fusarium oxysporum* f. sp. *dianthii* and *F. oxysporum* f. sp. *cylaminis*, showed a significant ability of pre-conditioned *B. subtilis* M51/rs to protect test plants by 75% in respect to control.

Key words: *Bacillus subtilis* M51, biocontrol ability, pre-adaptation, olive husk compost

Introduction

Successful applications of plant beneficial microorganisms (PBO) introduced into soil require that sufficiently high number of cells survive and remain active for long periods of time. The colonization and subsequent activity of these inoculants are restricted by their physiological status and by the physicochemical characteristics of the recipient soil. In fact, progressive decline in bacterial cell numbers after introduction into soil has often been observed. In vitro cultivation of these microorganisms may cause some change in their physiological traits that can lead to a loss in the viability in soil after introduction. Therefore, a physiological pre-conditioning of bacteria in a carrier matrix should be considered as a good strategy to improve colonization ability of a bacterial strain prior to release into the soil (Heijnene *et al.*, 1992; Filippi *et al.*, 1999; Van Dyke & Prosser, 2000). Moreover, the benefit of compost application as a soil amendment has been well documented (Filippi *et al.*, 1990; Lumsed *et al.*, 1993; Hoitink & Boehm, 1999). In this study an olive husk compost, produced with initials of microbial inoculum as a booster, has been used as a carrier for the biological agent *B. subtilis* M51, in order to improve its dissemination and survival in soil.

Material and methods

Bacterial strain

The fate of inoculant within composting and in soil, was assessed by a spontaneous mutant, antibiotic-resistant strain of *B. subtilis* M51. The mutant strain of *B. subtilis* M51/rs, resistant to streptomycin and rifampicin ($100\mu gL^{-1}$ and $75\mu gL^{-1}$, respectively), did not differ from the wild-type in growth rate and *in vitro* range of antagonistic activity.

Assessment of the survival and metabolic affinity of M51/rs in organic substrates

A compost produced from olive husk was tested for the physiological conditioning of *B. subtilis* M51/rs. The compost was tested at different maturity states of 90, 150 days of composting with TEC (Total Extract Carbon) values of 5,6 and 8,9% respectively. In order to evaluate the possible competition between M51/rs and microbiota the composts were steam sterilised. As a control, a sandy loam and greenhouse potting soils were used. The soils and composts (20 kg) were inoculated with 10^6 g^{-1} dw cells of strain M51/rs in its vegetative state and maintained for 120 days at 22°C and 60% humidity. For good colonisation, inoculated substrates were weekly mixed. Total population of viable cells were detected by decimal dilution on Waksman Agar (WA) containing streptomycin and rifampicin (100μgL^{-1} and 75μgL^{-1}, respectively). After incubation at 27°C for 5 days, the average number of colonies developing on the plate (n=3) was considered the viable cell number. Spore number in the total population of M51/rs was measured after the sample was treated at 80°C for 15 sec. Vegetative cell density was calculated as the difference between viable cells and spores.

Soil survival and colonising ability of pre-conditioned strain M51/rs

The olive husk compost resulting in higher metabolic affinity with *B. subtilis* M51/rs (150 days old) was mixed with three types of soil, a sandy loam (A), a clay soil (B) and a potting soil (C). The compost was mixed with soils in order to obtain a final population density of M51/rs of about 10^6 cell g^{-1} dw. As a control, soil was mixed with the same quantity of non-bacterized compost inoculated with a liquid suspension of M51/rs (10^6 cell g^{-1} dw).

Greenhouse test

Two soils (S1 and S2) naturally infected with *Fusarium oxysporum* f.sp. *dianthi* (3000 propagule g^{-1}), from Sanremo (Imperia, Italy) with different texture and structure and potting soil (P), artificially infected by *F. o.* f. sp. *cyclaminis* (3000 propagule g^{-1}), were tested *in planta* as follows: C = soil (control); M = soil + liquid bacterial suspension of M51/rs; MC = soil mixed with olive husk compost not bacterized + liquid bacterial suspension of M51/rs; BC = soil mixed with olive husk inoculated compost. In all mixes the final microbial density was about 10^6 cell g^{-1} dw. The mix with S1 and S2 soils were used to fill greenhouse benches (2 x 1 x 0,3) and each parcel planted with 200 rooted cutting carnation variety "Olga". Each mix of soil P was distributed in ten polycarbonate pot (1,5 L) with a cyclamin plant in full vegetative vigour. The greenhouse was conditioned at 16 h illumination at about 25°C, 8 dark h at about 15°C. Disease incidence was assessed by weekly visual observation of plant mortality . The basal parts of the steam of diseased plants were surface sterilized in 1% (w/w) sodium hypoclorite for 30 sec, rinsed twice in sterile water and placed on potato dextrose agar (PDA) (Difco) at 27°C for 5 days, in order to assess *F. oxysporum*.

Results and discussion

The results clearly indicate that *B. subtilis* M/51/rs cells pre-conditioned in an organic matrix survive and are more active than those added directly to the soil. Since the physiological state of bacterial cells at the moment of exposure to soil may affect their ability to establish themselves and to survive, a physiological adaptation on mature olive husk compost resulted to optimisation of inoculum survival.

Assessment of the survival and growth of M51/rs in organic substrates

M51/rs showed different behaviour in the compost in respect to soil (Fig. 1). In sandy loam soil, vegetative cells declined in the first 30 days from 10^6 to 10^3 cell g^{-1} dw., and M51/rs survived as spores until 120 days (Fig. 1A). In potting soil the population behaviour was the same as that observed in the sandy loam soil, however the final population density was higher, *i.e.* at 10^5 cell g^{-1} dw. (Fig. 1B). In compost however, 90-day-old M51/rs population survived similarly to that

observed in the potting soil (data not shown). In sterile and non-sterile mature compost (120 days of composting), viable cell number increased from 10^6 to about 10^8 cell g^{-1} dw. after the first 30 days from inoculation, reaching 10^9 cell g^{-1} in both composts at 120 days (Fig. 1C). These results show that only the 120-day-old compost was able to sustain the growth and the diffusion of M51 strain. Sterilisation of the composts did not improve colonisation by M51; the competition between inoculated bacteria and indigenous microbiota does not seem to be a limiting factor. Therefore the importance of maturity of the compost seems to play a key role in term of metabolic affinity of the substrate as a carrier.

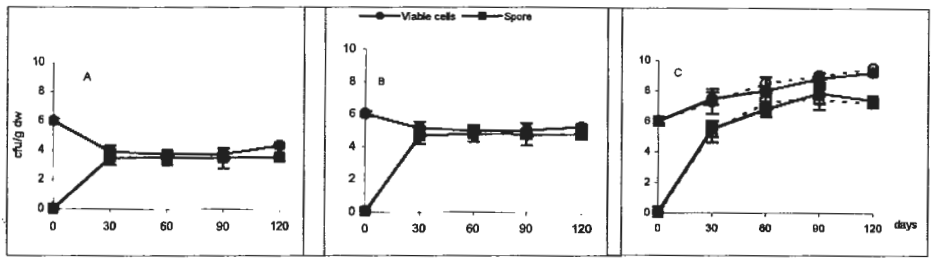

Figure 1. Survival of *B. subtilis* M51/rs in different substrates: A) sandy soil; B) potting soil; C) 120-day-old compost. Broken lines indicate sterilised compost, unbroken lines indicate non-sterilised compost.

Soil survival and colonising ability of pre-conditioned strain M51/rs

Population density of unpreconditioned M51/rs, introduced in the soil amendment with non-inoculated compost and inoculated with free bacterial suspension in soil A, decreased from 10^6 to 10^4 cell g^{-1} dw. during the first 30 days, and then remained almost constant. In soil B, viable cells remained at their initial value at all tested times whereas in soil C a slight increase was observed in the first 60 days and then decreased rapidly (Fig. 2). On the contrary, physiologically preconditioned *B. subtilis*, possessed a similar population level in soils. In this case, M51/rs was able to grow rapidly and attain a high population density at the 30th day and then decrease to values at the end of the trial (90 days) (Fig. 3).

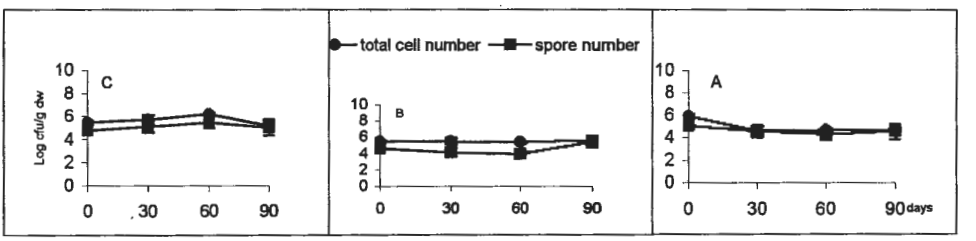

Figure 2. Evaluation of growth and survival ability of *B. subtilis* m51/rs inoculated in different soil by liquid suspension: A) sandy loam; B) clay soil and C) potting soil.

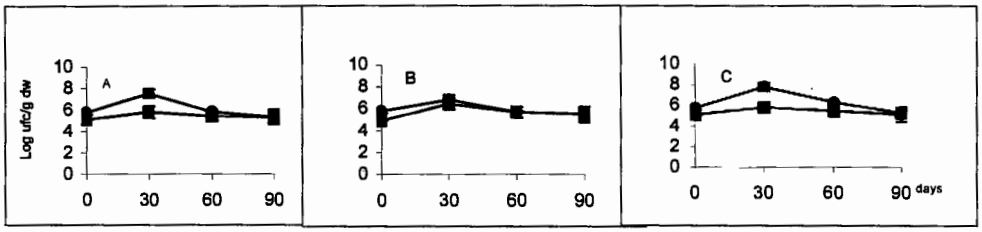

Figure 3. Evaluation of growth and survival ability of *B. subtilis* m51/rs inoculated in different soil by pre-conditioned compost: A) sandy loam; B) clay soil and C) potting soil.

Greenhouse test

The effectiveness of the release of M51/rs by a bacterized compost is clearly demonstrated in the greenhouse tests. In fact, survival of the plants in soils amendment with pre-conditioned M51/rs (BC) was complete in the first 30 days, and remained about 75% until the end of the test (90 days). In soils not amended with inoculated compost (C, M and MC), survival was about 85% of average in the first 30 days, rapidly decreasing to 0% by the 90th day.

On the bases of the results obtained, the practice of pre-conditioning of BCAs appear to be a promising strategy to overcome the problem of biotical and abiotical stress of the inoculant when released in recipient soil. The efficacy of inoculated compost is probably due to the formation of physical and nutritional microsites that act in sustaining colonisation in soil.

Acknowledgements

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Biological Control of Fungal and Bacterial Plant Pathogens
IOBC wprs Bulletin Vol. 24 (3) 2001
pp.259 - 263

Effect of seed priming in the presence of *Trichoderma koningii* on seed and seedling disease induced in tomato by *Rhizoctonia solani* and *Fusarium oxysporum* f. sp. *lycopersici*

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Abstract: Seed priming in the presence of *T. koningii* effectively protected seeds and seedlings in the nursery from attack by these pathogens with a control of 96% and 56%, respectively. Taking into account that these pathogens have also potential to affect plants after transplant, the seeds primed in the presence of *T. koningii* were grown in a seed bed sprayed once with a suspension of *T. koningii*. After transplant, soil was sprayed once or twice during the experiment with a suspension of this antagonist. The pronounced biocontrol effect against both plant pathogens was observed in seeds primed in the presence of *T. koningii* and in soil which was sprayed twice with *T. koningii*, resulting in 62 and 70% protection against *R. solani* and *F. oxysporum*, respectively. These results were significantly different to those presented in plants which emerged from unprimed and primed seeds without *T. koningii*, in which no protection and 30% protection, respectively, were observed in *R. solanii* infested soil. No protection was observed in plants that emerged from unprimed and primed seeds in *F. oxysporum*-infested soil. Plant height increased when the biocontrol agent was incorporated into the soil, both in the presence and absence of plant pathogens.

Introduction

Promising results have been obtained by combining biological agents in conjunction with physiological methods such as seed priming. This method consists of a presowing hydration treatment in which the germination is initiated, but stopped before radicle emergence (Bradford, 1984). Solid matrix priming has been developed as an alternative to priming seeds in osmotic solutions resulting in expressed in increased plant stand, disease resistance and seedling vigour in several crops (Harman *et al.*, 1989). This method was ideal for integration with biological control agents (Harman and Taylor, 1988). In a previous research we demonstrated that seed priming in the presence of a selected strain of *T. koningii* effectively protected bean and cucumber against damping-off agents (Mezui *et al.*, 1994). Taking into account that tomato plants are severely affected in nurseries and in the field by pathogens such as *R. solani* and *F. oxysporum* f. sp. *lycopersici*, the purpose of the present work was to evaluate the biocontrol effect of seed priming combined with *T. koningii* treatment in tomato against these pathogens.

Materials and methods

Priming of tomato seeds (*Lycopersicon esculentum*) was performed in a solid matrix consisting of wheat husks moistened to 80%, under a layer of etamine. Seeds were then incubated for 48 h at 20°C. Seeds were immersed for 10 min in 10^7 *T. koningii* conidia/ml before introduction in the priming matrix. Unprimed and primed seeds were sown in seedbeds

containing non-infested soil and soil artificially infested with the pathogens. Soil contained 10^2 *R. solani* propagules/g or 10^3 *F. o.* f.sp. *lycopersici* microconidia/g. Stands were counted daily and counts were expressed as % of emergence. There were 40 replicates for each treatment and three repetitions.

Seedlings obtained from seed beds of unprimed seeds, primed seeds and seeds primed in the presence of *T. koningii* (TH11) in absence of the pathogens, were planted in soil artificially infested with the concentrations of plant pathogens previously selected for producing above 80% incidence and high severity. Soil planted with seedlings obtained from seeds primed in the presence of *T. koningii* were treated with a suspension of this antagonist containing 10^7 conidia/ml 30 and 60 days after transplant. Studies were conducted in a greenhouse at 22°C. Weekly counts were carried out 1-12 weeks after planting. Counts were expressed as percentage of protection which was expressed as (T/Th) x (Pd- Pda) /Pd (T= total number of plants in absolute control treatment, Th= total number of healthy plants in absolute control treatment, Pd= number of diseased plants on control (unprimed or primed) planted in the presence of the pathogen, Pda= number of diseased plants on treatment with antagonist planted in the presence of the pathogen). In order to determine the effect of the treatments on the vigour of plants, plant height from soil to apical buds was measured.

Results

Priming resulted in a faster emergence and higher final stands as compared to unprimed seeds. Combining seed priming with *T. koningii* dressing resulted in further increased of emergence and higher protection against the evaluated pathogens. In non-infested soil, emergence was 90% for unprimed seeds, 100% for primed seeds, and 100% for seeds primed in the presence of *T. koningii*. In *R. solani* infested soil the respective emergence was 53%, 83.2% and 90.4%. The last two treatments, as compared with unprimed seeds, represented 64.3% and 78.6% protection, respectively. In *F. oxysporum* infested soil, protection as compared with unprimed seeds, was 83.2% for primed seeds, and 56% for seeds primed in the presence of *T. koningii* (Fig. 1).

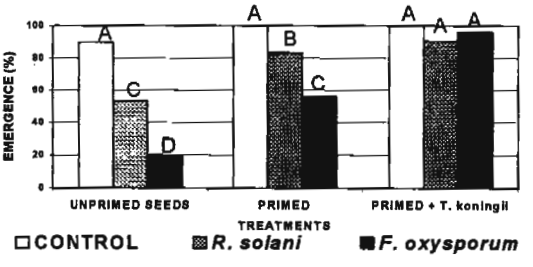
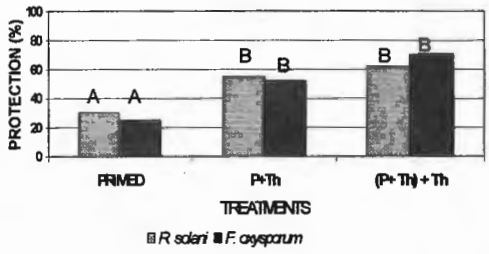


Figure 1. Emergence of healthy tomato seedlings from primed or unprimed seeds dressed or not with *T. koningii*. Columns followed by a common letter are not significantly different at $P \leq 0.05$ according to Tukey multiple range test.

Under greenhouse conditions, after transplanting, plants obtained from unprimed seeds in the presence of the pathogens increased in disease incidence and severity compared with plants obtained from both primed seeds and seeds primed in the presence of *T. koningii*. Acute symptoms of disease were observed only four and six weeks after the transplant for

both pathogens. Priming resulted in some protection as compared to plants obtained from unprimed seeds, nevertheless priming in the presence of *T. koningii* appeared more effective than priming alone. In *R. solani* and *F. oxysporum* infested soils, all plants obtained from unprimed seeds were affected by the pathogens after 12 weeks. When compared with results obtained in plants from unprimed seeds, protection of 55% and 52% was obtained for plants from seeds primed in the presence of *T. koningii*. When *T. koningii* was applied twice after the transplant, increased protection was observed, in that case, 62% and 70% against both pathogens respectively, whereas only 30% and 25% protection, respectively, was observed in plants obtained from primed seeds without pathogens (Fig. 2).



P = Primed Th = *T. koningii*

Figure 2. Biocontrol effect of seed priming in the presence of *T. koningii* on control of *R. solani* and *F. oxysporum*. Columns followed by a common letter are not significantly different at $P \leq 0.05$.

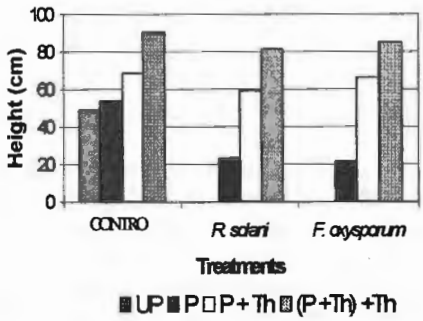


Figure 3. Increased growth response of tomato plants induced by seed priming (P) combined with *T. koningii* (Th), as compared with obtained in plants from unprimed seeds (UP) and primed seeds (P). (UP = Unprimed P = Primed Th = *T. Koningii*)

When compared with the growth observed in plants obtained from seeds primed and non treated with biocontrol agents, plant growth increased by 91% when plants were seeded in

soil inoculated with *R. solani*, by 95% when seeded into soil inoculated with *F. oxysporum* and by 99% when planted in the absence of plant pathogens. From these results it is concluded that seed priming combined with the utilization of biocontrol agents constitutes a promising alternative for control of *Rhizoctonia solani* and *Fusarium oxysporum* in tomato (Fig. 3).

Seed priming alone (undressed) induced some protection against pathogens, expressed in reduced symptoms and a lower number of infected plants as compared with unprimed seeds (Harman & Taylor, 1988), which could be explained by the release of seed exudates during priming, thus decreasing attraction of the pathogens, as observed by Osburn *et al.* (1989) in the interaction of sugar beet with *Pythium*. Another explanation may be due to increased emergence and rapid growth of seedlings that could escape infection by the pathogens.

Seed priming combined with *T. koningii* dressing enhanced emergence and protected tomato efficiently against *R. solani* and *F. oxysporum* under seed bed conditions as well as after transplant, relative to priming alone (undressed). Benefits resulting of this treatment might be linked to occupation by *T. koningii* during the 48 h of priming of possible sites of infection, removal of exudates, and induction of plant defence mechanisms as shown by Cotes et al 1996 in the interaction of bean with *Pythium*.

The increased growth response observed following application of *Trichoderma* spp. to a pathogen-free soil and in soil artificially infested with pathogens, has been documented (Inbar *et al.*, 1994). In the present work, we demonstrated enhanced growth response when *T. koningii* was applied to soil sown with plants obtained from seeds primed in the presence of *T. koningii*, than in plants obtained from seeds primed in the presence of *T. koningii* without addition of the antagonist to soil. Whereas plants obtained from seeds primed in the absence of the antagonist, plant vigour was not significant, thus demonstrating that plant growth enhancement was produced by *T. koningii*.

In conclusion, seed priming in the presence of *T. koningii* represents an attractive method for introducing the antagonist to potential infection courts of the pathogens and constitutes a promising alternative to control *Rhizoctonia solani* and *Fusarium oxysporum* in tomato. This method also has a positive effect in plant growth enhancement.

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Biological Control of Fungal and Bacterial Plant Pathogens
IOBC wprs Bulletin Vol. 24 (3) 2001
pp.265 - 269

Effect of *Trichoderma harzianum* treatments on systemic resistance in pepper plants (*Capsicum annuum*) to *Phytophthora capsici* and its relation with capsidiol accumulation

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Abstract: The effect of pepper seed and root treatments with *Trichoderma harzianum* spores on the necrosis caused in stems by *Phytophthora capsici* inoculation and on the course of capsidiol accumulation in the inoculated sites were studied *in vivo*. The results obtained indicate that necrosis developed significantly more slowly in treated than non-treated plants. Isolation from stem zones immediately contiguous with the necrotic zones revealed that the presence of *P. capsici* mycelium was greater in non-treated plants than in treated plants. This behaviour seems to be related with slower pathogen growth in the tissue of treated inoculated plants. These results suggest that *T. harzianum*, introduced into the subterranean part of the plant, induces a systemic defense response against *P. capsici* in the upper part of the plant. However, necrosis continued to develop slowly in treated inoculated plants, so that any resistance must be considered as quantitative rather than qualitative. Analysis of capsidiol in the stems of treated inoculated plants by the end of the sixth day after inoculation, revealed that its concentration was more than 7-fold greater than in non-treated and inoculated plants. The increase in capsidiol concentration on day 6, was related with the treatment of the seeds by *T. harzianum*.

Introduction

Trichoderma harzianum is a biocontrol agent of several pathogens, among them *Phytophthora* spp. (Sid Ahmed *et al.*, 1999; Smith *et al.*, 1990). Its principal antagonistic action was attributed to its direct interaction with the pathogens involved through antibiosis and/or mycoparasitism associated with competition for nutrients and space. However, the effect of *T. harzianum* on the metabolic changes of plants that result in enhanced resistance to pathogens has not been investigated in depth. Research into the resistance of pepper to *P. capsici* indicates that such resistance is related to metabolic changes in the plant, among them the production of phytoalexin capsidiol (Candela *et al.*, 1995). In an experiment, in which varieties of pepper were inoculated with *P. capsici*, Egea *et al.* (1996) observed a correlation between capsidiol accumulation and the degree of resistance in plants.

The objectives of this study were to evaluate the effect of pepper seed treatment or root drenching with *T. harzianum* on necrosis in stems inoculated with *P. capsici* and to analyse the accumulation of capsidiol in the inoculated sites and its relation with the *T. harzianum* treatment.

Material and methods

Seed treatment and root drenching with* T. harzianum *spores

Pepper seeds were disinfected with 2% sodium hypochlorite for 5 min. and rinsed three times in sterile distilled water before being introduced into the *T. harzianum* suspension. As a control, seeds were treated with a sterile saccharose-water solution. The density of *T. harzianum* was $2.5x10^4$ CFU/seed. The treated seeds were sown in plug trays containing peat and sand sterile mixture (3:1 v/v). The trays were arranged in a randomized complete block in a chamber with 16 h photoperiod at 25°C and 75-85% r. h. In another treatment, the plant roots were drenched with four concentrations of spore suspension at $2x10^3$, $2x10^5$, $3x10^7$ and $5x10^8$ spores/ml, ten millilitres of each suspension being added to the substrate surface around the stem base of each plant. After seven days, the plants were inoculated with *P. capsici* as described below.

***Evaluation of* T. harzianum *treatments on necrosis caused by* P. capsici**

Pepper plants with 5-7 true leaves were stem-inoculated with *P. capsici.* Five mm diameter discs of *P. capsici* were placed on wounded sites on the stems and covered with aluminium foil containing moist cotton wool. These plants were designated treated and inoculated (T&I). Non-treated and inoculated plants (NT&I), treated plants inoculated with PDA (T&NI) and non-treated and non-inoculated plants (NT&NI) were used as controls. The extent of necrosis was measured at 3, 6 and 9 days in the plants grown from treated seeds and at 3, 6, 9, and 15 days in the plants treated by root drenching. To detect the presence of *T. harzianum* and the extent of *P. capsici* within the stem, two sections 1.5 cm long were cut, from either side of the necrotic zone on days 3, 6 and 9. The sections were disinfected and sown in TSM medium (Askew & Laing, 1993) in the case of *T. harzianum* and P5VPP-BH (Papavizas *et al.*, 1981) in the case of *P. capsici.*

Capsidiol extraction from plants

To detect capsidiol, stem sections were taken from the inoculated sites at 3, 6 and 9 days. Capsidiol was analyzed following the method described by Egea *et al.* (1996). All results were analyzed by one-way ANOVA and the means were separated using Fisher's LSD test at $P \leq 0.05$.

Results and discussion

Effect of *T. harzianum* treatments on necrosis caused by *P. capsici*

Three days after inoculation with *P. capsici,* the NT&I and T&I plants showed a small dark brown stretch of necrosis, which continued to develop more rapidly in NT&I plants. The mean length ofnecrosis was 48.6 mm in NT&I plants after day 9, almost twice the length of that observed in T&I plants, resulting in almost all cases in stem withering and plant death. The progress of necrosis was lower in T&I plants, although it did not cease (Fig. 1 A).

Drenching the roots with suspensions of *T. harzianum* spores had a variable protective effect, which did not depend on the spore concentration used (Fig. 1B). There was no statistically significant relation between the doses of spores added and the length of necrotic tissue recorded. However, there was a significant difference between the infection which developed in T&I plants and that in NT&I plants, which was observed three days after inoculation. The reduction in necrosis length obtained by drenching roots with *T. harzianum* spores was less pronounced than that obtained by treating the seeds (Fig. 1 A and B). Generally, necrosis developed more slowly in T&I than in NT&I plants.

The isolations from T&I and NT&I plants are shown in figure 2. No significant difference was observed between mycelium growth towards the top or towards the stem base,

and so the mean of both values was taken. Isolation showed that no stem contained *T. harzianum* while the percentage of *P. capsici* isolated was lower in NT&I plants than in T&I plants at day 3, 6 and 9 (Fig. 2). This behaviour seems to be related with slower pathogen growth in the tissue of T&I plants. This latter response was probably mediated at a distance by the presence of *T. harzianum* on the root surface. However, necrosis continued to develop slowly in T&I plants, so that any resistance must be considered as quantitative rather than qualitative.

Capsidiol accumulation

As shown in table 1, three days after inoculation, the amount of capsidiol detected in necrotic zones of the NT&I plants was statistically similar to that detected in T&I plants, but significantly above that detected in T&NI plants. On day 6, the amount of capsidiol accumulated in T&I plants was 7.4-times greater than in NT&I plants. The amount accumulated in T&NI plants was less than in either of the above. On day 9, levels of capsidiol were significantly greater in NT&I than T&I plants. Levels in T&NI plants were similar to those detected in T&I plants.

The increase in capsidiol concentration on day 6, was related to the *T. harzianum* treatment of the seeds. Capsidiol accumulation in the necrotic zone might contribute to a decrease in the vegetative growth of *P. capsici* and to a reduction in infection (Egea *et al.*, 1996). However, high phytoalexin concentrations may be toxic to the same plant and so it seems reasonable to presume that plants may have a mechanism for regulating the production of such compounds (Hammerschmidt, 1999).

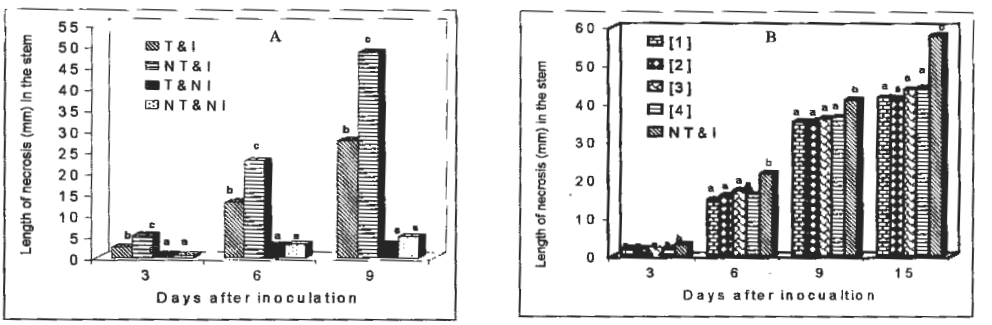

Figure 1. Progress of necrosis in stems of pepper plants grown from seeds treated with *T. harzianum* (A) or treated by drenching the roots with four concentrations of *T. harzianum* (B) and inoculated with *P. capsici*.

Table 1. Accumulation of capsidiol, in the necrotic zones of the stems of pepper plants grown from seeds treated with *T. harzianum* and inoculated with *P. capsici*.

	Concentration of capsidiol in µg/g of fresh tissue		
	Day 3	Day 6	Day 9
T&I	46.72 c [a]	241.97 d	33.03 b
NT&I	44.93 c	32.68 b	121.76 c
T&NI	35.15 b	63 c	32 b
NT&NI	15.89 a	19.9 a	13.25 a

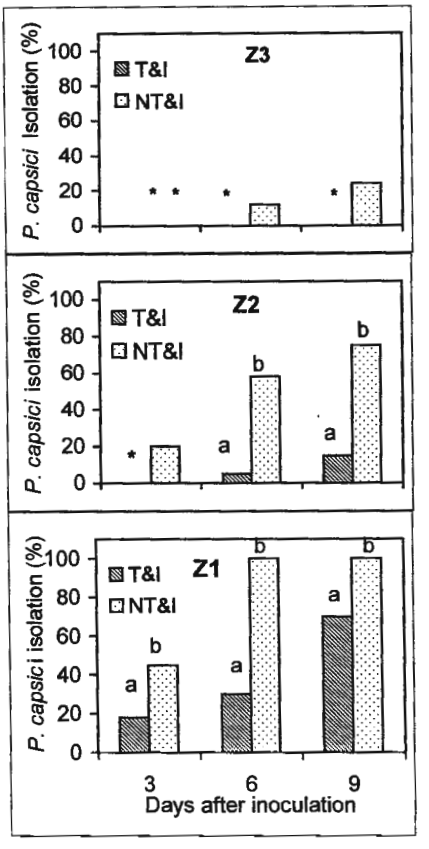

Figure 2. Percentage of stem zone sections in which *P. capsici* was recovered from pepper plants grown from seeds treated (T&I) or not treated (NT&I) with *T. harzianum*. Values represent the mean percentages of corresponding zones (Z1, Z2, Z3) above and below necrotic areas in which *P. capsici* was recovered. * *P. capsici* was not recovered. The percentages are the means from two experiments. Bar values at 3, 6 or 9 days with the same letter are not significantly different.

Acknowledgements

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Biological Control of Fungal and Bacterial Plant Pathogens
IOBC wprs Bulletin Vol. 24 (3) 2001
p.271

Effect of organic amendments on survival and pathogenicity of sclerotia of *Sclerotinia minor*

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Lettuce drop, caused by *Sclerotinia minor* Jagger is a worldwide disease. Losses of 10 to 50% have been reported. The disease is one of the main adversities affecting lettuce drop in La Plata green belt. A pathogenicity test, two sclerotia survival tests and an associated mycoflora study were performed in order to assess the effect of three organic amendments on the pathogenic ability of *Sclerotinia minor* on *Lactuca sativa* L. Meat powder, chicken layer and alfalfa hay were assayed at two concentrations each: 2.5 % and 7.5 % W/W. In the first experiment with one of sclerotia survival tests, ten sclerotia were fixed on a slide with a silicon adhesive. One of such slides was placed with the side containing the sclerotia face down into each of the treatments. For the second test, 50 sclerotia were placed into a little mesh bag. One bag was buried in each one of the trays used for the pathogenicity test.

Epidemics parameters such as final incidence of wilted plants and standardized area under disease progress curve were recorded in the pathogenicity test, while recovery and proportion of destroyed, nonviable and parasitized sclerotia were assessed in sclerotia survival tests. The mycoflora study recorded quantitative and qualitative aspects of the fungal population of the most efficient treatments, in order to assess the role on suppressiveness. Meat powder amendments were phytotoxic and could not be evaluated. Alfalfa hay and chicken layer exhibited a significant reduction in epidemic parameters. Significant correlation occurred between epidemic parameters and recovery of viable sclerotia in both the survival tests. Neither the sclerotia survival test nor the associated mycoflora study, allowed the establishment of a consistent association between suppression and direct fungal antagonism. A possible role of both physical and chemical factors such as concentration of oxygen, carbon dioxide and inorganic nitrogenous forms, are phenomena discussed in disease suppression.

Disinfection of soil by steaming: effect on *Sclerotinia sclerotiorum, S. minor* and *Trichoderma* spp.

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A preliminary study was conducted in order to evaluate the effectiveness of steam treatments on the soil pathogens *Sclerotinia sclerotiorum, S. minor* and the main antagonist *Trichoderma* spp. A TX 40 of 3 bar and 400 kg/h steam generating engine (Atheliers Chappaz S. A., France) was used. The experiment was developed in a greenhouse at Estación Experimental de Gorina, Ministerio de Agricultura, Ganadería y Alimentación, Provincia de Buenos Aires, Argentina. The soil of the greenhouse was inoculated with sclerotia of *S. sclerotiorum* and *S. minor* placed in small nylon mesh bags in order to detect them easily after the treatment.

The four treatments were: 1.45, 3.30 and 7 min of steam and the untreated control. Soil temperature was recorded for each treatment at several depths, 8cm and 13cm depth. The bags were removed the day after steam application. Sclerotia viability was assessed by germination tests in 2% PDA + chloromycetin 250g/l plate. *Trichoderma* spp. population were analyzed using a *Trichoderma* selective medium (Elad *et al.*, 1981) and colony forming unit number (cfu/g) was recorded.

An effective reduction of sclerotia viability was recorded, 90% for *S. minor* and 72% for *S. sclerotiorum* obtained with the 7 min treatment. On the other hand, *Trichoderma* spp. populations decreased 69% with 1.45min, 96% with 3.30 min and 97% with the 7 min steam treatments, compared to the untreated controls.

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Elad, Y., Chet, I. & Henis, Y. 1981: A slective medium for improving quantitative isolation of *Trichoderma* spp. from soil. Phytoparassitica 9: 59-67.

Biological Control of Fungal and Bacterial Plant Pathogens
IOBC wprs Bulletin Vol. 24 (3) 2001
p.275

Biological activity of an antibiotic produced by Frankia Aips1

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Frankia is a symbiotic actinomycete forming nitrogen-fixing root nodules in actinorrhizal plants like alder *(Alnus* sp.*)* and *Casuarina* sp. However, this microbe is common also in soils without host plants. *Frankia* is known to produce antibiotics, siderophores and plant hormones, compounds that evidently help this slowly growing microbe to survive in non-symbiotic conditions.

The studied antibiotic was purified from the culture broth of *Frankia* strain AiPs1 isolated from a Finnish Scots pine *(Pinus sylvestris* L.) stand. The purification of the ethyl acetate extract was carried out by using thin layer chromatography (TLC) and high performance liquid chromatography (HPLC). The antimicrobial activity was tested against bacterial (*Brevibacillus laterosporus, Staphylococcus aureus, Streptococcus pyogenes, Clavibacter michiganensis* subsp. *sepedonicus, Enterococcus faecalis, Bacillus subtilis, Pseudomonas aeruginosa, Escherichia coli*) and fungal strains (*Phytophthora* spp., *Botrytis cinerea, Fusarium culmorum, Rhizoctonia solani, Heterobasidion annosum, Candida albicans*), using cell suspension assays and a disc diffusion method. The clonal rat pituitary tumor cell line (GH_4C_1) was used to determine the effect of the antibiotic on $^{45}Ca^{2+}$ fluxes through cell membrane.

The antibiotic was active against all the tested Gram+ bacteria and most tested fungi. A concentration of only 125 ng/ml inhibited growth of the actinomycete *C. michiganensis.* The compound also inhibited the oomycete *Phytophthora.* In the GH_4C_1 cell line the studied antibiotic inhibited the influx of $^{45}Ca^{2+}$ with an IC_{50} value of 5.3 µg/ml. The activity was comparable to verapamil hydrochloride, a common calcium antagonist in clinical use.

Since the study shows that the antibiotic produced by *Frankia* AiPs1 affects the growth of several bacterial and fungal strains, this compound would certainly be useful for *Frankia* competing for nutrients in soil. Furthermore, the fact that the studied antibiotic significantly inhibited the influx of $^{45}Ca^{2+}$ in GH^4C^1 cells indicates that besides being an antibiotic the purified compound may have other specific activities as well.

Biological Control of Fungal and Bacterial Plant Pathogens
IOBC wprs Bulletin Vol. 24 (3) 2001
p.277

Organic soil amendments as a biological control of soilborne pathogens

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The effects of soy meal, of high protein content in organic soil amendment, soil solarization and crop rotation were tested in micro-plots. Soil from two fields known as naturally infested by soilborne pathogens of potato and peanuts were used in this experiment. The plots with the two types of soils were treated, once during the experiment in summer before the first crop as follows: untreated control; solar heating for 60 days; soy meal 2 kg per 1 m^2; soy meal under shade and soy meal in combination with solar heating. The crops during the rotation were: potato then sorghum then wheat (green manure) and finally peanut. The main soilborne pathogens in the control plots were: *Streptomyces* spp. (potato scab and peanut pod wart disease), *Verticillium, Rhizoctonia* and nematodes. During the experiment, soil samples were taken before and after each crop for the estimation of pathogens. It was found that all the treatments with soy meal were significantly different from the control in the following parameters: pathogens per gram soil, yield and disease incidence.

Soy meal, as a representative of amendment with high protein content, has in parallel two modes of action: liberation of ammonia into the environment that is toxic to parts of the soil micro-flora and micro-fauna and changes in the population of fungi and bacteria in the soil. These activities control some of the soilborne pathogens and in some cases caused the phenomenon of soil suppressiveness.

Antagonistic bacteria tightly associated to spores of three arbuscular mycorrhizal fungal species

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Spores of many species of arbuscular mycorrhizal (AM) fungi have been reported to harbour different taxa of microbes. Ultrastructural studies have shown bacteria and actinomycetes attached to and within spore walls, and their ecological role is under investigation. In this work, quantitative and qualitative analyses of microorganisms occurring in spores of *Glomus mosseae, G. caledonium* and *G. coronatum*, grown in pot cultures, were performed.

Spores were vigorously shaken and rinsed 15 times in sterile distilled water, then crushed, homogenized and plated on media for isolation of heterotrophic bacteria, actinomycetes and chitinolytic microbes. A large number of microorganisms were isolated from spore homogenates, while the rinsing water originating from the 15th washing was devoid of colony forming microbes. All the strains isolated were tested for their antagonistic activity against *Fusarium oxysporum* f.sp. *dianthi* and *Phytophthora cinnamomi*. Depending on the AM fungal species, different numbers of bacteria showed *in vitro* antagonistic activity, and the majority of strains was active against *P. cinnamomi*.

Our findings demonstrate that many microbial species, showing antagonistic in vitro activity against plant pathogens, are tightly associated with spores of AM fungi, and further studies aimed at understanding their physiological and ecological role could pave the way for their biotechnological utilization.

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Biological Control of Fungal and Bacterial Plant Pathogens
IOBC wprs Bulletin Vol. 24 (3) 2001
p.281

Accurate and rapid assessment of viability of the biocontrol agent *Trichoderma harzianum* using fluorescence-based digital image analysis

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Currently, there is no quantitative, rapid and reliable method for the determination of fungal viability, especially for those of the filamentous type. Accurate viability assessment can help largely for improving filamentous fermentation process design, monitoring and control or physiology-related studies. *Trichoderma harzianum* is an important mycoparasitic filamentous fungus, commonly used for biological control against a great variety of fungal phytopathogens. Viability and survival of *Trichoderma* in soil are among the most important factors influencing its effectiveness. Indeed, process optimization for the production of highly efficient biocontrol agents based on *T. harzianum*, requires of a reliable method to evaluate the effect of process parameters over fungal viability. In this work, a novel, rapid and reliable method for the estimation of *T. harzianum* viability and its application, is described.

Fluorescence microscopy and image analysis was used for the viability assessment of *T. harzianum*. After the evaluation of the two most commonly used fluorochromes, acridine orange (AO) and fluorescein diacetate (FDA) as viability stains, AO turned out to give ambiguous results and therefore FDA was chosen. The lower stability at room temperature and fast fluorescence intensity decay (50% after only 30 seconds of illumination in UV light) could be overcome by the use of a digital image acquisition by a frame grabber and a computer controlled video camera. Fresh (live) fungal hyphae emitted bright green fluorescence when stained with this dye (7.5 µg/L) whereas a total absence of fluorescence was observed when using sterilized (dead) fungal cells. Fresh cells were subjected to different lethal and sub-lethal treatments (heating, milling, microwaves) and the percentage of FDA stained fluorescent hyphae was then measured over the total hyphal area (% of FDA activity) by image analysis. At the same time, treated samples were reinoculated in shake flasks in order to assess viability in terms of fungal growth performance (i.e., the biomass produced after 30 h of fermentation). The FDA activity of treated cells correlated linearly with its growth capacity ($r = 0.968$). The regression equation was: Biomass (g/L) = 14.3 (FDA activity [%]). Finally, this method was used to evaluate fungal viability under two different fermentation conditions in a 10 L bioreactor. Viable biomass during fermentation was strongly influenced by the process conditions. The use of FDA, with computer-aided quantitative image analysis has made it possible to rapidly and reliably quantify the viability of *T. harzianum*.

The viability technique described here, is a very useful tool that, independently of the process conditions and fungal morphology, provides rapid and accurate physiological information of fungal cultures.

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Biological Control of Fungal and Bacterial Plant Pathogens
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Can enhanced susceptibility to pathogens of ethylene-insensitive plants be overcome by biocontrol agents that induce systemic resistance?

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Abstract: The plant hormone ethylene can both affect the development of disease symptoms and enhance resistance in many plant-pathogen interactions. When transgenic ethylene-insensitive tobacco plants (Tetr) are grown in potting soil, they develop symptoms of wilt and stem base necrosis within 3-10 weeks. From stem tissue of diseased Tetr plants, three oomycetes and four fungi were isolated that caused similar disease symptoms when inoculated on non-diseased Tetr plants. The isolates were identified as *Pythium sylvaticum*, *Pythium* sp. "group HS", *Pythium* sp. "group G", *Fusarium oxysporum*, *F. solani*, *Thielaviopsis basicola* and *Rhizopus stolonifer*. Ethylene-insensitive plants of both tobacco and *Arabidopsis thaliana* were more susceptible than wild type plants to six different *Pythium* isolates. Ethylene insensitivity also appeared to increase susceptibility to *T. basicola* and the two *Fusarium* spp. We aim at restoring resistance in ethylene-insensitive plants by treatments with specific hormones or rhizobacteria.

Key words: ethylene, tobacco, Arabidopsis, *Pythium*, *Thielaviopsis*, *Fusarium*, resistance

Introduction

The gaseous plant hormone ethylene is involved in developmental processes and in reactions of the plant to its environment. However, the role of ethylene in different plant-pathogen interactions seems ambiguous. Ethylene can aggravate development of disease symptoms, but ethylene signaling is also involved in inducing resistance against certain pathogens. Salicylic acid (SA) is an important signal molecule for inducing resistance in many plant-pathogen interactions (Dempsey *et al.*, 1999). Tobacco plants require intact ethylene signaling in addition to SA accumulation for induced resistance to tobacco mosaic virus (Knoester, 1998). In *Arabidopsis thaliana* however, ethylene signaling is not involved in SA-dependent systemic acquired resistance (Lawton *et al.*, 1995). In addition, SA-independent but ethylene/jasmonate-dependent resistance to bacterial (Norman-Setterblad *et al.*, 2000) and fungal (Thomma *et al.*, 2000) pathogens has been demonstrated in Arabidopsis, suggesting the existence of different signal transduction routes.

Ethylene can increase disease severity by causing, or accelerating, symptoms of chlorosis and necrosis. Indeed, some ethylene-insensitive plants appear to develop less disease symptoms after infection by the fungal pathogen *Fusarium oxysporum* (Lund *et al.*, 1998) or the bacterial pathogens *Pseudomonas syringae* and *Xanthomonas campestris* (Bent *et al.*, 1992; Lund *et al.*, 1998). Recently, Knoester *et al.* (1998) reported that tobacco plants transformed with the mutant *etr1-1* gene from Arabidopsis (Tetr) manifest strongly reduced sensitivity to ethylene and show wilting symptoms when grown in commercial potting soil. In this study, several fungi and oomycetes that appear to be responsible for the symptoms observed were isolated from wilting Tetr tobacco plants.

Material and methods

Isolation of fungi and oomycetes from plant material
Stems of tobacco plants showing symptoms of wilting and stem base necrosis were surface sterilized with 70% ethanol and 1% $NaClO_3$, transferred to water agar (WA) medium, potato dextrose agar (PDA, Difco) medium, or malt extract agar (2% ME, Difco), and incubated at 23°C for 1-5 days. Outgrowing fungi were transferred to fresh medium.

Inoculum cultivation
Identified *Pythium* isolates and *P. sylvaticum* (Knoester *et al.*, 1998), *P. irregulare* (Staswick *et al.*, 1998), *P. jasmonium* (Vijayan *et al.*, 1998; strain 101876, CBS, Baarn, The Netherlands) and *P. aphanidermatum* (Postma *et al.*, 2000) were grown in V8 medium (20% (v/v) V8-juice + 0.05 M $CaCO_3$, pH7.3) at 25°C for 14 days. Mycelium was washed with distilled water, homogenised in a blender and adjusted to 15 g mycelial mass/ liter. *Fusarium* isolates were grown in Czapek Dox medium at 24°C for 7 days, shaking at 150 rpm. Suspensions were filtered through glasswool, conidia were washed and suspended in 10 mM $MgSO_4$ to a final concentration of 10^7 spores/ml. *Thielaviopsis basicola* was grown on PDA at 24°C for two weeks. Spore suspensions were obtained by rinsing the plates with 10 mM $MgSO_4$ and filtering through Miracloth. Final concentration was 10^6 conidia/ml.

Bioassays
Tobacco cv. Samsun NN and ethylene-insensitive transformed Tetr or Arabidopsis wild type (Col-0) and ethylene-signaling mutants *etr1-1* and *ein2-1* and jasmonate-signaling mutant *jar1-1* were grown on river sand for two weeks. Seedlings were transferred to pots containing a soil/sand mixture. Before planting, roots were immersed in a suspension of the pathogen for 1-2 s. Disease development was scored for a period of at least two weeks after infection and percentages diseased plants were statistically analysed using analysis of variance (GLM Repeated Measures, SPSS 8.0 for Windows).

Results and discussion

When grown in non-autoclaved potting soil, ethylene-insensitive Tetr18 plants developed symptoms of wilting and stem base necrosis within 3-10 weeks. Non-transformed plants never showed symptoms of disease under these conditions. Autoclaving the soil prior to sowing prevented disease development in Tetr18 plants. From plants showing moderate to severe disease symptoms, 15 morphologically different fungi and oomycetes were isolated. These isolates were tested for pathogenicity by placing agar plugs with mycelium at the stem base of non-transformed and Tetr18 tobacco plants growing in autoclaved soil/sand mixture. Six of the tested isolates caused symptoms of disease in Tetr18 plants and were isolated again from diseased stem tissue. These isolates were identified as *Pythium* sp. "group HS" (isolate Nt15d), *Pythium* sp. "group G" (Nt59d), *Rhizopus stolonifer* (Ehrenberg: Fries) Vuillemin (Nt60c), *Fusarium oxysporum* Schlechtendahl: Fries (Nt32d), *F. solani* (Martius) Saccardo (Nt34b) and *Thielaviopsis basicola* (Berkeley & Broome) Ferraris (Nt29). No isolate caused wilting symptoms on non-transformed tobacco, suggesting that ethylene insensitivity results in a defect in defense mechanisms that is active against these pathogens.

Disease development was investigated in more detail in both tobacco and Arabidopsis. Bioassays were performed in which seedling roots were immersed in a suspension of mycelium (*Pythium* spp.) or spores (*Fusarium* spp. and *T. basicola*) and percentages of wilted plants were monitored. The *Pythium* isolates that were isolated from diseased Tetr18 tobacco (Nt15d and Nt59d) caused symptoms of disease on both non-transformed and Tetr18 tobacco (Table 1). Apparently, inoculation of seedlings by immersing the roots results in such a high

disease pressure that non-transformed plants are affected as well. However, the disease incidence was significantly higher in Tetr18 than in non-transformed tobacco. Four additional *Pythium* isolates were tested for their ability to cause disease in the tobacco plants. Root inoculation with *P. irregulare*, *P. jasmonium* and *P. sylvaticum* caused symptoms of wilting in Tetr18 plants but not in non-transformed plants. For *P. jasmonium* and *P. sylvaticum* the disease incidence was only minor. *P. aphanidermatum* has been described as pathogenic on tobacco. Indeed, almost all non-transformed and Tetr18 plants wilted after inoculation. When disease pressure was reduced by diluting the pathogen suspension, the level of disease development was higher in Tetr18 plants than in non-transformed plants. These results indicate that reduced ethylene sensitivity in tobacco plants results in enhanced susceptibility to *Pythium* spp.

Table 1. Disease development in ethylene-insensitive tobacco (Tetr18) or Arabidopsis (*ein2-1*) compared to their wild-type plants. 0: no disease symptoms, +: moderate disease development, ++: strong disease development.

	Tobacco		Arabidopsis	
Isolates	non-transformed	Tetr18	wild type	*ein2-1*
Pythium sp. Nt15d	+	++	+	++
Pythium sp. Nt59d	+	++	+	++
P. aphanidermatum	++	++	+	+
P. irregulare	0	+	+	++
P. jasmonium	0	+	+	++
P. sylvaticum	0	+	+	++
Thielaviopsis basicola	++	++	+	++
Fusarium solani 34b	0	+	n.d.	n.d.
F. oxysporum Nt32d	0	+	n.d.	n.d.

In the experiments with Arabidopsis two ethylene-signaling mutants, *etr1-1* and *ein2-1*, were used. Inoculation of the *etr1-1* mutant with the *Pythium* spp. never resulted in a higher percentage of wilted plants than in wild-type plants. This might be a result of the lower level of ethylene insensitivity of the *etr1* mutant compared to the *ein2* mutant, as suggested by Roman *et al.* (1995). Indeed, mutant *ein2-1* was significantly more susceptible to the isolates Nt15d, Nt59d, *P. irregulare* and *P. jasmonium* than WT Arabidopsis (Table 1). These results indicate that also in *A. thaliana* ethylene insensitivity results in enhanced susceptibility to *Pythium* spp.

Inoculation of tobacco with the isolate of *T. basicola* resulted in a high number of diseased plants in both non-transformed and Tetr18 plants (Table 1). Inoculation of Arabidopsis with *T. basicola* resulted in a significantly higher number of wilted *etr1-1* and *ein2-1* mutants compared to the wild-type plants. Inoculating seedling roots of tobacco with the isolates of *Fusarium* resulted in wilting plants in Tetr18 but not in non-transformed plants (Table 1). These results indicate that ethylene insensitivity also results in enhanced susceptibility to *Thielaviopsis* and *Fusarium*.

Several strains of rhizobacteria have been demonstrated to enhance resistance in different plant species (Van Loon *et al.*, 1998). Treatments with selected resistance-inducing bacteria and with the resistance inducing chemicals salicylic acid and jasmonic acid will be performed to investigate if the reduced resistance in ethylene-insensitive plants can be restored.

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Biological Control of Fungal and Bacterial Plant Pathogens
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Pythium oligandrum-mediated induced resistance against grey mould of tomato is associated with pathogenesis-related proteins

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Abstract: The biocontrol fungus, *Pythium oligandrum* was inoculated onto tomato roots to test its ability to induce systemic resistance against the foliar pathogen, *Botrytis cinerea*. Among different compounds liable to induce resistance to pathogens, the involvement of PR protein induction in tomato protection against grey mould was investigated. Ten days after inoculation with *B. cinerea* alone, leaves from tomato plants showed either severe necrosis or complete senescence, leading to defoliation from the stem. On the other hand, *P. oligandrum* root pre-inoculation before *B. cinerea* challenge significantly reduced grey mould severity and defoliation was not observed. Leaves from controls or plants inoculated with either *P. oligandrum* or *B. cinerea* alone synthesised few amounts of PR-5 and PR-3. Amplification of PR-3 and PR-5 synthesis and induction of a new PR-3 isoform were observed in the leaves of plants first colonised by *P. oligandrum*, and then challenged with *B. cinerea*. Thus, these investigations provided evidence that resistance against grey mould is expressed in tomato plants further to pre-inoculation with *P. oligandrum*. In addition: i) plant colonisation by *P. oligandrum* alone induced weak PR protein synthesis, and ii) significant host response occurred only upon pathogen attack in *P. oligandrum*-pre-inoculated plants. It is likely that such a defence system is very efficient in term of reduction of cost energy for tomato plants.

Keywords: induced resistance, PR proteins, biocontrol agent

Introduction

Botrytis cinerea-incited grey mould is one of most important foliar diseases observed in greenhouse-cultivated tomatoes. Control of this pathogen by antagonistic fungi (Elad, 1996) offers an alternative or a supplement to the use of fungicides. Among the most promising biocontrol agents, *Pythium oligandrum* has been described as an aggressive mycoparasite of numerous fungi including many plant pathogens (Benhamou *et al.*, 1999). Evidence was provided that *P. oligandrum* could penetrate and colonise tomato root tissues without causing host-cell damage (Rey *et al.*, 1998), but instead inducing local resistance against *Fusarium oxysporum* f. sp. *radicis lycopersici* (Benhamou *et al.*, 1997). Recently, it was shown that *P. oligandrum* produces a proteinaceous molecule that contributed to plant resistance against *Phytophthora parasitica* (Picard *et al.*, 2000). However, the possibility that *P. oligandrum*-mediated induced resistance could be systemic has not been investigated. The study reported here was designed to determine whether *P. oligandrum*, when applied at the root level, could induce resistance against a foliar pathogen, *Botrytis cinerea*. We focused on the development of disease symptoms in *B. cinerea*-infected leaves of tomato plants inoculated or not with *P. oligandrum*. In addition, expression of PR-3 and PR-5 proteins was determined in the

infected leaves. The correlation between enhanced protection against *B. cinerea* and production of PR proteins is discussed.

Materials and methods

Plant material and fungal inoculations

Tomato plants (*Lycopersicon esculentum* Mill. cv Prisca) were grown for 6 weeks in a greenhouse under a 14-h-light 16°C/10-h-dark 25°C photoperiod. Plants were inoculated three times with a *P. oligandrum* inoculum composed of an oospore-mycelium homogenate (35 000 oosp.mL^{-1}) and deposited at the collar of each plant. Inoculation with *B. cinerea* was performed by depositing 20-µL droplets of conidial suspension onto the upper surface of each leaf (500 spores/droplet, 10 droplets/leaf). Infections were scored according to a disease index with a relative scale of 0 to 10 on the basis of necrotic-lesion development. The lesions restricted to the deposition area had a score from 0 to 4; those spreading out of it were scored from 5 to 10.

Protein analysis and immunodetection by western blotting

Acid-soluble proteins were extracted from infected leaves (Renault *et al.*, 2000), and resuspended in SDS-sample buffer thereafter. SDS-PAGE was carried out using the mini protean II system (Biorad). Samples were applied to stacking and resolving gels usually containing 5 and 13.5% (w/v) of polyacrylamide, respectively. After electrophoresis, proteins were directly transferred onto 0.2-µm nitrocellulose filters. After electrotransfer and subsequent incubation of the blots with tobacco PR-3 (Q) and PR-5 (S) antisera, the antigen-first antibody complexes were detected with a second antibody phosphatase-conjugated goat anti-rabbit.

Results

***Assessment of* P. oligandrum-*mediated induced protection against* B. cinerea**

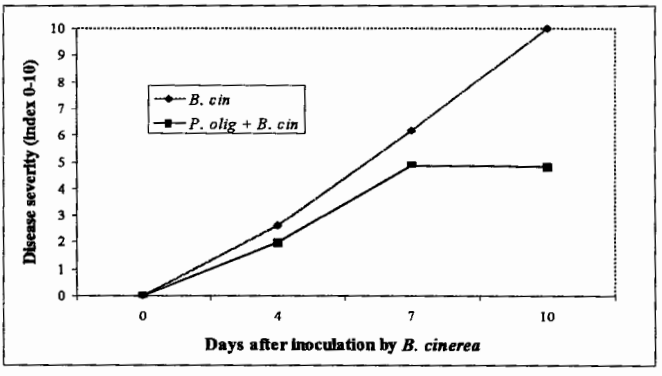

Figure 1. Effect of *P. oligandrum* on *B. cinerea* disease severity by 1-10 days after inoculation.

About 80% of the roots from plants inoculated with *P. oligandrum* were colonised by the fungus. Ten days after inoculation of tomato leaves with *B. cinerea* alone, severe necrosis

leading to complete senescence was recorded, resulting in defoliation of the leaves from the stem. Pre-inoculation of tomato roots with *P. oligandrum* before *B. cinerea* challenge on the leaves significantly reduced grey mould severity (Fig. 1) and defoliation was not observed.

Synthesis of PR-3 proteins

Western blotting revealed that the leaves of both control and *P. oligandrum*-inoculated plants (Fig 2a, 2b) synthesised only one protein of 27 kDa. This result was observed with each tested sample (Fig 2a). When plants were inoculated with *B. cinerea* alone, one additional 30 kDa band was detected (Fig. 2a). The synthesis of this PR-3 protein increased in plants pre-inoculated with *P. oligandrum* and challenged by *B. cinerea*; while a third protein (~32 kDa) was detected (Fig. 2a).

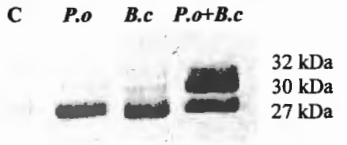

Figure 2a. Accumulation of PR-3 proteins in different plants. The experiment was carried out 10 days after inoculation of plants with *B. cinerea*.
C: control plants;
P.o: *P. oligandrum*-treated plant;
B.c: *B. cinerea*-inoculated plant;
P.o + *B.c*: plants treated first with *P. oligandrum*, then with *B. cinerea*.

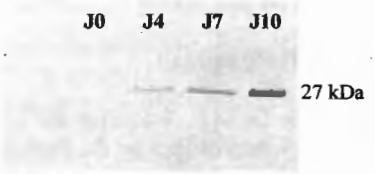

Figure 2b. Time course induction of PR-3 protein in leaves of plants treated three times with *P. oligandrum* alone. J0 to J10 correspond to 0 to 10 days after the last inoculation with *P. oligandrum*.

Synthesis of PR-5 proteins

A 24-kDa protein was detected in *B. cinerea*-infected leaves, whereas an increase in the synthesis of this protein was noticed in infected leaves upon pre-treatment with *P. oligandrum* (Fig. 3).

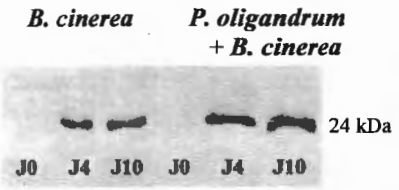

Figure 3. Time course induction of PR-5 proteins in plants treated with either *B. cinerea* alone or *P. oligandrum*, then challenged with *B. cinerea* at 0, 4 and 10 days thereafter.

Discussion

In agreement with previous reports (Benhamou *et al.*, 1997; Picard *et al.*, 2000), the present study confirms the high potential of *P. oligandrum* to induce plant resistance against pathogens and provided the first evidence that this resistance can be systemic. Synthesis and

accumulation of PR proteins differed with respect to tomato plant treatments. Increase of PR-3 and PR-5 protein syntheses and induction of a new PR-3 protein were observed only in leaves of plants pre-inoculated with *P. oligandrum*, and challenged by *B. cinerea*. Although, PR proteins are not usually considered as an early defense event set up by plants to prevent pathogen development (Sticher *et al.*, 1997), their rapid accumulation following pathogen challenge suggests that they play an important role in the plant defence strategy. However, induced resistance mechanisms may differ according to the inducing agent. For instance, Garcia-Lepe *et al.* (1998) observed that induced resistance against Fusarium wilt of tomato by *Penicillium oxalicum* did not correlate with increased PR-protein synthesis. Our investigations demonstrate that: pre-inoculation of tomato plants with *P. oligandrum* triggers the synthesis of PR proteins that are amplified upon pathogen attack. It is likely that such a defence response is very efficient in terms of reduction of cost energy for tomato plants.

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Biological Control of Fungal and Bacterial Plant Pathogens
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Soil conditions and regulatory genes modulate persistence and cell culturability of biocontrol agent *Pseudomonas fluorescens* CHA0

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Abstract: The biocontrol agent for soilborne diseases *Pseudomonas fluorescens* strain CHA0 can lose its ability to grow on agar plates during residence in soil under field conditions. As a consequence, the inoculant persists as a mixed population of culturable and non culturable cells. The aim of the present experiments was to examine in CHA0 the role of the major metabolic regulation system GacA/GacS on persistence and appearance of non culturable cells when introduced into soil microcosms prepared with two field soils and one forest soil, and in the rhizosphere of cucumber. The inoculants were monitored with culturable counts and total immunofluorescence microscopy counts. Results indicate that functional *gacA* and *gacS* genes prevented CHA0 cells to switch into a non-culturable state in all three soils. Moreover, more non culturable cells of the *gacA* and *gacS* mutants were found in the forest soil than in the field soils. In contrast, the two-component regulatory system GacA/GacS had no influence upon colonization of, and survival on, roots of cucumber.

Key words: biocontrol, *Pseudomonas fluorescens*, persistence in soil, non-culturability, *gacA* and *gacS* regulatory genes

Introduction

The soil bacterium *Pseudomonas fluorescens* strain CHA0 efficiently protects different plant species from soilborne diseases. Upon introduction of the strain into soil in field, mixed populations of culturable and non culturable cells appear (Troxler *et al.*, 1997). The switch of previously culturable cells into a non culturable state is governed by environmental stresses (Mascher *et al.*, 2000) and might be controlled by general metabolic regulatory systems. Several regulatory elements have been identified in strain CHA0. The global activator complex GacA/GacS controls the production of antimicrobial and other secondary metabolites. The purpose of the present experiments was to measure how the survival of strain CHA0 is modulated by the interaction of these regulatory genes with specific soil conditions. This was achieved by comparing the wild-type with *gacA* and *gacS* mutant derivatives.

Materials and methods

Strains and inoculum preparation

Strain CHA0-Rif (Natsch *et al.*, 1994) is a spontaneous rifampicin resistant mutant of strain CHA0. CHA96-Rif (Natsch *et al.*, 1994) is a spontaneous rifampicin resistant mutant derived from the *gacA* deficient mutant CHA96 (Laville *et al.*, 1992). Strain CHAS17-Rif is a spontaneous rifampicin resistant mutant of the *gacS* mutant CHAS17 (Duffy and Défago, 2000). The strains were stored at –80°C in 44% glycerol and grown routinely at 27°C with shaking (150 rpm) in King's B (KB; King *et al.*, 1954) broth containing 100 µg/ml rifampicin (Sigma, Steinheim, Germany). For inoculum preparation, overnight cultures were inoculated

on KB agar with rifampicin, and grown for 22 h at 27°C. After elution with sterile double distilled H_20 (dd H_20), cells were centrifugation and washed 3 times with dd H_20.

Soils, inoculation of soil microcosms and inoculation of cucumber roots

Soil material was collected from the A horizon of cambisols from fields in Eschikon (canton Zürich) and in Tänikon (canton Thurgau) and from forest in Mellstorf (canton Aargau). Before inoculation, sterile distilled water was added to achieve a soil water potential of –0.03 MPa after subsequent addition of inoculum. Soil microcosms consisted of 30 ml glass vials containing 10 g soil at a final concentration of 10^8 inoculant cells/g soil. The vials were stored protected from drought in a climate chamber at 12°C and 70% relative humidity. Throughout the 60-day experiment, soil water content remained essentially constant.

Seeds of cucumber (var. Chinesische Schlange) were surface-sterilized and incubated for 3 d on 0.6% tap water agar at 24°C for germination. Erlenmeyer flasks (1 l) were filled with 300 g artificial soil made up of quartz sand, quartz powder and the clay mineral vermiculite (Keel *et al.*, 1989) and subsequently sterilized. Prior to planting 5 cucumber seedlings, the soil was amended with 10 ml of bacterial suspension and 11 ml of Knop fertilizer solution (Keel *et al.*, 1989). The microcosms were incubated for 10 days in a climate chamber at 22°C with light (160 $\mu E/m^2/s$) for 16 h, followed by an 8-h dark period at 18°C.

Monitoring of inoculants

For the soil experiment, the entire content of each vial (10 g soil) was shaken at 300 rpm for 15' in 100 ml dd H_20. In the root colonization experiment, the entire root (without adhering soil) was shaken in 20 ml dd H_20 for 15' at 300 rpm. A dilution series was prepared from each sample and plated on KB plates containing 100 µg/ml rifampicin and 190 µg/ml cycloheximide (against fungal contaminants). Colonies were counted after incubation for 2 days at 27°C in the dark. The total number of inoculant cells was determined by immunofluorescence microscopy, as described elsewhere (Mascher *et al.*, 2000).

Statistical design and analysis

All treatments were replicated three times. Data were log transformed before processing data by analysis of the variance (Systats for Windows, ver. 5; SPSS Inc., Evanston, IL, USA). When appropriate, Tukey's HSD tests were used to compare treatments. Differences between treatments were retained significant at the $P<0.05$ level.

Results

Persistence in bulk soil and colonization of cucumber roots

In the field soils (Fig. 1), colony counts of the strain CHA0-Rif decreased moderately after inoculation and the vast majority of inoculant cells were culturable after 60 days. In contrast, colony counts of strains CHA96-Rif and CHAS17-Rif decreased by a few log units, and the number of culturable cells was a small fraction of the total number of inoculant cells still present in soil. In the forest soil, CHA0-Rif behaved similarly. However, for strain CHA96-Rif the number of culturable cells was at the detection limit (i.e. 10^3 cells/g soil) and for strain CHAS17- Rif it was below. The number of culturable cells represented less than 1% of the total inoculant population for the 2 mutants. At 10 days, comparable colonization of cucumber seedling roots was achieved by the 3 strains (Fig. 2). Culturable cells represented 35-50% of all inoculant cells.

Discussion

Strain CHA0-Rif persisted as culturable cells for 60 days in all 3 soils. Reduction of cell culturability in strain CHA0-Rif has been observed under stressful conditions in soil (Troxler

et al., 1997) and *in vitro* (Mascher *et al.*, 2000). Apparently, similar conditions of stress did not prevail in soil in the current work, even in the forest soil despite pronounced acidity (pH of 3.8). The *gacA* and *gacS* mutants were clearly compromised in their ability to maintain cell culturability in all 3 soils. In both strains, the inoculum persisted as a mixed population of culturable cells and predominant non-culturable cells. This suggests that *gacA* and *gacS* can influence stress protection mechanisms in addition to controlling secondary metabolite production. Which mechanisms of stress protection may be controlled by GacA/GacS remains to be established. In *P. fluorescens* Pf-5, *gacA/gacS* regulates positively the accumulation of the stationary-phase sigma factor σ^S and thus contributes to the ability of the cells to resist oxidative stress (Whistler *et al.*, 1999). The capacity to withstand oxydative stress seems important for bacterial root colonization (Kim *et al.*, 2000). Here however, root colonization did not depend apparently upon stress protection mediated by GacA/GacS. This suggests the existence of further cellular devices to cope with environmental stress, which might likewise influence survival in soil.

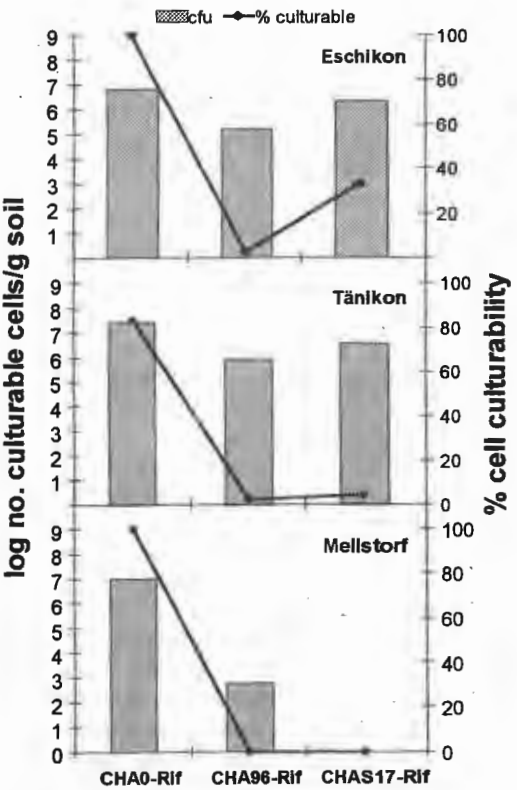

Figure 1. Persistence of culturable cells and their proportion among total cells of *P. fluorescens* CHA0-Rif and the regulatory mutants CHA96-Rif and CHAS17-Rif in soil. Cells were incubated in two field soils (Eschikon and Tänikon) and a forest soil (Mellstorf) for 60 days.

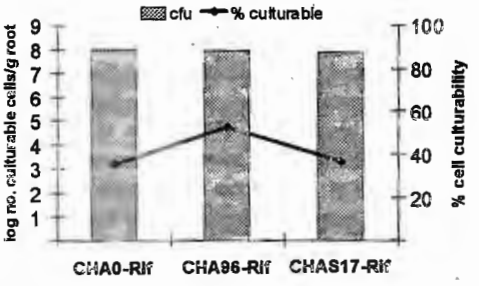

Figure 2. Number of culturable cells and their proportion among total cells of *P. fluorescens* CHA0-Rif and the regulatory mutants CHA96-Rif and CHAS17-Rif on cucumber roots. Cells were inoculated into soil prior to planting of the cucumber seedling and root colonization was assessed at 10 days.

Acknowledgements

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Induction of resistance to postharvest decay by the yeast biocontrol agent *Candida oleophila*

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Abstract: Results of this study show that application of *Candida oleophila* to surface wounds or to intact grapefruit elicited systemic resistance to *Penicillium digitatum.* The induction of resistance was pronounced already 24 h after elicitation, and was distance and concentration dependent, and was restricted to peel tissue surrounding the yeast application site. Only viable yeast cells were able to enhance fruit resistance to the pathogen. Application of *C. oleophila* cell suspensions to grapefruit peel tissue increased accumulation of chitinase and β-1,3-endoglucanase protein levels as indicated by Western immunoblotting analysis. Overall, this study provides evidence that induction of resistance mechanisms against postharvest decay of citrus fruit takes place and should be considered as an important factor of a multi component mechanism of action of the yeast *C. oleophila.*

Key words: Postharvest, biocontrol, resistance, yeast, *Candida oleophila, Penicillium digitatum*

Introduction

Several bacteria and yeasts have been shown to protect a variety of harvested commodities against a number of postharvest pathogens (Chand-Goyal & Spotts, 1996; Droby *et al.*, 1991; El Ghaouth *et al.*, 1998; Janisiewicz, 1994; Roberts, 1990; Teixidoi *et al.*, 1998). In particular, yeasts occurring naturally on fruits and vegetables have been targeted by many workers as potential antagonists of postharvest diseases because they exhibit a number of traits that confer greater potential for colonizing wound sites (Droby *et al.*, 2000).

Currently, four antagonistic microorganisms, two yeasts, *Candida oleophila*, and *Cryptococcus albidus* and two strains of a bacterium, *Pseudomonas syringae* are commercially available under the trade names ASPIRE (Ecogen Inc., Langhorn PA), YieldPlus (Anchor Yeast, South Africa), and BIOSAVE-110 (EcoScience, Orlando, FL), respectively. In general, the mode of action of many antagonists of postharvest diseases is poorly understood. It appears that the mode of action comprises a complex mechanism which could involve one or several of the following processes: nutrient competition, site exclusion, induced host resistance and direct interactions between the antagonist and the pathogen (Droby & Chalutz, 1994).

The objectives of our study were to: 1) determine and characterize the ability of the yeast antagonist *C. oleophila* to elicit systemic resistance to *P. digitatum* in grapefruit flavedo tissue following application of the antagonist to either surface wounds or whole intact fruit and (2) determine whether induction of antifungal proteins (chitinase and β -1,3-glucanase), ethylene, Phenylalanine ammonia lyase (PAL) and phytoalexins occurred in flavedo tissue, following application of the yeast cells to either surface wounds or intact fruit.

Materials and methods

Induction of resistance tests

Fruit were gently wounded with a dissecting needle (1 to 2 mm deep) at three different sites around their blossom end, and 20 µl of aqueous cell suspension of *C. oleophila* at the desired concentration, were pipetted into each wound site. Wounds treated with the same amount of distilled sterilized water served as a control. After 24 h incubation at 20°C, fresh wounds were made at a distance of 1 cm from either the yeast-treated or the water control wounds, and inoculated with 20 µl of *P. digitatum* (5×10^4 spores ml^{-1}). Fruits were then incubated at 20°C in plastic trays under humid conditions and the percentage of infected wounds was determined 4 days after inoculation. Eighteen fruits were used for each treatment (total of 54 wounds per treatment), and each experiment was repeated at least three times with similar results. To assess the effect of various concentrations of *C. oleophila* on eliciting resistance to *P. digitatum* similar experiments were performed as already described. The yeast cell concentrations tested were: 10^9, 10^8, 10^7 and 10^6 cells/ml. Development of the resistance was examined by inoculating wounds made 24 h after yeast application, as already described, at 1, 2, 4, and 8 cm from the yeast-treated sites. The ability of *C. oleophila* in eliciting resistance in whole intact fruit was tested by dipping the fruit in the yeast cell suspension (10^8 cells/ml) for 20 sec, allowing to air-dry and kept for 24 h at 20°C. Fruit were then surface sterilized by extensive wiping with pure ethanol to remove yeast cells from the surface and wound inoculated at three sites as already described.

Protein extraction and immunoblotting analysis

Protein extraction and immunoblotting was performed according to Lers *et al.* (1998). Proteins were separated on 12% SDS-PAGE using a mini PROTEIN II apparatus, and transferred to nitrocellulose membranes with a Bio-Rad Mini Trans-Blot electrophoresis unit. Immunodetection was performed with citrus chitinase and β-1,3-endoglucanase antibodies. The primary antibody reacting bands were visualized using commercial secondary antibodies conjugated to alkaline phosphatase.

Results and discussion

***Induction of resistance to* P. digitatum**

Results of the present study provide evidence that the yeast antagonist *C. oleophila* is capable of inducing resistance in wounded and non-wounded grapefruit peel tissue against *P. digitatum*, the cause of green mold of citrus fruit. When applied to surface wounds *C. oleophila* elicited systemic resistance in peel tissue at a distance of up to 4 cm around the challenged site. Maximum resistance, however, was evident in the close vicinity (1 cm) of the yeast-treated wound (Table 1). In addition, dip application of *C. oleophila* to whole intact fruit induced resistance in the entire surface of the fruit to *P.* digitatum (Fig. 1). Induction of resistance required high concentration of viable yeast cells (Table 1). Only viable yeast cells were needed to elicit resistance response (data not shown). This suggests that elicitor/s may be produced by the yeast cells and secreted to the nearby plant peel tissue and, thus, be responsible for the induction of resistance in the fruit. This hypothesis, however, still needs to be confirmed by further experimental data.

Induction of chitinase and glucanase

Application of *C. oleophila* cell suspensions to either peel disks or to the intact whole fruit induced the accumulation of chitinase and β-1,3-endoglucanase (Fig. 2). There was a moderate increase in chitinase after 48 h also in water-treated control peel disks, and that increase was probably related to the wounding of the tissue, since it was not observed in peel

tissue obtained from non-wounded fruit. In intact fruit, dipping in *C. oleophila* increased the accumulation of the same 22 and 24 kD chitinase proteins, already after 1 day of incubation, and this increase continued over a period of up to 7 days (Fig. 2). Accumulation of a 39 kD β-1,3-endoglucanase in *C. oleophila* cell suspension was only evident after 48 h of incubation as compared with control fruit (Fig. 1). In intact fruit, only a slight induction in the accumulation of the 39 kD β-1,3-endoglucanase protein was observed after 24 h, and continued to increase after 5 and 7 days of incubation (Fig. 2).

Table 1. Effects of distance from the *C. oleophila*-treated wounds, time of fruit inoculation and yeast cell concentrations on the resistance of grapefruit against *P. digitatum*.

Treatment	Infected wounds (% of control)	Rot diameter (mm)
Distance of the infected site from the *C. oleophila* treated wounds (cm)		
1	32 c*	24 b
2	49 b	29 b
4	60 b	32 ab
8	85 a	44 a
Time of inoculation after yeast application (h)		
0	82 a	43 a
24	40 b	37 ab
48	40 b	31 b
72	37 b	27 b
C. oleophila concentrations (cells mL^{-1})		
10^6	98 a	48 a
10^7	90 a	43 ab
10^8	55 b	36 b
10^9	41 c	31 b

*Data are means of 18 fruit per treatment (total of 54 wounds) in 3 different experiments. Values followed by different letters are significantly different at $P \leq 0.05$ according to a Student-Newman-Keuls one way ANOVA test on ranks.

Although the implication of induced disease-resistance in the mode of action of microbial antagonists remains to be determined, the observed accumulation of chitinase, ß-1,3-glucanase, and phytoalexins, in yeast-treated tissue suggests a putative involvement of these biochemical defense responses in the biocontrol activity of the antagonists. It is quite possible that yeast-mediated defense reactions play a supporting role in wound sites as well as in intact tissue in restricting fungal spread.

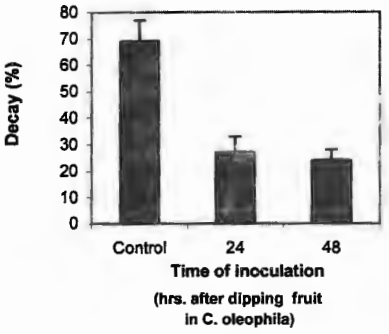

Figure 1. Induction of resistance in intact grapefruit.

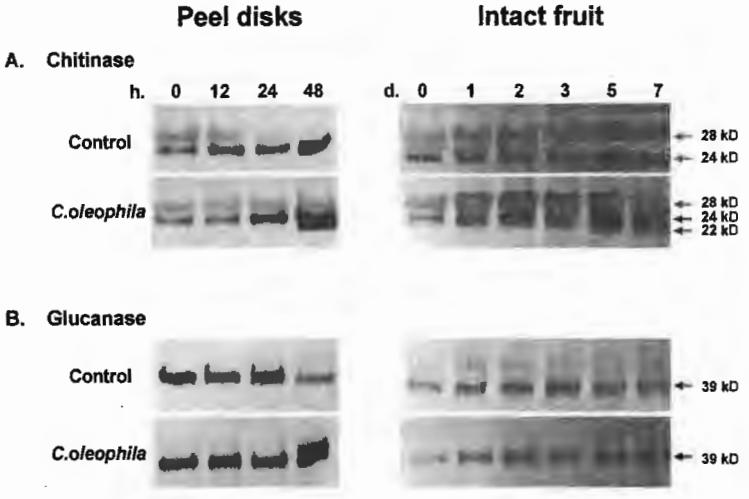

Figure 2. Induction of chitinase and glucanase in peel disks and intact grapefruit

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Resistance to oxidative stress and antagonism of biocontrol yeasts against postharvest pathogens

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Abstract: We analysed a model system consisting of two yeast isolates with higher (LS-28, *Cryptococcus laurentii*) and lower (LS-11, *Rhodotorula glutinis*) antagonistic activities against postharvest fungal pathogens. Our goal was to assess a possible correlation linking i) resistance of biocontrol yeasts to oxidative stress caused by reactive oxygen species (ROS) generated in apple wounds, ii) timely colonisation of wounds by these microorganisms and iii) antagonistic activity against postharvest fungal pathogens. Hydrogen peroxide (H_2O_2) in apple wounds is generated immediately after wounding, reaching its maximum after 60 min. LS-28 exhibits a faster and higher colonisation of apple wounds than that of LS-11. In contrast to LS-28, the number of LS-11 cells decreases 1 h after application (when H_2O_2 peaks), increasing only at following times. In *in vitro* experiments, LS-28 is more resistant than LS-11 to H_2O_2-generated oxidative stress. The combined utilisation of biocontrol yeasts, superoxide dismutase (SOD) and catalase (CAT) in apple wounds prevents the decrease of LS-11 cells observed 1 h after application and significantly enhances colonisation both by this isolate and LS-28. In antagonistic activity assays against *Penicillium expansum* the presence of SOD and CAT significantly enhances the activities of both LS-11 and LS-28. These findings suggest that resistance of biocontrol yeasts to oxidative stress could be involved both in their efficient colonisation of apple wounds, and,consequently, in successful antagonistic activity against postharvest fungal pathogens.

Key words: antagonist yeasts, biocontrol, resistance to oxidative stress, postharvest disease

Introduction

Postharvest fungal pathogens of horticultural and fruit crops invade host tissue mainly through wounds. Biocontrol agents, therefore, have to colonise these sites timely to act as an effective physical and/or biochemical barrier against these fungi. Wounding of plant tissue is associated with lypolitic acyl hydrolase and phospholipase activation, formation of free radicals (Thompson *et al.*, 1987, Doke *et al.*, 1991; Lee *et al.*, 1997) and, possibly, reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2) and superoxide anion ($O_2^{\cdot-}$), which are generated in plant tissue challenged by incompatible pathogens or treated with elicitors (Bolwell *et al.*, 1999). Both free radicals and ROS induce oxidative stress in biological systems. These considerations led us to test the hypothesis that an effective antagonistic activity involves an association between a timely colonisation of wounds and resistance to oxidative stress by biocontrol agents. For this purpose, we analysed a model system consisting of two yeast isolates with higher (LS-28, *Cryptococcus laurentii*) and lower antagonistic activities (LS-11, *Rhodotorula glutinis*) (Lima *et al.*, 1998). These biocontrol agents were compared for a) *in vitro* resistance to H_2O_2-generated oxidative stress, b) colonisation of freshly generated apple wounds and c) influence of ROS-deactivating enzymes both on their colonisation of wounds and their antagonistic activity against *Penicillium expansum*.

Material and methods

Apples (cv Annurca) kept in cold storage for one month from harvest were used in all experiments. Assays of resistance of LS-11 and LS-28 to H_2O_2-generated oxidative stress, time-course analyses of apple wound colonisation by yeast cells and antagonistic activity assays against *P. expansum* were performed as described elsewhere (Chaput & Sels 1987; Castoria *et al.*, 1997; Lima *et al.*, 1998). In the inherent experiments, SOD and CAT dissolved in sterile distilled H_2O were added to wounds (25 and 150 units/wound, respectively) at the same time as antagonists.

In antagonism assays wounds were treated also with bovine serum albumin (BSA) at the same total protein concentration (45 μg/wound) as SOD+CAT treatments, to assess the effect of protein (nutrient) addition on antagonistic activity.

Results and discussion

Hydrogen peroxide generation in apple wounds occurs immediately and peaks at 60 and 120 min after wounding. Interestingly, SOD addition causes a faster (peak at 30 min) and higher production of H_2O_2, indicating that a previous production of $O_2^{\cdot-}$ could be responsible for H_2O_2 generation (data not shown).

The more active antagonist LS-28 shows higher resistance to H_2O_2-generated oxidative stress than isolate LS-11 *in vitro* (Fig. 1A, B). Live cell of LS-11 decrease in a dose-dependent fashion as compared to the control, at all times following H_2O_2 treatment (Fig. 1A). A dose-dependent effect of H_2O_2 is observed also on LS-28 (Fig. 2B), but much lower than on LS-11. An analogous pattern is recorded also following *in vitro* incubation of LS-11 and LS-28 with an $O_2^{\cdot-}$ generating system (data not shown). The lower resistance of LS-11 to oxidative stress may be due to the presence in its polar

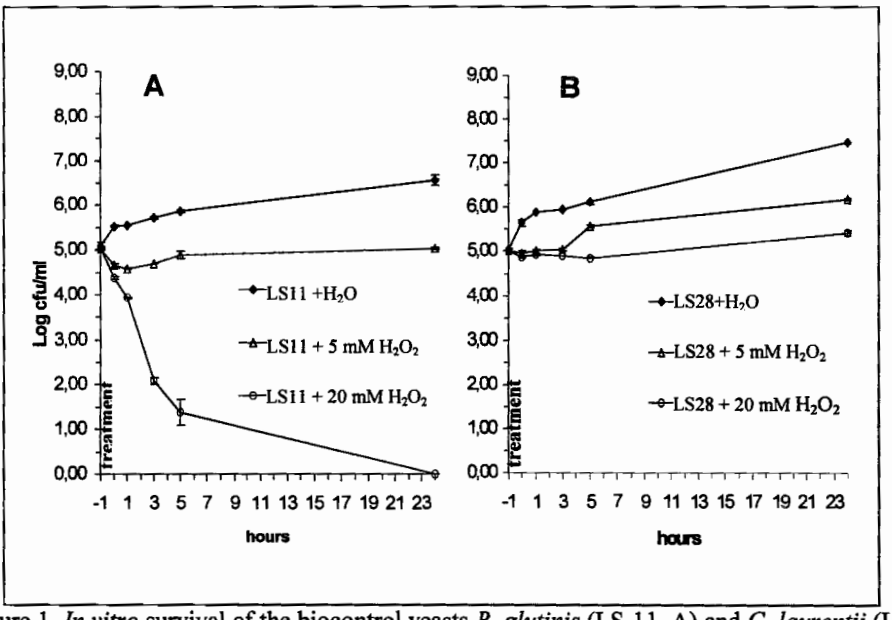

Figure 1. *In vitro* survival of the biocontrol yeasts *R. glutinis* (LS-11, A) and *C. laurentii* (LS-28, B), expressed as log CFU (Colony Forming Units), following 1h treatment with 5 and 20 mM H_2O_2. Values are the mean ± SD of one experiment. Numbers on *x* axis indicate times from treatment with H_2O_2

lipids of more unsaturated fatty acids than the ones detected in the polar lipids of LS-28 (data not shown).

Cells of the more active antagonist LS-28 colonise apple wounds more rapidly and at a higher degree than LS-11, steadily increasing from 0 to 4 h (Fig. 2A, B). Interestingly, number of LS-11 live cells decreases 1 h after application (Fig. 2A), when concentration of H_2O_2 is at its peak (see above), suggesting that such decrease could be due to oxidative stress caused by ROS generated in apple wounds. This is further supported by the positive effect of SOD + CAT, and to a lower degree, of CAT treatments, in which the decrease of LS-11 cells at 1 h is totally prevented and LS-11 shows a much higher rate of wound colonisation than respective control (H_2O) at all the times tested. As expected, SOD treatment does not have the same positive effect at 1 h (Fig. 2A), since it causes a rapid increase of H_2O_2 (see above) in apple wounds. Also in SOD treatment, however, a significant increase of LS-11 cells is recorded at 2 and 4 h. Analogous positive effects of SOD + CAT and CAT treatments are recorded also with LS-28 (Fig. 2B). The increase of H_2O_2 in SOD treatment is the most plausible explanation for the lowest rate of LS-28 colonisation observed at 1 h after application.

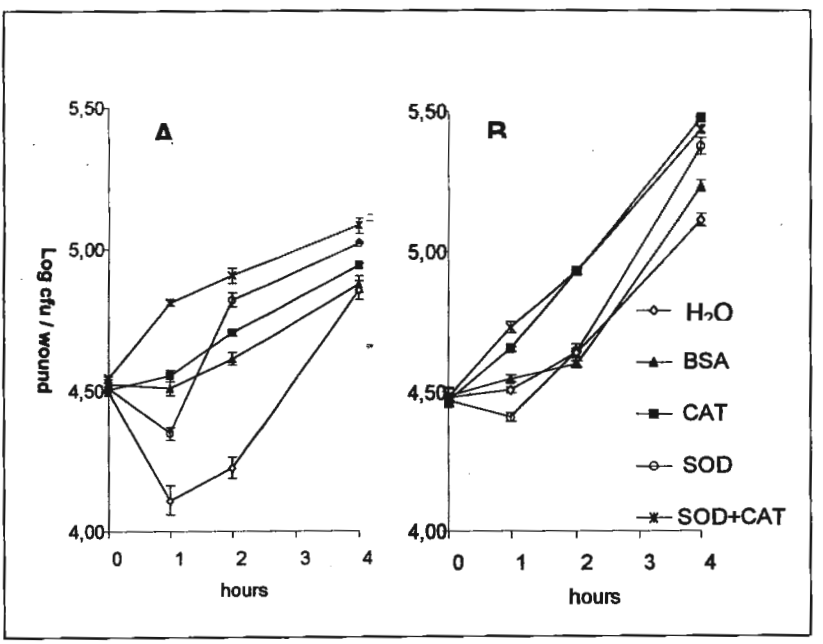

Figure 2. Time-course colonisation of apple wounds by the biocontrol yeasts *R. glutinis* (LS-11, A) and *C. laurentii* (LS-28, B), expressed as log CFU (Colony Forming Units), applied with or without ROS-deactivating enzymes (SOD and CAT). Values are the mean ± SD of one experiment.

Table 1 shows that the presence of SOD+CAT in apple wounds determines at least a four-fold increase of activity against *P. expansum* of both antagonists. Comparison of treatments *P. expansum*+H_2O and *P. expansum*+ SOD+CAT suggests that these enzymes do not significantly favour pathogen infection, at least in these experimental conditions. On the other hand, BSA slightly favours *Penicillium* infection and sensitively lowers the activity of both yeasts, This datum confirms the role of

competition for nutrients in the activity of these antagonists (Castoria *et al.*, 1997), and more importantly, that the enhancement of their activity is not due to a "nutrient" effect of SOD or CAT as a protein, but to their nature of ROS-deactivating enzymes.

Table 1. Antagonistic activity of the biocontrol yeasts, *C. laurentii* (LS-28) and *R. glutinis* (LS-11) against *P. expansum* on wounded apples, in the absence and in the presence of ROS-deactivating enzymes (SOD and CAT).

Treatment	Percentage of infected wounds
P. expansum	84.9 b
P. expansum + SOD + CAT	77.0 b
P. expansum + BSA	93.0 a
P. expansum + LS-11	33.0 e
P. expansum + LS-11 + SOD + CAT	6.7 g
P. expansum + LS-11 + BSA	58.0 c
P. expansum + LS-28	22.0 f
P. expansum + LS-28 + SOD + CAT	4.4 g
P. expansum + LS-28 + BSA	44.0 d

Values represent means ± SD of one experiment. Means with different letters are significantly different ($P \leq 0.05$, Duncan test). Experiments were repeated twice.

These findings suggest that resistance to oxidative stress could represent a pivotal mechanism of action for an efficient and timely colonisation of apple wounds. This would allow biocontrol yeasts to successfully compete for space and nutrients, thus preventing rots caused by postharvest fungi. Studies are in progress to assess the role of resistance to oxidative stress as a general trait of efficient yeast antagonists.

Acknowledgements

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Biological Control of Fungal and Bacterial Plant Pathogens
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Induction of systemic resistance in apple by the yeast antagonist *Candida saitoana*

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Abstract: The ability of *Candida saitoana* to induce systemic resistance in apple fruit against *Botrytis cinerea* was investigated. To separate the antagonistic activity of *C. saitoana* from its ability to induce resistance, the antagonist and the pathogen were applied in spatially separated wounds. In fresh apples, *C. saitoana*, applied 0 or 24 h before inoculation with *B. cinerea*, showed no effect on lesion development. When applied 48 or 72 h post-inoculation with *B. cinerea*, however, *C. saitoana* reduced lesion diameter by more than 50 and 70 %, respectively, in comparison to wounding. *C. saitoana* had no effect on lesion development on stored apples, regardless of the lag period between yeast treatment and inoculation with *B. cinerea*. *C. saitoana* increased chitinase and β-1, 3-glucanase activity with a higher induction in fresh apples than in stored apples. In fresh apples, the onset of systemic resistance to *B. cinerea* coincided with the increase in chitinase and β-1, 3-glucanase activities in protected tissue.

Key words: apple, chitinase, β-1,3-glucanase, biocontrol, disease resistance *Botrytis cinerea*

Introduction

Recently, several antagonistic microorganisms were shown to effectively control a number of postharvest pathogens on a variety of harvested commodities (Wilson & Wisniewski, 1994). The mode of action of antagonistic yeasts, however, has not been fully elucidated. The biocontrol activity of antagonistic yeasts may involve nutrient and site competition (Droby & Chalutz, 1994) and direct parasitism (Wisniewski *et al.*, 1991; El Ghaouth *et al.*, 1998). Additional modes of action such as induced resistance have been suggested (Droby & Chalutz, 19944). Antagonistic yeasts have been shown to induce the accumulation of phytoalexins (Rodov *et al.*, 1994) and antifungal hydrolases (Ippolito *et al.*, 2000), and the deposition of barriers in harvested commodities (El Ghaouth *et al.*, 1998). In these studies, the implication of induced resistance, in local protection provided by antagonistic yeasts was not clearly demonstrated since no attempt was made to separate the antagonistic activity of the yeasts from the fruit-mediated disease suppression. The present study was undertaken to determine if treatment of apple fruit with *C. saitoana* induces systemic resistance to *B. cinerea* and also determine the temporal and spatial induction patterns of chitinase and β-1,3-glucanase in apple fruit treated with *C. saitoana*.

Material and methods

Cell suspension of *C. saitoana* (10^8 CFU ml^{-1}) and spore suspension of *B. cinerea* (10^5 spores ml^{-1}) were obtained following the method described by El Ghaouth *et al.* (1998). Apples (cv Red Delicious) were hand-picked at harvest maturity and stored at 4°C for 1 week to 5 months before being used in biocontrol tests. Fresh apples (firmness = 72-80 N) and

stored apples (firmness =31-40 N) were wounded individually at two different locations, approximately 2 cm apart. On each fruit, the initial wound (3 mm in diameter by 5 mm deep) was treated with 35 µl of a yeast suspension or sterile water and, at various time intervals (0, 24, 48, and 72 h) after initial treatment, the second wound was inoculated with 10 µl of a spore suspension of *B. cinerea*. Treated fruit were stored at 20°C in enclosed plastic trays for 7 days. For each treatment at each inoculation time interval, four replicates of 10 fruit were arranged in a randomized complete block design and the entire experiment was repeated twice. For tissue analysis studies, fresh and stored apples were wounded, treated with a yeast suspension or sterile water, and stored at 20°C as described above. Tissue samples were collected at various time intervals (0, 12, 24, 48 and 72 h) after treatment from six fruit randomly selected from each treatment. From each fruit, tissue samples were taken from both yeast and water-treated wounds and from healthy tissue 2 cm away from the edge of the wound. Samples were individually extracted as previously described (Ippolito *et al.*, 2000). Chitinase was assayed using a dye-labeled carboxymethylchitin following the method described by Wirth & Wolf (1990). ß-1, 3-glucanase was determined following the method of Abeles & Forrence (1979).

Results and discussion

C. saitoana was more effective in inducing disease-resistance on fresh apples than on stored apples (Fig. 1, A&B). With fresh apples, *C. saitoana* when applied either immediately or 24 h before inoculation with *B. cinerea*, had no significant effect on lesion development. When applied 48 or 72 h prior to inoculation with *B. cinerea*, however, *C. saitoana* was very effective in reducing lesion size and the level of reduction was significantly higher than that observed with wounding alone (Fig. 1 A). With fresh apples, wounding alone caused a slight reduction in lesion size that was not significantly different from the control. On stored apples, *C. saitoana* showed no significant effect on lesion development, regardless of the lag period between yeast treatment and inoculation with *B. cinerea* (Fig. 1 B).

Analysis of the induction kinetics of chitinase and ß-1,3-glucanase activities in wounds of fresh apples showed that the yeast treatment caused a transient increase in chitinase and ß-1,3-glucanase levels starting 12 h after treatment, with the maximum accumulation occurring after 96 h (Fig. 2, A & B). A much smaller increase in chitinase and β-1,3-glucanase activity with time was also observed in wounded control in compared to non-wounded control fruit. With stored apples, the induction of chitinase and ß-1,3-glucanase activities in response to yeast and wounding followed the same pattern as seen in fresh apples, except the magnitude of accumulation of these enzymes was markedly decreased in stored vs. fresh apples (Fig. 2, C & D). With stored apples, the yeast treatment and wounding increased chitinase and ß-1,3-glucanase activities starting after a lag of 24 h and the level of the increase of both enzymes was slightly higher in yeast-treated tissue (Fig. 2, C & D). In fresh apples, the increase of chitinase and ß-1,3-glucanase activities by *C. saitoana* was not restricted to the treatment site. Analysis of the tissue area adjacent to the yeast-treated wounds showed a gradual increase in chitinase and ß-1,3-glucanase activity starting 24 h after treatment (Fig. 2, A, E & B, F). In wounded control fruit the induction of chitinase and ß-1,3-glucanase was mainly limited to the wound site (Fig. 2, E & F). With stored apples, the induction of chitinase and ß-1,3-glucanase activities by *C. saitoana* and wounding was mainly limited to the treatment site (data not shown).

Induction of disease-resistance by microbial and chemical elicitors is well documented in vegetative tissue (Kuc & Strobel, 1992). In the present study we showed that the treatment of apple wounds with *C. saitoana* induced a gradual systemic resistance against *B. cinerea* that

was highly dependent on fruit stage of ripeness. With stored apples, *C. saitoana* had no effect on lesion development and induced a comparatively low and localized accumulation of chitinase and β-1,3-glucanase. This may explain, in part, the inability of the yeast to induce systemic resistance in stored apples. In contrast, in fresh apples, the onset of the systemic resistance closely paralleled the increase in chitinase and β-1,3-glucanase activities locally in the treated-wound site and systemically in tissues distant from the initial wound. Although a causal connection between the accumulation of chitinase and β-1,3-glucanase and systemic protection has not yet been established, the occurrence of high levels of these enzymes in systemically protected tissue suggests their involvement in disease resistance. Chitinase and β-1,3-glucanase are known to inhibit the *in vitro* growth of several pathogenic fungi (Schlumbaum *et al.*, 1986) and in several plant-pathogen interactions, their induction is often correlated with the onset of induced resistance (Kuc & Strobel, 1992; Sticher *et al.*, 1997). The observed systemic protection conferred by yeast treatment shows that the activation of defense mechanisms in harvested crops is feasible and has potential in reducing postharvest diseases.

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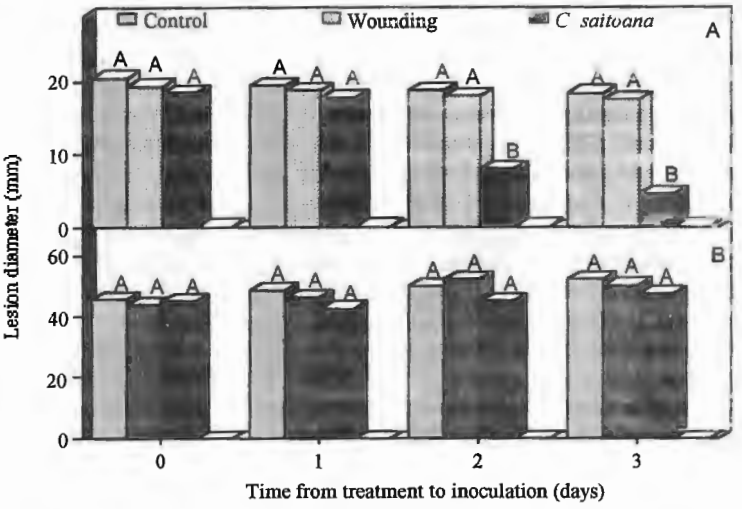

Figure 1. Effect of *C. saitoana* on lesion development caused by *B. cinerea* on fresh (A) and stored apples (B). Columns with the same letter within the same time interval are not significantly different according to Duncan's multiple range test, $P \leq 0.05$.

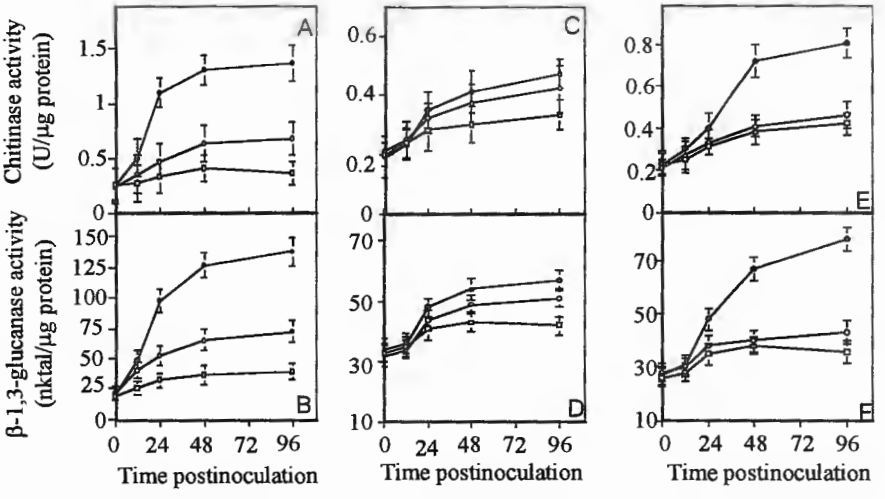

Figure 2. Time course (in hours) of lytic activity in extracts from wounds of fresh apples (A&B), stored apples (C&D), and tissues approx 1 –2 cm from wounds of fresh apples (E&F) that were treated with *C. saitoana* (•) or sterile water (○) and tissue of non-wounded control fruit (□). Bars represent standard deviations.

Biological Control of Fungal and Bacterial Plant Pathogens
IOBC wprs Bulletin Vol. 24 (3) 2001
pp.313 - 316

Thermo-therapy and microbiological control of storage fungi on acorns (*Quercus robur*)

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Abstract: Thermo-therapy with hot water is an efficient treatment against Ciborea in acorns. However, the treatment may favour growth of other pathogens or specific saprophytes. The aim of the present work was, therefore, to test the effect of combining hot water treatment and biological control on the mycofloral composition on acorns during storage. After four months, the hot water treatment was highly effective against several dematiaceous fungi while those belonging to Fusarium, Mucoraceae and Penicillium were registered in high numbers after thermo-therapy compared to the untreated control. In contrast, the biocontrol candidate, *Clonostachys rosea* IK 726, alone or combination with thermo-therapy reduced *Fusarium* spp., *Alternaria* spp., *Ulocladium* spp., *Mucoraceae* and *Penicillium* spp. significantly. The antagonist became well established on the acorns during the four-month storage period. The results show that biocontrol of storage fungi in combination with hot water treatment is a promising approach.

Key words: *Clonostachys rosea* IK 726, dematiacious fungi, seed storage, recalcitrant seed

Introduction

Acorns are temperate recalcitrant seeds, which have to be stored at high humidity and at temperatures between -2 to 0°C. These conditions favour infection by the pathogenic fungus *Ciborea batschiana.* In addition, many saprophytic fungi (typically 'moulds' belonging to *Penicillium* or *Aspergillus* etc.) grow intensively during storage that also can have a negative affect on seed quality. Thermo-therapy (hot water treatment) is often used to eliminate *Ciborea* in acorns. However, it has been observed that the treatment favours growth of "moulds" and some other saprophytes; therefore thermo-therapy is typically followed by a fungicide treatment before the acorns are stored. Since effective fungicides are being withdrawn from the market, alternative procedures are needed. The aim of the present work was to test the impact of combining hot water treatment and biological control agents (BCAs) on the composition of the mycoflora under short term storage of acorns.

Material and methods

Treatments

In October 1999 acorns collected from nets under alley trees were imported from the Netherlands. Dead and low quality acorns were removed from the batch by water skimming for ½ hour, with seeds being divided thereafter into the following treatments: 1) untreated control; 2) thermo-therapy; 3) thermo-therapy + sticker; 4) BCA + sticker or 5) thermo-therapy, BCA and sticker. For thermo-therapy a container with 300-400 kg acorns was

immersed in 800 l water, at 41°C, When the temperature again reached 41°C, the seeds were further treated at this temperature for 2½ h. The seeds were then surface dried for 2 hours on net trays. Immediately after drying acorns were moved to trays containing 15 kg seeds. All commercial products of BCAs were in the form of wettable powders and were mixed with water before use (see table 1 for quantities of inocula). *Clonostachys rosea* IK 726 was prepared in a peat/wheat bran formulation [1.8 x 10^8 colony forming units (cfu)/g] (Jensen *et al.*, 2000). Sepiret (1.2ml pr. kg seed) was added as sticker for all biocontrol treatments. The acorns were mixed with Sepiret and inocula in a big plastic bag for two minutes and transferred to net trays, and pre-incubated for 5 days at 15°C. Afterwards, all seeds were inoculated at -1°C, RH 90-95% in open plastic boxes.

Assessments

Seed germination: Four replicates of 50 acorns were placed in paper towels after removing the pericarp and 1/3 of the acorn at the distal end, and incubated at 15°C in 12/12 h dark/light. Germination was assessed during a six weeks period. Acorns were recorded as germinated when the radicle had the same length as the seed. Ciborea: Pericarps were removed from 100 acorns, the seeds were surface disinfected and incubated on wet filter-paper at 10°C for 7 days, thereafter C. batschiana-infected seeds were recorded. This test was conducted by the Danish Plant Directorate. Inoculum survival: Samples of three randomly selected seeds of each biocontrol treatment were washed with 30 ml water and 30 glass beads on a whirlmixer for 1 minute. Series of 10-fold dilutions were plated on PDA, amended with 2.2 g/l TritonX-100 and antibiotics (chloramphenicol 0.5 mg per l and chlortetracyclin 0.25 mg/l). Colonies were counted daily for 5 days. The test was carried out before storage (after pre-incubation) and repeated again after 4 months of storage. Microflora: After 4 months of storage, 20 acorns per treatment were placed in plastic boxes (11 x 17cm^2) on blotter paper folded in 2.5 cm pleats and placed on a wire-net. The paper had contact with 100ml water. The boxes were covered with transparent lids and placed under NUV light for 8 days at 20°C and a 12 hours light period. After incubation the acorns were inspected by stereomicroscopy for identification of microflora.

Results

Establishment of BCAs

Before storage the cfu's ranged from 5.3 x 10^4 to 5.3 x 10^9 corresponding to the characteristics and/or dosage of the microorganism in the product (Table 1). After 4 months a lower cfu was registered in all treatments compared to the level after 5 days. Both Rotstop and Binab without hot water treatment had especially very low number of cfu's (below detection level).

Microflora

The hot water treatment was effective in eliminating *Ciboria.* After 4 months of storage, 16% of the acorns were infected in the untreated control compared to 0% in the hot water treatment. All hot water treated acorns germinated much better than acorns in the untreated control (table 2). After storage for four months, all treatments except control + sticker (Sepiret) had a positive effect on germination. Hot water treatment had a significant effect on several dematiaceous fungi e.g. *Acremoniella atra, Cladosporium* spp. and sterile isolates with black mycelia. However, some fungi were generally registered at higher numbers in hot water treatments. These fungi belonged to *Fusarium, Mucoraceae, Penicillium* and also dematiaceous fungi such as *Alternaria* spp. and *Ulocladium* spp. (Table 2). In contrast, treatment with the antagonistic fungus *Clonostachys rosea* IK 726 alone or in combination with thermo-therapy resulted in a significant reduction of these fungi compared to untreated or hot water treatment, respectively (Table 2).

Table 1. Biocontrol treatments and number of colony forming units (cfu's) of fungi on acorns after coating and preincubation for 5 d at 15°C before storage and after 4 months of storage.

Formulae	Treatment of 15 kg of acorns	Hot water treatment			Untreated		
		Time 0[1]	After 4 months[2]		Time 0[1]	After 4 months[2]	
		Cfu	cfu	Freq.[3]	Cfu	cfu	Freq.[3]
C. roseum	60g in 300ml water	2.2×10^6	1.3×10^5	100	3.6×10^6	1.4×10^5	100
Supressivit	30g in 125ml water	4.6×10^7	9.9×10^6	85	-[4]	-	-
Binab TF.	50g in 125ml water	5.3×10^4	2.7×10^4	80	5×10^4	0[5]	60
Rotstop	25g in 125ml water	3.5×10^7	0[5]		3.7×10^7	0[5]	
Mycostop	50g in 125ml water	5.3×10^9	3.9×10^8		-	1.7×10^9	

[1]Measurement after coating and 5 days of pretreatment at 15°C, before storage at –1°C;
[2]Measurement after coating, pretreatment and 4 months of storage at –1°C;
[3]Frequency of *Clonostachys rosea* and *Trichoderma* spp measured on acorns (see table 2) for *C. rosea* coated and Supressivit/ Binab TF coated seeds respectively
[4]not measured; [5]Numbers below the level of detection ($<2 \times 10^4$).

Discussion

A diverse saprophytic microflora dominated by *Acromoniella atra*, *Cladosporium*, *Mucoraceae* and *Penicillium* were registered during low temperature storage of the acorns. Potential pathogenic fungi including *Ciborea*, *Colletotrichum*, *Fusarium* and *Verticillium* were found in limited numbers. It was found that hot water treatment increased germination, reduced the occurence of *Ciborea* and was highly effective against dematiaceous fungi such as *Cladosporium* and fungi with black sterile mycelia. However, moulds were registered in higher numbers after thermo-therapy compared to the untreated control. *Clonostachys rosea* IK726 appeared to increase seed germination and decrease natural saprophytic colonization in general. It is well known that saprophytes may cause rotting of the seeds, or influence germination or seedling survival negatively (Rees & Phillips, 1986) and therefore, use of antagonistic, saprophytic fungi (BCAs) under new extreme conditions is unpredictable. However, the results presented here show that biocontrol against storage fungi is a promising approach, but more research is needed to prove the effect during the complete storage period.

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Table 2. Incidence of fungi on acorns in blotter tests, 20 acorns per treatment. Test of acorns treated with biocontrol agents and/or thermo-therapy following 4 months of storage at -1°C.

	Treatment	Frequency (%) of taxon on seeds																		Seed germination (%)
		Acremoniella atra	*Acrospeira mirabilis.*	*Acremonium/ Cephalosporium*	*Alternaria* spp.	*Cladosporium* spp.	*Codinaea simplex*	*Cfr. Cylindrium*	*Epicoccum purpurea*	*Fusarium* spp	*Mucoraceae*	*Papulaspora* spp.	*Penicillium* spp.	pyknider/cfr. *Phoma*	*Pyrenochaeta* spp.	Sterilt mycelium	*cfr. Subramanomyces*	*Ulocladium* spp.	Unidentified	
Untreated	Untreated control	60	5	5	30	100	10	55	25	50	90	5	65	[1]		45	10	30	5	10
	Control + sticker	45	15	15	20	95	15	50	65		60	15	55		5	25	10	45	10	10
	Clonostachys rosea	10			5	95		5	5	10	15		35	10	15		25	15		72
	Binab TF	30	5	20	50	100		50	5	50	55	35	90		5	30	10	50	5	60
	Rotstop	45	15	10	40	95		60	5	35	60	25	65	5	20	45	5	60	20	39
	Mycostop	40	35	20	10	65		10		60	35	50	55		5		10	20	25	72
Thermo-	Thermo-therapy control	40	5		70	10		5	25	55	95	5	100	20	5	15		50	10	89
	Clonostachys rosea				15	20			15	20	45		45	5	20		10	5		96
	Binab TF	25	20		75	50	10	35	20	70	100		80		20	10	15	60	5	87
	Rotstop	5			70	10		10	5	65	80		85	5	20	5		70		77
	Supressivit	15	10		70	10	5	15	5	55	45	15	75		50	5	5	45		88
	Mycostop	10	15	20	55		5	5		45	95		75	15	5			75	5	89

[1] Taxon were not found

Biological Control of Fungal and Bacterial Plant Pathogens
IOBC wprs Bulletin Vol. 24 (3) 2001
pp.317 - 318

Modes of action of biocontrol agents of postharvest diseases: challenges and difficulties

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Numerous biological control agents (BCAs) against post-harvest diseases have already tentatively been used in the past. Unfortunately, practical application is still limited because BCAs don't meet biological, technological and economical requirements. In this context, better knowledge on the modes of action of BCAs are crucial for improving successful post-harvest biocontrol strategies in order to (1) develop rational selection procedures yielding a second generation for more effective antagonistic strains, (2) optimize the method and timing of antagonist application, (3) achieve appropriate production and formulation enhancing antagonistic efficacy, and (4) provide a quality control procedure.

The protective action of BCAs can rest on one or several of the following processes: nutrient or site competition, antibiosis, direct interactions between the BCA and the pathogen, and induced host resistance. Moreover, multiple interactions between antagonist, host, pathogen, and other components of natural epiphytic microflora can interfere with the elucidation of BCAs mode of action. And last but not least, this elucidation is also hampered by the difficulty of interpretation of *in vitro* studies. While several features of the pathogen-antagonist interactions are relatively easy to assess *in vitro*, it is much more problematic to prove the involvement of a mechanism at the site of action. Hence, most conclusions are often based on indirect evidence.

Different experimental strategies based on several techniques (microscopy, biochemical and molecular tools) have been developed to overcome these difficulties. For example, the following progressive steps are currently used to determine if a particular compound such as hydrolytic enzymes or toxins is directly involved in biological control of fungal pathogens: (1) the purified compound shows fungicidal or antimicrobial properties, (2) the compound may be detected *in situ*, when producing strains are present, (3) the biocontrol ability of mutants defective in compound of interest is reduced in the laboratory and in practical conditions, (4) the complementation of the mutant with DNA sequence restoring the synthesis of the compound of wild strain also restores biocontrol ability.

Since it is easier genetically manipulate prokaryotes, all of these approaches have been already adopted to determine the implication of metabolites produced by antagonistic bacteria. However, the steps involving molecular biology were rarely reported to analyze the implication of a compound produced by antagonistic yeast's due to their greater genetic complexity and the scarcity of molecular tools in comparison with bacterial agents. In this context, the modes of action potentially involved in the antagonistic activity of bacterial and yeast strains against post-harvest pathogens on fruits will be reviewed with respect to the methodology and the techniques used to their study. Special attention will focus on the mode of action of *Pichia anomala* strain K, an antagonistic yeast against *Botrytis cinerea* on apples. In this particular case, biochemical and molecular tools were used to determine the possible implication of 1,3-ß-glucanases in the antagonistic relationship (Jijakli *et al.*, 1998; Grevesse *et al.*, 1998).

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Biological Control of Fungal and Bacterial Plant Pathogens
IOBC wprs Bulletin Vol. 24 (3) 2001
p.319

Purification and properties of two chitinolytic enzymes of the biocontrol agent *Serratia plymuthica* C48

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The synthesis of chitinases is a common mechanism in antagonism of bacteria and fungi. The strain *Serratia plymuthica* C48 was previously isolated from the rhizosphere of oilseed rape as an antagonist to phytopathogenic fungi. *S. plymuthica* C48 was shown to produce and excrete a set of various chitinases. The aim of this study was to determine enzymatic properties of the endochitinase (E. C. 3.2.1.14) CHIT65 and the *N*-acetyl-β-1.4-D-hexoseaminidase (E. C. 3.2.1.52) CHIT100. For the endochitinase CHIT65 with an apparent molecular mass of 60.5 kD, a high homology was shown for chitinase A from *S. marcescens* by comparison of N-terminal amino acid sequences. CHIT65 displayed optimal activity at 55°C and pH 5.4. The activity was increased up to 250 % by 10 mM Ca^{2+}, Co^{2+} and Mn^{2+}. Inhibition up to 80 % by 10 mM Cu^{2+} was shown. CHIT100 appeared to be a monomeric enzyme with a molecular mass of 95.6 kD and an isoelectric point of pH 6.8. The enzyme displayed optimal activity at 43°C and pH 6.6. The activity was decreased by more than 90 % in the presence of 10 mM Co^{2+} and Cu^{2+}. Sodium chloride did not have any influence on enzymatic activity of both CHIT65 and CHIT100.

CHIT100 affected germination and germ tube elongation of spores of the phytopathogenic fungus *Botrytis cinerea*, causing inhibition of 28 % and 31.6 %, respectively, at 100 µg ml^{-1}. At the same concentration, CHIT65 was able to inhibit germination and germ tube elongation by 78 % and 63.9 %, respectively.

Biological Control of Fungal and Bacterial Plant Pathogens
IOBC wprs Bulletin Vol. 24 (3) 2001
pp.321 - 324

Isolation of genes from *Trichoderma harzianum* CECT 2413 expressed at different pHs

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Abstract: Using the differential display technique, we have isolated two genes from *Trichoderma harzianum* CECT 2413 which show changes in their expression levels when pH changes from 3 to 6. The first gene (*pst1)* shows high similarity with sugar transporters from yeasts, filamentous fungi and mammals. *pst1* is strongly derepressed by carbon starvation, and sligthly induced by pentoses, such as xylose. Its expression in yeasts does not complement any mutation in hexose transporters. The second gene (*pfp1*) seems to be homologous to L-amino acid oxidases of *Neurospora* and *Crotalus*, and other similar proteins. *pfp1* gene is highly expressed when using ammonium as a nitrogen source, and is strongly repressed when using leucine or tryptophane.

Key words: *Trichoderma harzianum*, pH, sugar transporter, L-amino acid oxidase

Introduction

pH is a key factor in understanding the relationships between organisms and their environment. Since many enzymatic activities, mainly extracellular are pH-dependent, it is not difficult to assume that the organisms have developed regulatory systems to avoid the expression of genes under conditions which are not favourable for the proteins they code for. Therefore pH may be used by the organisms to sense how advantageous the medium is for gene expression.

Aspergillus nidulans, which is able to grow over a wide range of pHs, from 2.5 to 9.0, has a well-known pH regulatory system: the transcription factor PacC, and *pal* genes act in the signal transduction pathway. Similar systems have also been found in other fungi and yeasts (Denison, 2000). However, little is known about the specific genes that are pH regulated. (Denison, 2000).

Some data indicates that *Trichoderma* has also a pH regulatory system. When *T. harzianum* is grown in glucose- and ammonium-containing cultures, pH decreases below 3. That is, when *Trichoderma* grows rapidly, pH decreases rapidly too. On the other hand, when *Trichoderma* grows with not easily assimilable carbon sources such as chitin or fungal cell walls, or it is carbon starved, pH is maintained around the initial pH values (between 5 and 6). This pH is, in fact, the optimum observed for several of the enzymes involved in mycoparasitism (Benítez *et al.* 1998). Moreover, *Trichoderma* secretes different extracellular proteins at different pHs, and the activity of an acidic protease is known to be pH regulated (Delgado-Jarana *et al.* 2000). Finally, *Trichoderma* genes involved in mycoparasitism show PacC putative boxes in their promoters. The question is whether or not *Trichoderma* senses not only fungal cell walls but also a nearly neutral pH which may indicate that the conditions are unfavourable, and it must secrete enzymes that degrade fungal cell wall. In order to answer these questions we have isolated several genes that are differentially expressed at pH 3

and 6, using a differential display technique. Their regulation patterns and probable functions are discussed below.

Material and methods

Culture conditions

T. harzianum CECT 2413 was inoculated in salt minimal medium supplemented with 2% glucose and 0.5% ammonium sulphate and grown for 40 h at 22°C and 180 rpm. Mycelium was then washed and transferred to the indicated media, where it was allowed to grow for 8 h. When buffered media were used, 0,2 M citrate buffer pH 3 or 0,2 M MES-KOH pH 6 were utilised. Mycelium was then filtered to obtain RNA by standard techniques.

Differential display

Mycelium grown for 40 h as indicated above was transferred to media buffered at pH 3 and pH 6 containing 2% or 0,2% glucose. DD-RT.PCR was performed as described by Liang & Pardee (1998). 0,2 µg RNA were subjected to reverse transcription using a set of three different anchored oligo-dT primers (GenHunter). Each of the three RT obtained from each RNA pool, was then subjected to a PCR, using the oligo-dT and one arbitrary 13-mer from a set of eight different primers (GenHunter). α-[^{33}P]-dATP was used in order to label the PCR products. The PCR was carried out as follows: 94°C for 30 s, 40°C for 2 min, 72°C for 30 s for 40 cycles, and 72°C for 5 min. Each RNA yielded 24 PCR. Each PCR was subjected to a 6 % denaturing polyacrylamide gel in TBE buffer in a Genomyx LR equipment for 3.5 h (Beckman). The dried gel was exposed to a Biomax MR film for 1 day. When the film was developed, it was oriented to the dried gel, and the differentially expressed bands were isolated. Each band was eluted in 100 µl of TE buffer at 37 °C for 30 min and was amplified using the corresponding oligos. This fragment was used to screen a cDNA library (λZAPII) in order to isolate the complete cDNA.

Results and discussion

In order to isolate genes that showed a differential expression pattern at pH 3 and 6, we carried out a differential display technique described in the materials and methods section. The isolated fragments were then used as probes to screen a cDNA library. Complete cDNAs were sequenced.

Isolation of a putative sugar transporter

The first clone analysed was highly expressed only at low glucose concentrations, but its expression was lower at pH 6 than at pH 3 (Fig. 1A). Its sequence showed high similarity with a fungal sugar transporter and therefore we called it *p*utative *s*ugar *t*ransporter *1* (*pst1*). Like other transporters described, *pst1* showed a typical structure of twelve transmembranal domains, with a large loop between domains I and II, another loop between domains VI and VII, and a large carboxy-terminal end. However, this end was not as large to consider *pst1* as a gene homologous to yeast *snf3* or *Neurospora crassa rco-3*, which are glucose sensor genes.

pst1 was also subjected to carbon catabolite repression (Fig. 1B). It was not expressed when 2% glucose was present in the cultures, but was partially derepressed when glycerol was used as a carbon source. In carbon starvation conditions it was highly expressed. When combining different sugars with glycerol the expression level was not elevated, except for arabinose and xylose. Thus, pentoses seemed to induce *pst1* expression.

We tried to complement sugar transport mutations in yeasts. In order to do so we utilised strain RE700A (*hxt1-7* null) which was not able to grow in glucose-containing medium, and strain EBY.VW4000 (*hxt 1-17 null*, *Δgal2*, *Δstl1*, *Δagt1*, *Δmph2*, *Δmph3*) which did not grow

in hexoses used as a sole carbon source (Wieczorke *et al.*, 1999). However, expression of *pst1* was not able to restore growth in any of the carbon sources tested (glucose, galactose, arabinose, xylose, fructose, mannose and raffinose, all of them at high or low concentration). Such lack of phenotype has already been described and was probably due to a lack of assembly between the permeases and the cell membrane. We are at present carrying out measurements of transport of hexoses and pentoses in a strain of *T. harzianum* which overexpresses the *pst1* gene in order to determine the function of Pst1.

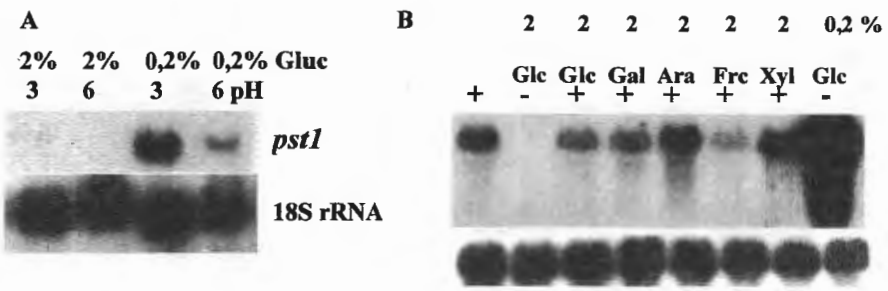

Figure 1. Northern-blot experiments using the putative sugar transporter 1 cDNA (*pst1*) as a probe. +/- indicates the addition or not of 2% glycerol to the cultures.

Isolation of a putative L-amino acid oxidase

The second clone analysed showed higher expression levels at pH 6 than at pH 3, and was not subjected to carbon catabolite repression (Fig. 2A). It shared high similarity with several proteins: i) with L-amino acid oxidases present (LAAO) in snake venoms (Suhr & Kim, 1996) and in *N. crassa* genome (Niederman & Lerch, 1990); ii) with an interleukin-induced protein, related to inflammatory process, and iii) with a tryptophane-2-monooxigenase from *Agrobacterium*. All of them are flavoproteins. For these reasons, we called our newly isolated gene *p*utative *f*lavo*p*rotein (*pfp1*). The putative protein deduced from the sequence showed a very similar amino acid content to that of the L-amino acid oxidases of *N. crassa* and *T. viride* (Niederman & Lerch, 1990, Kusakabe *et al.*, 1980). LAAOs oxidate mainly hydrophobic amino acids to obtain ammonium and H_2O_2, and are being currently studied as mitotic inhibitors.

Surprisingly, although the LAAO analysed (Sikora & Marzluf, 1982) was induced by the presence of L- and D-amino acids in culture medium, the *pfp1* gene was strongly repressed when L-leucine was used as the sole nitrogen source (Fig. 2B). However, its expression was induced when using ammonium or amino acids such as L-glutamine. *pfp1* was not regulated by carbon sources, and the pH did not relieve amino acid repression. One possibility is that *pfp1* may be the enzyme that catalyses the reverse reaction: consumption of ammonia to yield amino acids. The decrease of ammonia in the culture would lead to a decrease of pH. This can be the reason why *pfp1* is repressed by acid pH and by amino acids, and induced by ammonium. The use of LAAO has been proposed as an useful way to alter environmental pH to values appropiate for the growth of an organism (St. Leger *et al.*, 1999).

In order to check its activity we overproduced *pfp1* as a GST-fused recombinant protein in *Escherichia coli* cells. However, the scarce stability of Pao1 after removal of the GST tag impeded further purification. *pfp1* could not be detected after over-expression in *S. cerevisiae*

and we do not yet know if the protein is degraded because of its putative toxicity (growth rate is lower than that of the GST expressing control). We also over-expressed *pfp1* in *T. harzianum* and are at present carrying out experiments to detect changes in the internal pool of amino acids and in the external pH.

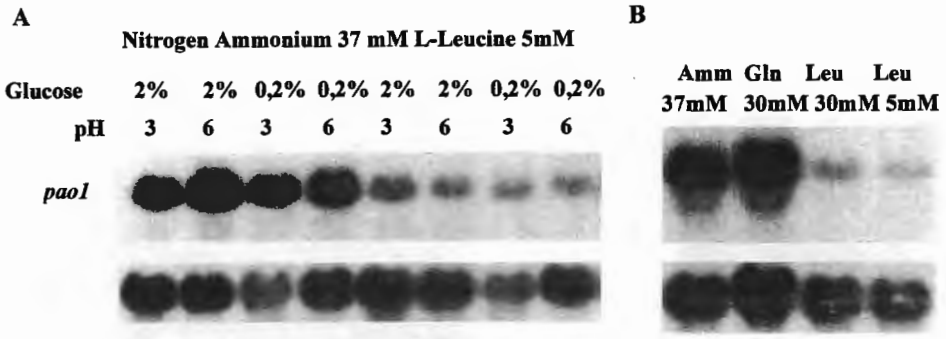

Figure 2. Northern blots experiments using the putative amino acid oxidase 1 cDNA (*pfp1*) as a probe.

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Biological Control of Fungal and Bacterial Plant Pathogens
IOBC wprs Bulletin Vol. 24 (3) 2001
pp.325 - 328

β-1,6-glucanase isozyme system in *Trichoderma harzianum* CECT 2413. Isolation of a new component

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Abstract: Hydrolytic enzymes secreted by *Trichoderma harzianum* seem to play an important role in mycoparasitism, one of the main mechanisms involved in antagonism of *Trichoderma* strains against other filamentous fungi. Among these enzymes, β-1,6-glucanases can be involved in the degradation of qualitatively important polymers maintaining the fungal cell wall structure. To date, only two endo-β-1,6-glucanases have been described in *Trichoderma harzianum* both belonging to strain CECT 2413 (BGN16.2 and BGN16.2). We have detected a third isozyme with different properties and regulation compared to two previously described β-1,6-glucanases. This new isozyme is produced in the presence of cell walls in the culture media but it is not detected when chitin is added. The protein has been purified and characterized, differing from both BGN16.1 and BGN16.2.

Keywords: *Trichoderma*, lytic enzymes, CWDEs, β-1,6-glucanase, isoenzymes

Introduction

Trichoderma harzianum is a filamentous fungus described as a potential biocontrol agent against phytopathogenic fungi. The antagonism displayed by *T. harzianum* is explained by different mechanisms, of which, one of the most interesting being mycoparasitism, a process involving degradation of the pathogen cell wall and penetration of the host hyphae as initial steps (Papavizas, 1985). *Trichoderma* strains produce a wide variety of extracellular enzymes, including chitinases, glucanases and proteases that are presumably involved in cell wall degradation. Proteins with β-1,6-glucanase activity could be important components of the cell-wall-degrading enzyme complex as their substrate (β-1,6-glucan) has been identified as a key component in the fungal cell wall architecture. In fungal model systems (*Saccharomyces cerevisiae*), β-1,6-glucan is responsible for binding of the two main components of fungal cell walls, chitin and β-1,3-glucan, and seems to be involved in linkage of these two components to the protein layer of the wall (Kapteyn *et al.*, 1997).

Previous studies in our group led to the purification of two β-1,6-glucanases, BGN16.1 and BGN16.2 (De la Cruz *et al.*, 1995; 1999) produced by *T. harzianum* CECT 2413 under chitin-induction conditions. BGN16.2 is the only β-1,6-glucanase cloned to date (Lora *et al.*, 1995). We have purified and cloned a novel acidic β-1,6-glucanase from supernatants of *Trichoderma* growing on *Botrytis cinerea* cell walls. This isozyme is not expressed under chitin-induction conditions, clearly differentiating it from previously purified BGN16.1 and BGN16.2.

Materials and methods

Microorganisms

Two different fungi have been used in this work: *T. harzianum* CECT 2413, which is the microorganism under study, and *Botrytis cinerea* isolated from strawberry crops. The latter being used as a source of fungal cell walls for induction.

Biochemical characterization

β-1,6-glucanase activity was determined by measuring the amount of reducing sugars released from pustulan. The standard assay included 0.2 ml of 5mg /ml of pustulan in 50 mM sodium acetate buffer, pH 5.5 with 0.05 ml of the enzyme solution, appropriately diluted. Reaction mixtures were incubated at 37°C for 30 min, terminated by boiling 10 min and assayed for reducing sugars content by the Somogyi and Nelson procedure, using glucose as a standard. One unit of β-1,6-glucanase activity was defined as the amount of enzyme that releases 1 μmol of reducing sugar equivalents, expressed as glucose, per min under the standard assay conditions.

Activity at optimum temperature was determined by performing the standard assay within the temperature range of 25-70°C. Thermal stability was determined by incubating the enzyme at temperatures from 30 to 70°C in 50 mM sodium acetate for 30 min and then measuring the remaining activity by adding pustulan as a substrate, followed by incubation at standard assay conditions. Inactivation temperature was defined as the temperature causing a reduction of 50% of the specific activity.

The optimum pH was determined using the standard assay with modifications in the reaction buffer: for pHs from 3 to 5, citrate-acetic acid buffer was used, for pHs from 6 to 8, phosphate buffer was used and finally, Tris-HCl buffer was used for pH 9. In all cases the buffer concentration was 50 mM.

Michaelis-Menten constants were determined with the Lineweaver-Burk representation using data from pustulan hydrolysis at different times and pustulan concentrations in the range of 0.5-10 mg/ml.

Detection of β-1,6-glucanase activity in agar replicas of SDS-PAGE gels, as well as specific β-1,6-glucanase activity staining after isoelectrofocusing, were done as described by Soler *et al.*(1999).

Purification steps

Production of extracellular enzymes was done using a two-step method. Firstly, *Trichoderma* was grown in Czapek minimal medium supplemented with 2% glucose for 48 hours. Mycelia was filtered, washed and transferred to the induction media containing 0.5% cell walls in minimal Czapek medium.

Three consecutive steps were used: pustulan adsorption-digestion, cromatofocussing in Mono P 5/20 from Pharmacia and gel filtration in Sephacryl S 200 HR (Pharmacia). Chromatographic conditions were similar to those previously described for BGN16.1 and BGN16.2 (De la Cruz *et al.*, 1995; 1999).

Results and discussion

The aim of our study was to detect new cell-wall-degrading-enzymes (CWDEs) produced by *Trichoderma* in induction conditions using fungal cell walls as the only carbon source. A new isozyme with β-1,6-glucanase activity was differentially produced under these conditions and was detected by specific activity staining after native isoelectrofocussing (Fig. 1). The new isozyme was not detected in culture media supplemented with chitin, a compound widely used as an inducer for the production and purification of *T. harzianum* CWDEs.

We attempted to purify this enzyme using a similar approach to that already used successfully in purifying BGN16.1 and BGN16.2. The three steps carried out were pustulan-adsorption-digestion (which is the key step for successful purification), chromatofocussing and finally, gel filtration in Sephacryl S 200 HR. The enzyme was purified to homogeneity as indicated by SDS-PAGE. Each purification step was followed by activity staining in agarose replicas containing pustulan after SDS PAGE and renaturing as described by Soler *et al.* (1999).

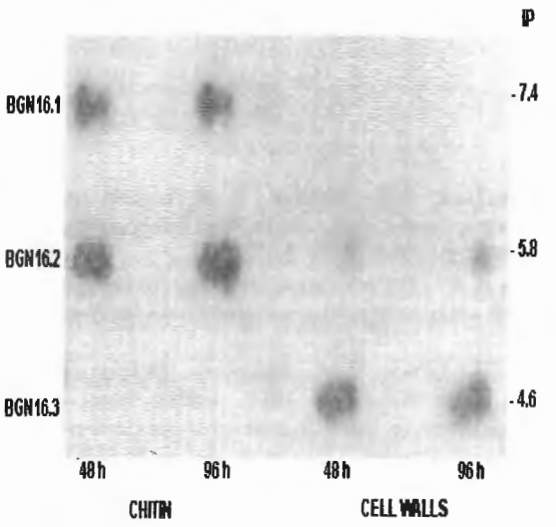

Figure 1. Specific β-1,6-glucanase activity staining after isoelectrofocussing. Samples were extracellular enzymes produced by *T. harzianum* CECT 2413 under two different induction conditions (chitin and cell walls as carbon sources), at two different times (48 and 96 h).

In order to study regulation of the enzyme, we used different carbon sources to culture *Trichoderma harzianum* CECT 2413. The acidic β-1,6-glucanase could be clearly detected in supernatants when the fungus was grown using cell walls or pustulan as carbon sources. However, it could not be detected when glucose, glycerol or chitin were used as carbon sources. The enzyme was also present in carbon-source-depletion conditions (starvation conditions), although the amount of enzyme was lower than in *Botrytis*-cell-wall inductions.

The molecular weight found for this acidic isozyme was 48 KDa and the isoelectric point determined by isoelectrofocussing was 4.6. A Km of 1.1 mg pustulan was determined. Both optimal and inactivation temperatures were 50ºC, suggesting protection of the enzyme by the substrate, as previously described for BGN16.2 (De la Cruz *et al.*, 1995).

An endohydrolytic mode of action was found when products from pustulan enzymatic degradation were applied to HPLC Aminex column (BioRad) for their identification by comparison to cellulose oligomers (2 to 5 glucose residues). The 2-glucose dimer was the most abundant product of pustulan hydrolysis.

Sequences of amino terminal and an internal peptide were obtained from purified protein samples. The sequences (Fig. 2) did not show any significant homology with that of

BGN16.2, the only one available coding for an endo-β-1,6-glucanase, or with any other hydrolytic enzyme sequence available in protein data bases.

Amino terminal: A A G A Q A Y A S N Q A G N

Internal peptide: G L N S N L Q I F G S P W

Figure 2. Amino terminal and amino peptide sequences obtained from the purified β-1,6-glucanase.

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Biological Control of Fungal and Bacterial Plant Pathogens
IOBC wprs Bulletin Vol. 24 (3) 2001
pp.329 - 332

Chitinases produced by the biocontrol agents *Verticillium suchlasporium* and *Verticillium chlamydosporium*

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Abstract: Nematophagous fungi *Verticillium chlamydosporium* and *V. suchlasporium* growing on colloidal chitin produced increasing chitinolytic activity over time. Maximum activity was found 21-28 days after inoculation. Both fungi displayed higher chitinolytic activity in cultures supplemented with yeast extract. Mayor endochitinases with optimum pH of 5.5 for both fungi were separated by affinity chromatography on a macroporous crosslinked chitin matrix.

Key words: Chitinases, nematophagous fungi, plant parasitic nematodes, biocontrol, affinity chromatography

Introduction

Plant parasitic nematodes are mainly controlled by chemical nematicides. Some of these have been withdrawn from the market for health or environmental reasons, including methyl-bromide, which will be banned from the market in the next years in different European countries. Nematophagous fungi are of great interest as possible biocontrol agents of plant-pathogenic nematodes and together with other biocontrol agents are good candidates to replace chemical products. Egg-parasitic fungi, such as *Verticillium suchlasporium* and *V. chlamydosporium*, must penetrate the nematode eggshell during infection (Lysek & Krajci, 1987). The nematode eggshell is formed of several layers. Of these, a chitinous layer, comprised of a protein matrix embedding chitin microfibrils, is probably the major barrier to egg infection. Evidence of nematode egg shell-degrading enzyme production by fungal parasites at the point of penetration was observed by ultrastructural and immunological studies (Lopez-Llorca & Robertson, 1992 a and b). An extracellular protease from the egg-parasite *V. suchlasporium* was immunolocalised during egg penetration by the fungus. Chitinase, proteases and other extracellular enzymes (lipases, amylases or pectinases) have been detected *in vitro* from fungal egg parasites (Dackman *et al.*, 1989). In the present paper, we describe the production of chitinases from *V. chlamydosporium* and *V. suchlasporium* and characterise the major chitinases produced.

Material and Methods

Growth conditions and enzymes assays

Production of enzymes by *V. suchlasporium* and *V. chlamydosporium* was carried out in 1000 ml Erlenmeyer flasks containing 150 ml of growth medium (colloidal chitin 0.2% w/v, NaCl

(0.03%), $MgSO_4.7H_2O$ (0.03%), K_2HPO_4 (0.03%), $CaCl_2$ (0.06%), $FeSO_4.7H_2O$ (0.03%), yeast extract (0.02% if any). Colloidal chitin was prepared according to Chigaleichik (1976). Sterilization was done at 120° C for 20 min. Each flask was inoculated with 7 agar plugs (5 mm in diameter) taken from the edge of 2-week-old colony and incubated at 22° C in the dark without shaking. Protein production, chitinolytic and proteolytic activities were determined. For protein purification, 3-week-old cultures were filtrated through Whatman paper and Durapore membranes of 0.22 µm pore size. Culture supernatants were lyophilised and kept at -20^0 C. Protein concentration was determined according to Bradford (1976).

Chitinolytic activity was assayed as follows: one ml of culture supernatant, 0.3 ml of 1 M sodium acetate buffer pH 4.7 (SAB) and 0.2 ml of colloidal chitin was mixed and incubated at 40^0 C for 24 h. After centrifugation, 0.75 ml of the supernatant were mixed with 0.25 ml of 1% dinitrosalicylic acid in 0.7 M NaOH and 0.1 ml of 10 M NaOH. The mixture was heated for 5 min at 100^0 C. Absorbance was measured at 582nm. Specific activity was defined as quantity of N-acetylglucosamine (µ mol) released by 1ml of enzyme solution for one hour. N-Acetyl-1, 4-β-D-glucosaminidase activity was measured as the release of p-nitrophenol (pNP) from p-nitrophenyl-N-acetyl-D-glucosaminide (NG) as follows: mixture of sample solution (from 1 to 25µl), 0.2 ml of NG 1mg/ml H_20 and 1 ml of 0.1 M SAB was heated at 40° C for 6 h. 0.3 ml of 0.125 M sodium tetraborate-NaOH buffer pH 10.7 was added to the sample. Absorbance at 400 nm was measured. Specific activity was defined as µ mol of pNP/h ml of enzyme solution. Thermal dependence of chitinolytic activity was assayed in 0.1 M SAB.

Affinity Chromatography

Proteins were precipitated with 40% (w/v) ammonium sulphate (AS); pellets were dissolved in 1 ml 0.1M sodium phosphate buffer pH 7.7 (SPB) and 100 mg of AS were added (sample buffer). One ml of the sample was applied onto a 2-ml column with macroporous cross-linked chitin (MPC-chitin) equilibrated with sample buffer. The column was washed with 10 ml of sample buffer, 30 ml of SPB and 6 ml of 0.2 M sodium carbonate buffer pH 9.1 (SCB). Chitinase and N-Acetyl-β-D-glucosaminidase activities were determined in 1ml fractions. Endochitinolytic activity was determined by a viscometric method with chitosan as a substrate. Chitosan with a degree of acetylation of 20% and MW of 390 kDa was a product of BioChit (Moscow).

Results and discussion

V. suchlasporium and *V. chlamydosporium* growing in the presence of 0.2% of colloidal chitin produced a complex system of hydrolytic enzymes. Several proteases, chitinases, 1,3-β-glucosidases and N-acetyl-1,4-β-D-glucosaminidase were detected in the culture supernatants (data not shown). Protein production increased over time for both fungi and peaked after 3 weeks of growth. (Fig. 1) Chitinolytic activity in the cultures was detected after one week of incubation and reached a maximum after 3 weeks for both fungi (Fig. 2) Maximum activity was observed at 37^0 C, level of activity decreased above 40^0 C (Fig. 3).

Nematophagous fungi must break down eggshell in order to penetrate the host; proteases and chitinases can be the key factors for fungal penetration. When *Globodera pallida* eggs were incubated with both enzymes the eggs-shell showed scars and peeling (results not shown). These results supported those of Lopez-Llorca & Robertson (1992 b) for *V. suchlasporium*.

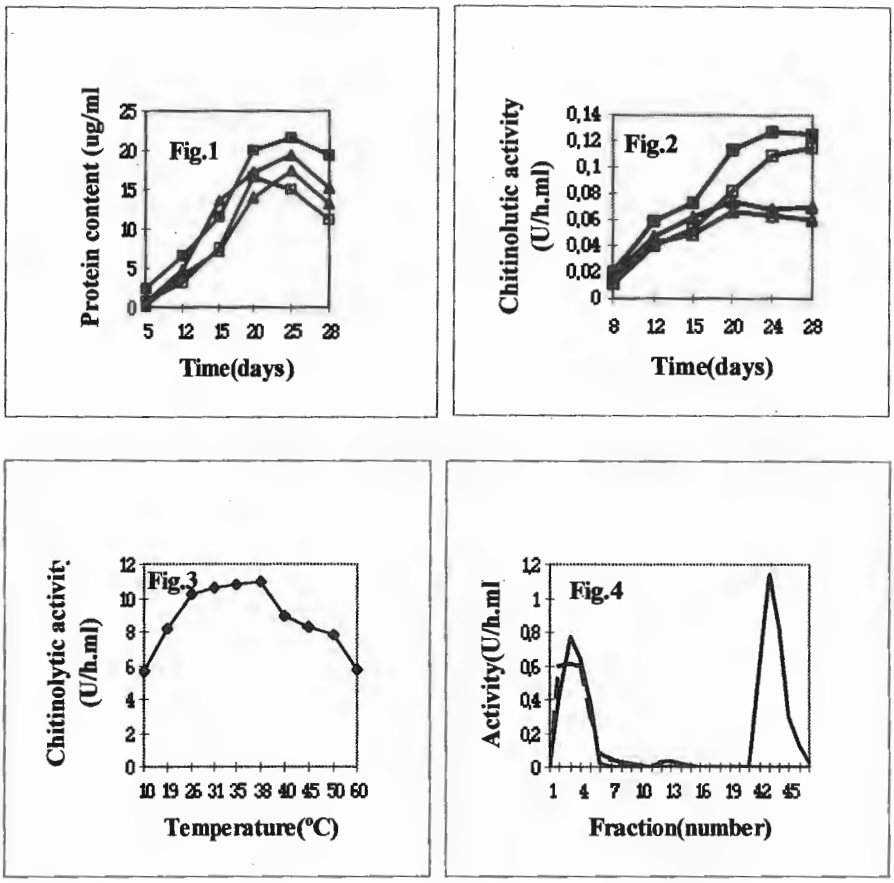

Figure 1. Protein content in culture filtrates. Figure 2. Chitinolytic activity in culture filtrates. Figures 1-2. *V. suchlasporium* with (–■–) and without (–□–) yeast extract; *V. chlamydosporium* with (–▲–) and without (–Δ–) yeast extract. Figure 3. Temperature dependence of chitinolytic activity of *V. suchlasporium*. Figure 4. Elution profile of chitinolytic (—) and N-acetyl-1,4 –β-D-glucosaminidase (– –) activities. Fractions eluted with (fraction number/eluent): 1 11/ 10% AS in 0.1M SPB; 12 40/ 0.1M SPB; 41 47/ 0.2M SCB.

Chitinolytic enzymes present in the culture supernatant were precipitated by 40% (w/v) of AS. Proteins were loaded on the column and 50-60 % of initial activity was retained. The fractions contained unbounded chitinases of low affinity towards MPC-Chitin, proteases and most of N-acetyl-1,4-β-D-glucosaminidase. Thorough washing with 0.1 M SPB eliminated the rest of N-acetyl-1, 4-β-D-glucosaminidase. A peak containing 50-60% of initial chitinolytic activity was eluted with 0.2 M SCB pH 9.1 (Fig 4). SDS-PAGE showed a 43 kDa single protein band (results not shown). This data was consistent with a major role of hydrophobicity in affinity interaction among chitinases and MPC-Chitin, (Tikhonov *et al.*, 1998). No elution of C43 by SPB and elution of this enzyme by SCB of higher pH, confirmed

an input of another type of affinity interaction (probably hydrogen binding) among the sorbent and the protein. Chromatographically, chitinases of *V. suchlasporium* and *V. chlamydosporium* have shown identical behaviour. SDS-PAGE and pH activity dependence of C43s revealed their equality in MW and pH 5.2-5.7 optima. Purified chitinase lost its activity after 30 min at 80^0C.

Endo-mode of C43s action was demonstrated by viscometric assay using soluble chitosan of high molecular mass as a substrate. Relative viscosity of chitosan solution decreased 7.6-fold in 30 min. No detectable level of reducing saccharides was found during the time of chitosan splitting.

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Biological Control of Fungal and Bacterial Plant Pathogens
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Molecular genetics and pathogenicity of biocontrol and mushroom *Trichoderma*

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Abstract: Molecular genetic analysis and pathogenicity trials were carried out on isolates of biocontrol and mushroom isolates of *Trichoderma harzianum*. Both randomly amplified polymorphic DNA PCR and sequence specific PCR analyses discriminated the biocontrol isolates from the highly pathogenic *T. harzianum* biotypes 2 and 4 (Th4) on cultivated mushrooms (*Agaricus bisporus*). We propose that our sequence specific PCR primers (ThF/ThR) might have utility in predicting the pathogenicity of biocontrol *T. harzianum* on *A. bisporus*. Phylogenetic analysis based on a ~441 bp sequence in beta tubulin gene 1 suggested that biocontrol and pathogenic isolates share a recent common ancestry, but have diverged considerably. An actual determination of pathogenicity on mushrooms substantiated the molecular data; both a commercial biocontrol *T. harzianum* (*TRICHODEX* T39) and a related *T. harzianum* biotype 1 from mushrooms were not pathogenic on *A. bisporus*, whereas Th4 caused extensive crop loss.

Key words: mushroom, *Agaricus bisporus*, green mold, beta tubulin gene 1, molecular genetics

Introduction

The recent global escalation of Trichoderma green mould to epidemic proportions on the cultivated button mushroom, *Agaricus bisporus*, has heightened concern for the nontargeted consequences of deploying biocontrol isolates of *Trichoderma* on plants. *T. harzianum* biotypes 2 (Th2) and 4 (Th4) are newly recognized mushroom pathogens causing devastating crop loss throughout Europe and North America, respectively (Castle *et al.*, 1998; Chen *et al.*, 1999a, 1999b; Muthumeenakshi *et al.*, 1994; Ospina-Giraldo *et al.*, 1998, 1999). Numerous nonpathogenic or opportunistic *Trichoderma* spp. also inhabit the cultivated mushroom ecosystem, including *T. harzianum* biotype 1 (Th1) and *T. atroviride* [formerly *T. harzianum* biotype 3 (Th3)]. Herein, we have conducted molecular genetic and pathological analyses on biocontrol and mushroom *T. harzianum*, so as to bring into focus the actual threat posed by biocontrol genotypes to commercial mushroom production.

Materials and methods

Fungal cultures

Biocontrol and mushroom *T. harzianum* used in this study were described previously (Chen *et al.*, 1999b). Briefly, biocontrol isolates 209, 210, 211, and 212, originating in England, U.S./Colombia, U.S., and U.S., respectively, were obtained from the American Type Culture Collection (ATCC). Biocontrol isolate 201 is a commercial formulation (TRICHODEX T39, Makhteshim Agan Inc.) originating in Israel. Isolate T27 and isolates T97 and T99 of *T. harzianum* were from D. A. Seaby (Department of Agriculture of Northern Ireland, U.K.) and G. J. Samuels (USDA, ARS, U.S.), respectively. *Trichoderma longibrachiatum* T46 (#18648), *T. reesei* T208 (#56765), and *T. pseudokoningii* T48 (#64400) were obtained from

the ATCC. *Trichoderma citrinoviride* T193, *T. koningii* T197, and *T. atroviride* T195 were kindly supplied by D. L. Rinker (University of Guelph, Ontario, Canada).

PCR and Beta tubulin gene 1 sequence analysis

RAPD PCR amplification using decamer primers (University of British Columbia, Vancouver, Canada) was carried out as described by Chen *et al.* (1999a). Sequence specific PCR was done with primer pair ThF/ThR targeting a 444 bp sequence unique to the Th2 and Th4 genomes (Chen at al., 1999b). Primers BTF (5' GTTGGTTCTGCCTTCTGG 3') and BTR (5' AACAGCTGGCCAAAGGG G 3') were tailored using the Amplify Program (University of Wisconsin, Madison, WI) and the published sequence data for beta tubulin gene 1 in *T. viride* (Goldman *et al.*, 1993). These primers defined a 498 bp PCR product spanning two introns, corresponding to nucleotides 678 to 1175. Primers were synthesized by The Pennsylvania State University (PSU) Nucleic Acid Facility (NAF).

PCR was carried out according to Chen *et al.* (1999b). DNA products were subjected to electrophoresis in low melting agarose gel and purified using the Wizard PCR Preps Kit (Promega, Madison, WI). Products were sequenced bidirectionally by the PSU NAF. Sequences (~441 bp long) were aligned with the Clustal W algorithm (Lasergene software, DNAStar). Neighbor joining (NJ) trees (Saitou & Nei, 1987) were generated by the computer program MEGA (Kumar *et al.*, 1993) and supported by bootstrapping (Felsentein, 1985).

Pathogenicity trials

Cropping trials were done at the Mushroom Research Center, PSU as before (Romaine & Schlagnhaufer, 1992) except that 1 ml of water containing *Trichoderma* spores was applied to 150 g of spawn, which was then mixed with 23 kg compost and packed into a 0.6 m^2 tray.

Results and discussion

RAPD PCR

Figure 1 compares the RAPD profiles obtained with primer 232 for biocontrol isolates 201, 210, 211, and 212 of *T. harzianum*, the three mushroom biotypes, and *T. atroviride*. Biocontrol isolates had profiles that were distinctly different from those of pathogenic Th2 and Th4. The two isolates of Th1, biocontrol isolate 201 and, to a lesser extent, isolate 211 shared a similar RAPD profile.

Sequence specific PCR

PCR amplification using primer pair ThF/ThR with biocontrol isolates of *T. harzianum* failed to generate the 444 bp DNA product that is diagnostic for pathogenic Th2 and Th4 (Chen *et al.*, 1999b). This finding corroborates the RAPD data indicating that biocontrol and mushroom pathogenic *T. harzianum* are genetically disparate. We propose that primer pair ThF/ThR may be a useful aid in typing biocontrol *T. harzianum* for pathogenicity on *Agaricus* mushrooms.

Beta tubulin gene 1 sequence analysis

Partial sequence analysis of beta tubulin gene 1 revealed that biocontrol isolates nested with pathogenic Th2 and Th4, Th1, and other *T. harzianum*. However, biocontrol genotypes were more closely related to Th1 with an ~2% nucleotide difference (nd) than to the pathogenic genotypes (~5% nd), which suggests that biocontrol and pathogenic genotypes share a recent common ancestry, but have diverged considerably. We also found that Th3 clustered with *T. viride* and *T. atroviride*. Our findings, based on beta tubulin gene sequence data, agree with the relationships inferred by ribosomal DNA (rDNA) sequence analysis (Castle *et al.*, 1998; Grondona *et al.*, 1997; Ospina-Giraldo *et al.*, 1998, 1999).

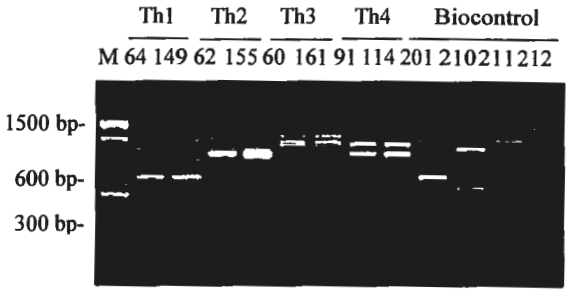

Figure 1. RAPD PCR profiles generated with primer 232 for two isolates each of Th1, Th2, Th3 (= *T. atroviride*), and Th4, and biocontrol isolates 201 (TRICHODEX T39) and 210 to 212. M, DNA markers (sizes appear on left).

Th2 and Th4 were found to be closely related but distinct genotypes, separated by a 0.2% nd within the partial sequence of beta tubulin gene 1. No variation in nucleotide sequence was detected among isolates within each pathogenic genotype. The close genetic relationship between these two genotypes and their clonal nature are also supported by RAPD and biotype specific PCR analyses as well as rDNA analysis (Castle *et al.*, 1998; Chen *et al.*, 1999a, 1999b: Ospina-Giraldo *et al.*, 1998).

Table 1. Effect of several *Trichoderma* isolates on mushroom production by *Agaricus bisporus* [a].

Treatment	Inoculum rate (no. viable spores)	Mushroom Yield (kg/m^2)
Control	0	14.59 a
Th1-64	3,900	14.30 a
	39,000	14.16 a
TRICHODEX T39	5,900	15.02 a
	59,000	14.83 a
Th4-114	5,500	3.07 b
	55,000	2.06 b

[a] Water (1 ml) containing the number of spores indicated was applied to 150 g of mushroom spawn, which was then mixed with 23 kg of compost and packed into a 0.6 m^2 tray. Means followed by the same letter are not significantly different by the Waller Duncan K ratio *t* test, $P \leq 0.0001$. Each mean is based on 8 replicate trays for two experiments.

Pathogenicity trials

In a replicated cropping trial, inoculation of *A. bisporus* spawn with spore suspensions of biocontrol isolate 201 (*TRICHODEX* T39) or Th1 (isolate 64) had no measurable effect on mushroom yield (Table 1). Green mould was not visible in the compost or peat casing layer following inoculation with these isolates. In contrast, inoculation with Th4 (isolate 114) resulted in an extensive development of green mould throughout the compost and casing, and

an 80 to 85% crop loss. Thus, a direct measure of pathogenicity on mushrooms substantiated the molecular data; a biocontrol *T. harzianum* and closely related Th1, which is commonplace in the cultivated mushroom ecosystem, were not pathogenic on *A. bisporus*.

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Biological Control of Fungal and Bacterial Plant Pathogens
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Extracellular enzyme profiles of mycoparasitic *Trichoderma* strains

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Abstract: This work was undertaken to investigate and compare the extracellular enzyme profiles in three mycoparasitic *Trichoderma* strains belonging to different species groups (*T. aureoviride*, *T. harzianum* and *T. viride*). The lyophilized culture filtrates were separated by Sephadex G150 gel filtration chromatography, and the extracellular β-glucosidase (EC 3.2.1.21), cellobiohydrolase (EC 3.2.1.91), endo-1,4-β-D-glucanases (EC 3.2.1.4), β-xylosidase (EC 3.2.1.37), endoxylanase (EC 3.2.1.8), trypsin-like and chymotrypsin-like protease (EC 3.4.21.1; EC 3.4.21.4) and β-1,4-*N*-acetyl-glucosaminidase activities (EC 3.2.1.30) were detected in the fractions by the use of natural and chromogenic substrates. Based on the results, izoenzymes belonging to all investigated enzyme-systems could be detected in the case of each investigated strain.

Key words: *Trichoderma*, gel filtration chromatography, extracellular enzymes

Introduction

Strains belonging to the genus *Trichoderma* are well known to produce many different extracellular enzymes. Some of these enzymes, like cellulases and xylanases take part in the decomposition of plant litter and are responsible for the survival and competitive abilities of the strains in the (Papavizas, 1985). Others, e.g. β-1,3-glucanases, chitinases and proteases play a role in mycoparasitism, during the degradation of fungal cell walls (Elad *et al.*, 1982). The quality and amount of these enzymes are important in the biofungicide efficacy, as they may determine the antagonistic properties of the strains.

The aim of this work was to examine the extracellular enzyme systems of strains *T. aureoviride* T122, *T. harzianum* T66 and *T. viride* T228, all of them effective antagonists of phytopathogenic fungi.

Materials and methods

Strains and culture media

Trichoderma strains were isolated by Manczinger *et al.* from Hungarian forest soil. These are cold tolerant strains antagonistic to *Pythium, Fusarium* and *Rhizoctonia* species (Antal *et al.*, 2000). For the induction of β-glucosidase, cellobiohydrolase and endo-1,4-β-D-glucanase; β-xylosidase and endoxylanase; trypsin-like and chymotrypsin-like protease and β-1,4-*N*- acetyl-glucosaminidase activities, microcrystalline cellulose; xylan; skim milk powder and colloidal chitin was incorporated as inducers into 20 ml liquid media (0.5% KH_2PO_4, 0.1% $NaNO_3$, 0.1% $MgSO_4 \cdot 7H_2O$), respectively. Conidia of the strains were inoculated into the appropriate inductive liquid media. The incubation was carried out in 50 ml Erlenmeyer flasks on a shaker at 150 rpm. After culturing at 25°C for 4 days, the mycelial pellets were removed by centrifugation and the supernatants were vacuum concentrated.

Gel filtration chromatography and measurement of extracellular enzyme activities
Amounts of 1.5 ml from the samples were fractionated on a 0.9 x 60 cm Sephadex G-150 column (Pharmacia). The column was equilibrated and eluted with a solution containing 0.1% NaCl and 0.1% $MgCl_2$, and 0.5 ml fractions were collected from all samples. β-Glucosidase, cellobiohydrolase, β-xylosidase, *N*-acetylglucosaminidase, trypsin-like and chymotrypsin-like protease activities were assayed using pNP-β-D-glucopyranoside, pNP-β-D-cellobioside, pNP-β-D-xylopiranoside, pNP-*N*-acetyl-β-D-glucosaminide, *N*-benzoyl-Phe-Val-Arg-pNA and *N*-Suc-Ala-Ala-Pro-Phe-pNA (Sigma) substrates, respectively as follows: 100 µl of each fraction was incubated with 100 µl substrate (200 µg/ml) at 25°C for 1 h. The reactions were terminated by adding 50 µl 10% Na_2CO_3, and optical density of the samples was determined with a Labsystems Uniskan II microtiter plate spectrophotometer at a wavelength of 405 nm. Endo-1,4-β-D-glucanase and endoxylanase activities were assayed with dinitrosalicylic acid reagent (Miller, 1959).

Results and discussion

The β-glucosidase profile of the examined strains (Fig. 1A) proved to be similar, at least two isoenzymes were detectable in all of the strains corresponding with the data in literature: two β-glucosidases are described in *T. pseudokoningii* (Dong *et al.*, 1997). However, based on the β-glucosidase profile of strain *T. aureoviride* T122, the presence of further isoenzymes is likely. Cellobiohydrolase activities (Fig. 1B) are probably composed of two isoenzymes, they can be easily distinguished in the case of strain *T. viride* T228. CBHI was found at 54 kDa in *T. koningii* (Wey *et al.*, 1994) with CBHII at 49.6 kDa in a *T. viride* strain (Wang *et al.*, 1995). The endo-1,4-β-D-glucanase enzyme profiles of the strains were complex, possibly consisting of several isoenzymes (data not shown). Four and five endo-1,4-β-D-glucanases have been reported for *T. viride* and *T. pseudokoningii* cellulase systems, respectively (Ilmén *et al.*, 1997; Dong *et al.*, 1997). The β-xylosidase activities (Fig. 1C) were detected in the high molecular weight fractions. In *T. reesei*, one β-xylosidase was found which proved to be a rather large protein with molecular mass around 100 kDa (Poutanen & Puls, 1988). The endoxylanase activities were found in the low molecular weight fractions (data not shown), and the endoxylanase system proved to be complex. In a *T. reesei* strain, two endoxylanases 20 and 19 kDa in size, were observed by Tenkanen *et al.* (1992). The profiles of trypsin-like (Fig. 1D) and chymotrypsin-like (Fig. 1E) proteases were similar between the strains, both systems consisting of more isoenzymes. In case of trypsin-like protease, high molecular weight isoenzymes were also detected in the strain *T. viride* T228. Geremia *et al.* (1993) isolated and purified an extracellular alkaline protease from *T. harzianum* with supposed role in mycoparasitism, however, the extracellular proteolytic system of *Trichoderma* species is relatively unknown. The β-1,4-*N*-acetyl- glucosaminidase activities (Fig. 1F) were present in the low molecular weight fractions. In *T. harzianum* two β-1,4-N-acetyl-glucosaminidases were described, 73 and 102 kDa in size, respectively (Lorito *et al.*, 1994; Haran *et al.*, 1995).

The extracellular enzymes important for antagonism were produced by *Trichoderma* strains in a large amount under inductive conditions. In general, the number of cellobiohydrolase and β-1,4-*N*-acetyl-glucosaminidase izoenzymes was the lowest, while the enzyme profiles of endo-1,4-β-D-glucanases , endoxylanase, trypsin-like and chymotrypsin-like proteases were more complex, indicating the presence of several isoenzymes. The molecular weight of β-1,4-*N*-acetyl-glucosaminidases and endoxylanases was determined as the lowest, while in the high molecular weight fractions, β-glucosidase and β-xylosidase activities could be detected. Although some slight differences were detected in number and

molecular weight of the izoenzymes among the strains, their extracellular enzyme profiles seems to be rather similar by this method.

Since the antagonistic abilities of the biocontrol strains depend on synergism between different enzyme systems, further detailed comparisons of the extracellular enzyme profiles of different *Trichoderma* strains effective against plant pathogens may be useful in the research into the factors underlying their modes of action.

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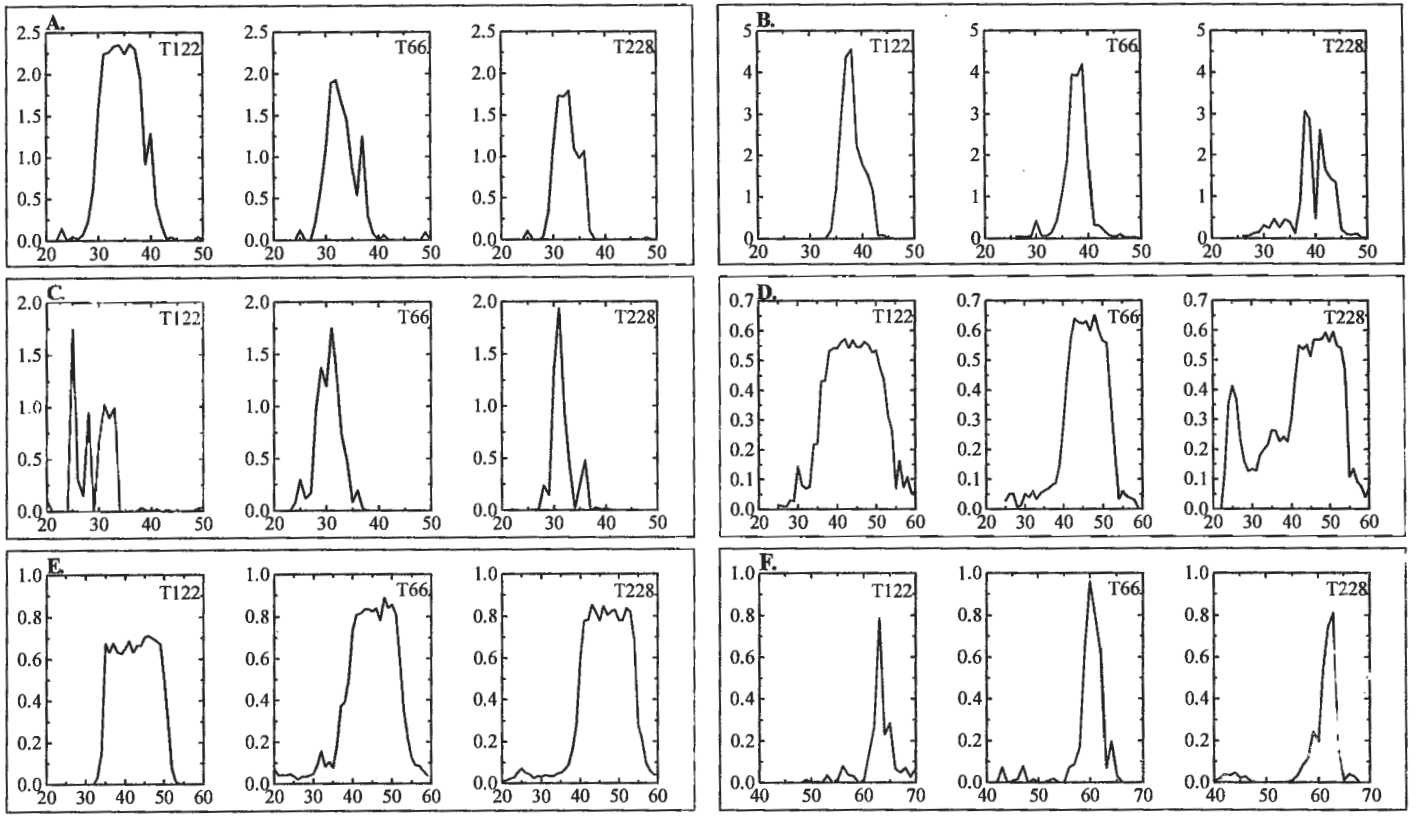

Figure 1. Extracellular enzyme profiles of *Trichoderma* strains. A. β-glucosidase, B. cellobiohydrolase, C. β-xylosidase, D. trypsin-like protease, E. chymotrypsin-like protease, F. β-1,4-*N*-acetyl-glucosaminidase activities X axis: fraction number, Y axis: OD_{405}

Biological Control of Fungal and Bacterial Plant Pathogens
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Phylogenetic analyses of *Trichoderma harzianum* associated with mushroom culture or used for biological control of plant pathogens

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Abstract: Nucleotide sequences of internal transcribed spacers (ITS-1 and ITS-2) and the 5.8S gene of the nuclear ribosomal DNA repeat were examined in 61 isolates of *Trichoderma* spp. associated with mushroom culture or used for biological control of plant pathogens. Phylogenetic analyses revealed that the biocontrol isolates were more closely related to an isolate of *T. harzianum* biotype 1 (Th1) than to the aggressive biotypes 2 and 4. Th1 has been isolated from mushroom compost, but is not the cause of widespread mushroom green-mould epidemics that have occurred during the last 13 years in Europe and North America. Three isolates of *T. harzianum* obtained from shiitake (*Lentinula edodes*) and maitake (*Grifola frondosa*) substrates were placed within the biocontrol group.

Key words: mushroom green mould, mushroom diseases, *Agaricus bisporus*

Introduction

Trichoderma green mould has been associated with *Agaricus bisporus* culture since the mushroom was first domesticated in France. Within the last 15 years, however, growers in Ireland, England, Canada, United States, France and Spain have experienced outbreaks of Trichoderma green mould that have resulted in millions of dollars in crop losses (Ospina-Giraldo *et al.*, 1999). Four biotypes of *T. harzianum*, designated Th1-Th4, have been found associated with mushroom compost. Biotype Th3 previously identified as *T. harzianum*, recently was recognized as a strain of *T. atroviride* (Castle *et al.*, 1998; Ospina *et al.*, 1998). Biotypes Th2 and Th4 are the most aggressive, causing the majority of crop losses (Seaby, 1987). In North America, the predominant biotype associated with the green mould epidemic is Th4 (Qi *et al.*, 1996). Several species of *Trichoderma* are known mycoparasites and have been used successfully against certain pathogenic fungi. Among these, *T. harzianum* is the species most often used for biological control of pathogens. Genetic relationships among these strains and biotypes Th2 and Th4 only were examined recently by Ospina-Giraldo *et al.* (1999). Concern had emerged regarding the potential role of biological control strains in the development of mushroom green mould epidemics. Therefore, we wanted to compare nucleotide sequences from the ribosomal DNA transcriptional unit (rDNA) of isolates comprising green-mould and biocontrol-related isolates of *T. harzianum*. We found that although the isolates known for biological control properties and green-mould associated isolates share a recent ancestor, they constitute different phylogenetic groups.

Materials and methods

Cultures

Sixty-one isolates of *Trichoderma* spp., representing *T. harzianum* Th4 as well as other green-mould associated biotypes, were used. All biological control strains of *Trichoderma* spp. available in the ATCC were included in this analysis.

DNA extraction, PCR and sequencing

Fungal genomic DNA was extracted using the Puregene kit, following the manufacturer's directions. DNA (10 ng) was amplified in 15-µL PCR reaction mixtures containing 0.4 U of *Taq* DNA polymerase, 0.2 mM of each dNTP, 0.5 µM of primers ITS-1 (**5'** TCTGTAGGTGAACCTGCGG **3'**) and ALR0 (**5'** CATATGCTTAAGTTCAGCGGG **3'**) and 2 mM $MgCl_2$. Reactions were performed in capillary tubes using a Rapidcycler® with the following program: 94□C/15 s, for one cycle; 94□C/0 s; 56□C/15 s; 72□C/45 s for 40 cycles and 72□C/2 min. PCR products from eight 15-µL reactions of each sample were purified with the Wizard® PCR system and the concentration was adjusted to 20 ng/µL. Samples were sequenced in both directions with the same primers that were used in the amplification step. Consensus sequences were determined using the Seqman module from the Lasergene software package.

Data analysis

Sequences were aligned following the Clustal W algorithm (Felsenstein, 1985) included in the Megalign module. Phylogenetic analyses were completed using the MEGA package version 1.01. Nucleotide distances were estimated by the Jukes-Cantor model (Jukes & Cantor, 1969) and phylogenetic inference was performed by the Neighbor-Joining (NJ) method (Saitou & Nei, 1987). Bootstrap tests with 1,000 replications were conducted to examine the reliability of the interior branches and the validity of the trees obtained. SE test was performed with the data set using the same characters in order to evaluate the statistical confidence of the inferred phylogeny.

Results

The phylogenetic tree of all isolates (Fig. 1) revealed the presence of multiple clusters. One cluster contained *T. atroviride* (Th3 isolates) and the biocontrol strain ATCC 64263. This cluster shares the oldest ancestor of the isolates studied. At least six different subgroups are derived from this ancestor. Isolates obtained from shiitake substrate and *A. bisporus* compost, clustered together and share the most recent ancestor with an isolate of Th1. A specimen of *T. virens,* isolated from a commercial growth promoter, is closely related to this cluster. Biocontrol- and green-mould related strains that share a very recent common ancestor, formed two other subgroups. This distinction, however, was not very well supported by bootstrap tests. The biocontrol subgroup included 16 isolates (out of the 17 used in this study) of *T. harzianum* and one isolate of *T. inhamatum,* known for their antagonistic properties. However, this subgroup also included one specimen of Th1 and several samples obtained from green-mould infested shiitake, maitake and *A. bisporus* substrates. The sister subgroup, constituted by the aggressive Th4 isolates, contained further subdivisions. Isolates 120, 122, 123 and 169 of Th4 are part of an independent lineage. The other subdivision contained the remaining confirmed Th4 isolates and one additional sample, respectively, from *Pleurotus ostreatus* and *A. bisporus* substrates.

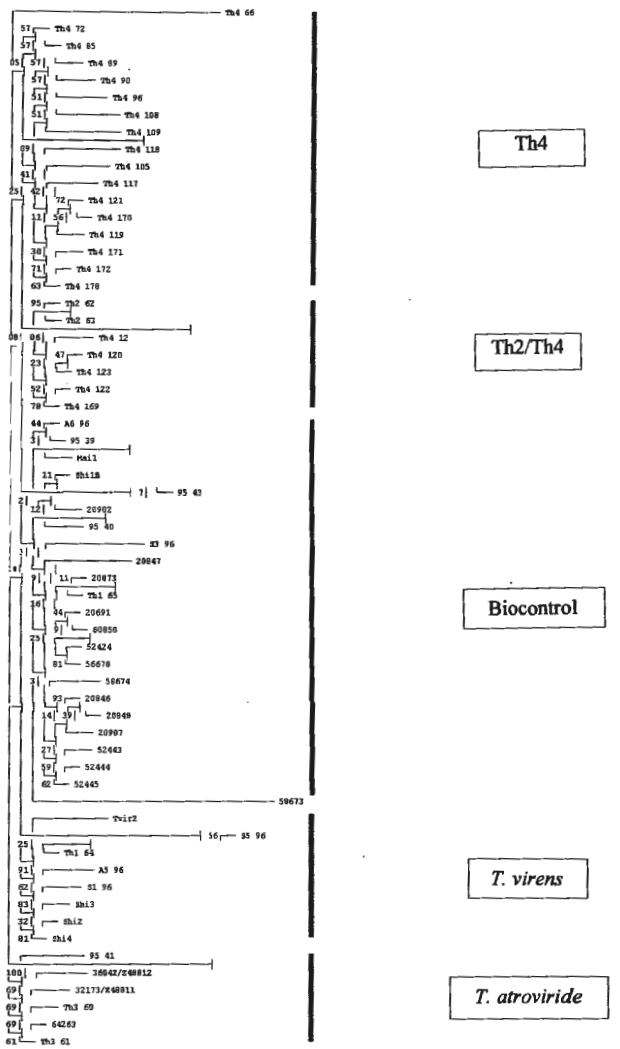

Figure 1. Phylogenetic relationships of 61 isolates of *Trichoderma* spp., inferred from rDNA sequence analysis. The phylogenetic tree was obtained by the Neighbor-Joining method using the Jukes-Cantor distance. The number of nucleotides in the subset was 572. Gaps and equivocal sites were excluded from the analysis. The numbers above the branches represent the values obtained after a bootstrap test with 1,000 replications. GenBank accession number Z48811 represents *T. atroviride* strains ATCC 32173 and 42831.

Discussion

Green-mould causing biotypes and biocontrol isolates are very closely related and share the most recent common ancestor (Fig. 1). Bootstrap analysis, using 1000 replications, of the NJ tree provides weak evidence to place both groups in distinct clades. However, no biocontrol strain was ever placed as the most recent ancestor of an aggressive biotype. The standard error, a statistically robust test in which the null hypothesis of branch-length-equal-to-zero is tested, also supported the results obtained after bootstrapping. In one case, a non-aggressive isolate of the Th1 biotype was found to be almost identical in sequence to the biocontrol strain ATCC 20873. This strain was reportedly obtained by protoplast fusion of ATCC 20737 and ATCC 60850. In addition, three other green-mould associated isolates (Mai1, Shi1B and S3-96), found on substrates of specialty mushrooms shiitake and maitake were placed within the biocontrol group.

Most of the Th4 isolates appear to be very closely related to each other, confirming the results obtained by Qi *et al.* (1996) who found very similar RAPD patterns in a population of Th4 isolates collected in Pennsylvania and Canada. The presence of several subgroups within the Th4 biotype (cluster A) suggests that some members of this biotype may have diverged along the evolutionary pathway (Fig. 1). In this group, it should be noted that the evolutionary rate is not constant for all isolates and several show an increased evolutionary distance. This suggestion is supported by bootstrap values as high as 72% (Fig. 1). In conclusion, we have found phylogenetic evidence suggesting that aggressive biotypes Th2 and Th4 are very closely related to biocontrol strains. However, the biocontrol strains do not appear to be responsible for the emergence of green-mould causing *T. harzianum*. Instead, Th2 and Th4 have evolved from a recent common ancestor for both biocontrol and green-mould related biotypes.

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Biological Control of Fungal and Bacterial Plant Pathogens
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Trichoderma, Collembola, pathogenic fungus, plant interactions

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Abstract: Interactions between the collembolan *Onychiurus armatus* and *Trichoderma harzianum* were studied in a simple experimental system with wheat plants. The fungus was applied to seed or mixed with substratum. The presence of *T. harzianum* was compatible with the survival and reproduction of animals, independent of the mode by which it was applied. In a more complex system including *Gaeumannomyces graminis* var. *tritici*, the mode of *T. harzianum* application significantly influenced the ability to control disease of *O. armatus*. In fact, when the mycoparasitic fungus was coated to seed its presence was compatible with biocontrol activity of Collembola against the disease caused by *G. graminis* var. *tritici*, whereas when *T. harzianum* was mixed with the substratum, these animals were not able to reduce the disease severity.

Key words: Springtails, *Trichoderma harzianum*, *Gaeumannomyces graminis* var. *tritici*, biocontrol

Introduction

Collembola, a well-represented taxon of soil animals, are predominantly mycophagous and largely dependent upon fungal food sources. Through their grazing activity they can profoundly influence the composition of fungal communities. Collembola cohabitate in the soil and rhizosphere with root infecting and natural or applied mycoparasitic fungi. Numerous studies carried out with different soilborne plant pathogenic fungi and Collembola species demonstrate that springtails significantly decreased the severity of disease caused by these fungi (Curl *et al.*,1988; Nakamura *et al.*, 1992; Lartey *et al.*, 1994; Scholte & Lootsma, 1998; Sabatini & Innocenti, 2000). Interactions between Collembola and biocontrol fungi are, on the contrary, scarcely investigated with contrasting results. Laboratory feeding experiments have demonstrated that springtails are more attracted to pathogenic fungi than to non sporulating *Trichoderma harzianum* cultures (Curl *et al.*, 1988; Innocenti *et al.*, 1997). Moreover, Curl *et al.* (1988) observed that Collembola were also attracted to conidia of this biocontrol fungus, and their activity reduced spore populations in the rhizosphere. In contrast, Lartey *et al.* (1994) reported that conidia of *T. harzianum* were lethal to springtails when ingested. Results of glasshouse experiments where the inoculum of *T. harzianum* fungus was mixed with substratum also containing *Rhizoctonia solani* inoculum, suggested a condition of coexistence between springtails and the mycoparasitic fungus in soil where a preferred food source (pathogen) was available (Curl *et al.*, 1988) No data are available on interactions between springtails and *Trichoderma* inoculum applied to seed, which represents the most economical and often effective application method for biological control of plant diseases.

Material and methods

Test organisms

Trichoderma harzianum Rifai ITEM 908 isolated from soil and maintained by cryo-conservation in water and glycerol (18%) at -80°C in the collection of the "Istituto Tossine Micotossine da Parassiti Vegetali" CNR Bari was used. It was characterised by low antibiotic production and good biocontrol activity in field experiments. The isolate *Gaeumannomyces graminis* Von Arx et Olivier var. *tritici* Walker (*Ggt*) LM 15.95 from the collection of the "Dipartimento di Protezione Valorizzazione Agroalimentare," University of Bologna was used. The fungus was isolated from wheat roots and stored in tubes on Difco potato dextrose agar (PDA) under mineral oil at 5°C. From these sources the fungi were transferred onto plates of PDA and cultured at 23°C for preparation of inoculum as described below. The collembolan species *Onychiurus armatus* (Tullberg) was used. Specimens were reared for several generations in the laboratory of the "Dipartimento di Biologia Animale," University of Modena and Reggio Emilia. They were maintained in glass jars containing a clay bottom saturated with distilled water, and kept in a thermostatic chamber at 20°C. The original stock of *O. armatus* was obtained from a cultivated cereal field located in the Po Valley (Italy). Tests were performed with sexually mature animals of the same age which had been starved for 48 h. Creso durum wheat (*Triticum durum* Desf.) cultivar susceptible to *Ggt* was employed.

Fungal inoculum preparation

T. harzianum was cultured on 200 g of autoclaved rice kernels in 500-ml Erlenmeyer flasks and inoculated with 10 ml of an aqueous suspension containing approximately 10^7 conidia/ml. Cultures were incubated at 25°C under a 12/12 h day/night photoperiod for 4 weeks. The harvested culture material was dried in a forced draft oven at 35°C for 48 h and finely ground to 0.2 mm grain size. Autoclaved wheat and millet seed colonised by the mycelium constituted the inoculum of *Ggt*.

Tube assay experiment

Glass tubes (35-mm diameter by 300-mm height) were used as experimental containers. *Trichoderma harzianum* power formulation was applied in two different ways: to seed (treatments 2, 4, 7, 10) or mixed with the substratum (1% w:w) at planting time (3, 5, 8, 11). Seeds were coated with *T. harzianum* by suspending 1 g of fungal inoculum in 20 ml of sterile distilled water containing some drops of TWEEN 20 and mixing with seeds for approximately 5 min. Coated seeds were air dried under a sterile air stream and planted within 24 h. The final population of *T. harzianum* was 10^8 CFU. Inoculum of *Ggt* was mixed (1% w:w) with the substratum constituted by sterile sand, at planting time (6, 7, 8, 9, 10, 11). Three kernels of wheat were sown in each tube. Fifty springtails were then released onto the substratum surface in treatments 4, 5, 9, 10, 11. Six replicates for each treatment were prepared. Tube assays, were placed upright in the racks in a randomised complete block design and incubated in a growth chamber at 20-25°C under 12h-day/night photoperiod and a relative humidity of 70-80%. Each tube was watered with 5 ml of tap water at the start of the trial and at weekly intervals.

Evaluation of the treatments

Five weeks after planting the substratum and plants were carefully extracted from each tube. Disease severity was rated on a scale from 0 to 8 and the index was then calculated (Sabatini & Innocenti, 2000) The plants were dried at 80°C for 24 h before being weighted. Springtails collected from the substratum of each tube by floating were grouped into adults and juveniles and counted. The animals were fixed in Gisin's fluid and the gut content was examined under

the light microscope. Data were subjected to analysis of variance (ANOVA) and Student-Newman-Keuls test.

Results and discussion

The effects of *T. harzianum* and *O. armatus* on emergence and dry weight of wheat seedlings are reported in Table 1. No statistical differences were observed between the data. Treatment effects on seedlings supplemented with *Ggt* are reported in Table 2. The disease index values confirm the biocontrol activity of Collembola. Isolate *T. harzianum* ITEM 908 did not exhibit any biocontrol activity against the disease caused by *Ggt*; this allowed verification of the biocontrol effect of springtails against the pathogen in presence of *T. harzianum* without interference from this fungus.

All Collembola collected from the substratum (treatments 4, 5, 10, 11) at the end of the experiments were alive, and live juveniles were found in addition to adults. Analysis of gut content showed that conidia of *T. harzianum* were palatable for springtails, in agreement with the findings of Curl *et al.* (1988). Application type of the mycoparasitic fungus significantly influenced suppression of disease by animals. The lowest disease index was observed when wheat seeds coated with *T. harzianum* were planted in substratum containing pathogen inoculum and springtails. In contrast, when both fungi were mixed with the substratum, the disease index was the highest. Mechanisms promoting these differences might be related to the formulation used, consisting of conidia and ground rice kernel. Laboratory feeding tests (unpublished data) have showed that specimens of *O. armatus* challenged with *T. harzianum* formulation or *Ggt* fed on both. Not only conidia, but probably also ground rice kernels attracted animals. When equal quantities of *T. harzianum* and *Ggt* inoculum were mixed in the substratum, springtails fed on both, without significantly reducing the development of *Ggt*, and thus disease. When the formulate was coated on seed, it was applied in much smaller amounts and more highly localized than the pathogen, and the animals turned mainly to the pathogen as food sources, significantly reducing *Ggt* growth and disease severity. These results suggest compatibility between the mycophagous animals and mycoparasitc fungus, both beneficial organisms for plant health, when seed dressing was used as the fungus application mode.

Table 1. Effects of interactions between *Trichoderma harzianum* and *Onychiurus armatus* on emergence and dry weight of wheat seedlings five weeks after planting.

	Treatment	Seedling emergence (%)	Root dry weight (mg)	Shoot dry weight (mg)	Collembola adults (n)
1	Untreated	88.9	33.2	41.6	
2	*T. harzianum* seed coated	83.3	41.3	53.5	
3	*T. harzianum* soil applied	94.4	35.1	50.7	
4	*T. harzianum* seed coated+ *O. Armatus*	94.3	34.3	54.9	39.0
5	*T. harzianum* soil applied +*O. Armatus*	74.9	27.9	44.5	36.5
	Significance	N.S.	N.S.	N.S.	N.S.

Table 2. Effects of interactions between *Trichoderma harzianum* and *Onychiurus armatus* in the presence of *Gaeumannomyes graminis* var. *tritici* on emergence, dry weight and disease index of wheat seedlings five weeks after planting.

	Seedlings emergence (%)	Root dry weight (mg)	Shoot dry weight (mg)	Disease index (%)	Collembola adults (n)
6 *Ggt*	94.4 a	26.4 a	39.3 ab	82.6 b	
7 *Ggt* + *T. harzianum* seed coated	88.9 a	32.4 a	37.2 ab	79,1 b	
8 *Ggt* + *T. harziamun* soil applied	93.3 a	37.3 a	39.1 ab	71,7 b	
9 *Ggt* + *O. armatus*	100 a	33.1 a	53.0 bc	44.8 a	30.8 a
10 *Ggt* +*T. harzianum* seed coated + *O. armatus*	86.7 a	35.6 a	63.3 c	24.2 a	27.0 a
11 *Ggt* + *T. harzianum* soil applied + *O. armatus*	83.3 a	28.1 a	29.1 a	98.9 b	33.5 a

Data followed by the same letter within a column are not significantly different at $P \leq 0.05$ according to the Student-Newman-Keuls test.

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Biological Control of Fungal and Bacterial Plant Pathogens
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Heterologous expression of a fungal β-1,3-glucanase in plants

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Abstract: β-1,3-glucanase BGN13.1 from the mycoparasitic fungus *Trichoderma harzianum* CECT 2413 was expressed in *Nicotiana tabacum* under the control of the cauliflower mosaic virus 35S promoter. Expression of the fungal gene was detected in transgenic plants by western blot analysis. The precursor protein seemed to be correctly processed in the transgenic plants, and the protein was active. No correlation was observed between the levels of BGN13.1 in leaf extracts and β-1,3-glucanase activity. Furthermore, some of the transformants displayed alterations in growth and development, and were sterile.

Key words: β-1,3-glucanase, *Trichoderma harzianum*, transgenic plants, tobacco, *Nicotiana tabacum*

Introduction

Mycoparasitic fungi have been studied to develop alternative strategies to chemical pesticides to control fungal plant pathogens. The mechanisms of antagonistic activity of *Trichoderma* spp., the best known biocontrol fungus, involve fungal cell wall degrading enzymes (CWDE) such as chitinases and glucanases. BGN13.1, an endo ß-1,3-glucanase from *T. harzianum* CECT 2413, showed cell-wall-degrading activity in *in vitro* assays (De la Cruz *et al.*, 1995). Furthermore, *T. harzianum* strains over-expressing this glucanase have improved antifungal activity against *Rhizoctonia solani*, *Botrytis cinerea* and *Phytophthora citrophthora*.

The plant defense system can be modified to produce high constitutive levels of antimicrobial compounds against microbial pathogens. Plant genes encoding CWDE, mainly chitinases, have been used to increase plant resistance to fungal pathogens, but so far not a single gene has produced an adequate level of resistance. For this reason, genes encoding CWDE from other organisms, such as mycoparasitic fungi are being utilised to obtain plants that over-express proteins, structurally different from their own. These proteins could contribute to an improved defence system. In fact, disease resistance in transgenic tobacco plants has been improved by heterologous expression of a chitinase from *T. harzianum* CECT 2413 (Lorito *et al.*, 1998).

The isolation of *Nicotiana tabacum* plants that overproduce the BGN13.1 glucanase from *T. harzianum* is reported in this paper.

Material and methods

Culture conditions

Nicotiana tabacum cv. Xhantii seeds were propagated *in vitro* in Murashige-Skoog basal salt medium (Sigma) containing sucrose 3%. Kanamycin (300 mg/L) was added in the case of transgenic plants. Germlines were grown for three weeks and transferred to soil in pots.

Construction of a transformation vector

bgn13.1 cDNA was translationally fused to the PR1 (tomato pathogenesis-related protein) signal peptide using intermediate vectors. The chimeric gene was subcloned in pBI121 under the control of the cauliflower mosaic virus 35S subunit promoter region (CaMV35S-pro) and the nopaline synthase terminator (NOS-ter). This construction was subcloned in *Eco*RI/*Hind*III sites of the binary vector pBin19. The resulting plasmid was named pBin-PRB13 (Fig. 1).

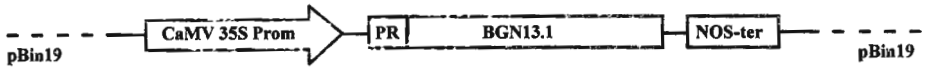

Figure 1. Schematic presentation of the construct used for plant transformation. CaMV 35S Prom: cauliflower mosaic virus 35S subunit promoter region. NOS-ter: *Agrobacterium* nopaline synthase terminator. BGN13.1: *bgn13.1* cDNA without fungal putative activation peptide. PR: tomato pathogenesis-related protein signal peptide.

Plant transformation

Agrobacterium tumefaciens carrying the vector pBin-PRB13 or the corresponding empty pBin19 were used to transform leaf disks of *N. tabacum* cv. Xhantii.

Preparation of total proteins from tobacco leaves

Leaf tissue was powdered in liquid nitrogen with a mortar and pestle, then homogeneized in ice-cold water and centrifuged at 4°C for 10 min at 7800*g*. The supernatant was dialyzed through a Sephadex G50 column. The resulting total leaf protein extract was used for Western blotting and β-1,3-glucanase activity was determined.

Western blot analysis

Proteins were separated by SDS-PAGE and transferred to Immobilon filters (Millipore) with a Semi-Dry Electrophoretic Transfer Cell (Bio-Rad) following manufacturer's instructions. Membranes were blocked for 2 h with a solution of skimmed milk in 10 mM Tris-HCl (pH 8), 1 mM EDTA, 150 mM NaCl and 0.1% Tritón X-100. Filters were incubated overnight at room temperature with the rabbit polyclonal antibody against BGN13.1, diluted appropriately in blocking solution, and then washed with blocking buffer. Alkaline phosphatase-conjugated anti-rabbit immunoglobulin G antibody (Sigma) was used as a secondary antibody.

Determination of β-1,3-glucanase activity

The protein extracts were diluted and incubated with 0.5% laminarin (Sigma) for 30 min at 37°C. The reaction was terminated after boiling for 5 min and the reducing sugars that were released were determined by the procedure of Somogyi (1952) and Nelson (1957).

Results and discussion

Tobacco transformants express fungal β-1,3-glucanase

Thirty seven kanamycin-resistant primary transformants were screened for expression of the fungal glucanase polypeptide by immunoblot analysis (Figure 2A). Most of them contained two bands that were immunoreactive with polyclonal antibodies against BGN13.1 protein from *T. harzianum* in total leaf extracts. These bands corresponded to the protein with or without the 28 aa signal peptide (Figure 2B). These data suggest that the precursor protein was correctly processed in the heterologous tobacco system.

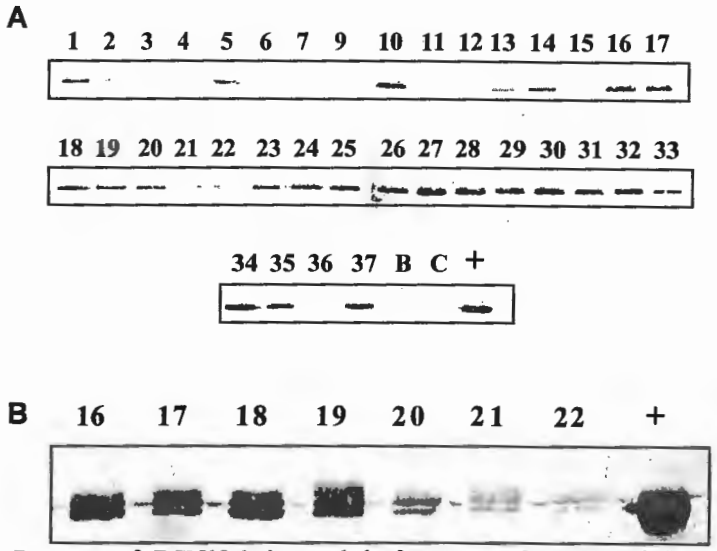

Figure 2. Presence of BGN13.1 in total leaf extracts. 20µg protein/lane. **B**: control transformed with pBin19. **C**: *Nicotiana tabacum* wild type. **+**: purified BGN13.1.

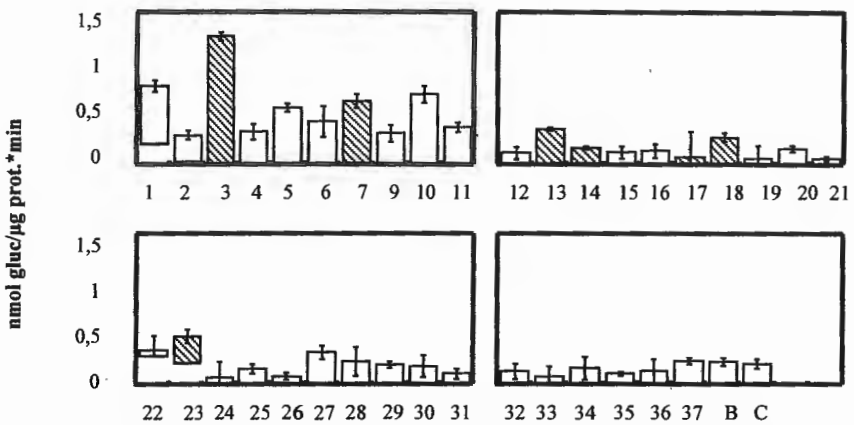

Figure 3. β-1,3-glucanase activity in transgenic plants. B: control transformed with pBin19. C: *Nicotiana tabacum* wild type. ▨ Sterile transformants.

β-1,3-glucanase activity

β-1,3-glucanase activity was measured in total leaf extracts. The levels of activity found in the transformants were not significantly higher than that of the wild type strain except for lines 1,3,5,7 and 10 (Figure 3). There was no correlation between β-1,3-glucanase activity and the level of expression of transgenic protein. For instance, protein BGN13.1 was not detected in lines 3 and 7 (Figure 2A). This results indicated that BGN13.1 activity in leave extracts was probably masked by those of the plant glucanases. Different levels of activity found in

transgenic lines might be due to different development stages or to response to some kind of stress.

Phenotypes observed

Whereas most transgenic plants grew and developed like the wild type, some of the transformants obtained (Figure 3) showed a delay in growth and development when compared to the control. Furthermore, these plants were sterile. These results were not surprising, given the role that β-1,3-glucanases may play in plant development. Alternatively, this phenotype could be due to a position effect caused by the integration of the ectopic gene. At present, we are carrying out greenhouse experiments in order to determine the resistance of transgenic plants to *Rhizoctonia solani* infection.

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Biological Control of Fungal and Bacterial Plant Pathogens
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The expression of enzymes involved in biological control of tomato phytopathogens by *Trichoderma* depends on the pathogen and biocontrol isolate

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Abstract: Four isolates of *Trichoderma harzianum* (N3, Th11, Th12 and Th16) showed different abilities to control the tomato root pathogens *Rhizoctonia solani*, *Fusarium solani* and *Pyrenochaeta lycopersici*. Analysis of the mechanisms involved showed that the differential expression of chitinases and proteases correlated well with the different ability of each *Trichoderma* isolate to control these phytopathogens. Glucanases and/or production of antibiotics contributed to a lesser extent in biological control.

Key words: biological control, tomato root diseases, *Trichoderma harzianum*

Introduction

Rhizoctonia solani, *Fusarium solani* and *Pyrenochaeta lycopersici* causes root diseases in tomato (Latorre, 1987). Control of these pathogens is presently accomplished through fumigation of soils with methyl bromide plus chloropicrine, carbamates and fungicides. Methyl bromide, although very effective in controlling the pathogens mentioned above, is highly toxic, contributes to environmental pollution, and its use will be forbidden from year 2010 (Ristaíno & Thomas, 1997). An alternative to reducing the use of chemicals for controlling phytopathogens, is their replacement, at least in part, with antagonistic microorganisms. Fungi from the genus *Trichoderma* are well known as biocontrol agents, and more specifically, those belonging to *T. viride* and *T. harzianum* species (Dennis & Webster, 1971a, b, c). Nevertheless, it appears that each *Trichoderma* isolate behaves in a different manner when confronted by the same phytopathogen. The differences observed could depend on the ability of each isolate to produce antibiotics or express genes that code for chitinases, β -1,3-glucanases and/or proteases involved in biocontrol activity or in the specific isoenzyme pattern (Grondona *et al.*, 1997). Our objective is the characterization of several *Trichoderma* isolates on the basis of the parameters already mentioned, in order to select *Trichoderma* strains with the best characteristics for their use as biocontrol agents of tomato phytopathogens.

Materials and methods

Trichoderma *isolation*

Trichoderma strains were isolated from soils and cultured on potato-dextrose-agar (PDA, DIFCO) until pure cultures were obtained. Four isolates (N3, Th11, Th12 and Th16) were identified as *T. harzianum* strains, based on their morphology after cultivation on PDA, malt-agar and Czapek agar.

Tomato pathogens

R. solani, *F. solani* or *P. lycopersici* were isolated from tomato plants showing symptoms of disease.

Dual cultures

Each *Trichoderma* isolate was grown on the same plate (PDA) opposite each tomato pathogen (*R. solani*, *F. solani* or *P. lycopersici*). Growth diameter of each pathogen was measured and compared to that of the pathogen, in the absence of the bio antagonistic. Each experiment was run in triplicate and repeated at least three times. Results are expressed as means $\pm$ S.D., and a Student test was performed at $P \leq 0.05$.

Production of volatile antibiotics

Half plates, one containing a *Trichoderma* isolate and the other containing one of the three pathogens were placed opposite each other preventing any physical contact between the fungi. They were sealed isolating the inside atmosphere and preventing loss of the volatiles formed. Plates were incubated at 28°C and at different time periods growth of the pathogen was measured and compared to controls developed in the absence of the bioantagonistic. Each experiment was run in triplicate and repeated at least three times. Results are expressed as means $\pm$ S.D., and a Student test was performed at $P \leq 0.05$.

Production of volatile antibiotics

Plates containing PDA, covered with a dialysis membrane, were grown with each of the antagonistic *Trichoderma*. After incubation for 72 hours at 28°C, the membrane with the *Trichoderma* strain was removed, and the plate was then inoculated with one of each pathogen. Plates were further incubated at the same temperature and growth of the pathogen was measured. Each experiment was run in triplicate and repeated at least three times. Results are expressed as means $\pm$ S.D., and a Student test was performed at $P \leq 0.05$.

Submerged cultures of* Trichoderma *and quantification of enzyme activities

Each isolate was grown in liquid Mandels medium (Mandels *et al.*, 1974) using either crab chitin, yeast glucans, or cell walls from *R. solani*, *F. solani* or *P. lycopersici* as the sole carbon source. Supernatants from 8-day-old cultures were used for the analysis of chitinases, β-1,3-glucanases and proteases after native electrophoresis of proteins (Pan *et al.*, 1991).

Results and discussion

Antagonistic effect of* Trichoderma *isolates on tomato pathogens

All four *Trichoderma* isolates (N3, Th11, Th12 and Th16) controlled development of each pathogen in dual cultures. Nevertheless, the order of control was Th11 > Th12 > N3 > Th16 for the three pathogens tested. When biocontrol was analyzed in terms of the effect of volatile and non-volatile metabolites, development of pathogens was much less affected by volatile metabolites than by non-volatile metabolites produced by the *Trichoderma* isolates (Table 1). Therefore, it appears that volatiles from these *Trichoderma* isolates are not the main mechanism of control. On the contrary, production of non-volatile antibiotics are present in all four isolates, suggesting that this mechanism could be important in the biocontrol of tomato

Table 1. Growth of tomato pathogens in the presence of different *Trichoderma harzianum* isolates: effect of volatile and non-volatile metabolites.

a) Effect of volatile metabolites

	Growth (diameter in cm)[1]		
Isolate	*R. solani*	*F. solani*	*P. lycopersici*
None	$3,3 \pm 0,2^{a}$	$1,4 \pm 0,1^{a}$	$0,4 \pm 0,05^{a}$
N3	$3,1 \pm 0,1^{a}$	$1,0 \pm 0,1^{b}$	$0,4 \pm 0,1^{a}$
Th11	$3,2 \pm 0,1^{a}$	$1,2 \pm 0,1^{b}$	$0,4 \pm 0,1^{a}$
Th12	$2,4 \pm 0,1^{b}$	$1,3 \pm 0,1^{a}$	$0,2 \pm 0,1^{b}$
Th16	$3,3 \pm 0,1^{a}$	$1,4 \pm 0,1^{a}$	$0,3 \pm 0,1^{a}$

b) Effect of non-volatile metabolites

	Growth (diameter in cm)[1]		
Isolate	*R. solani*	*F. solani*	*P. lycopersici*
None	$2,9 \pm 0,1^{a}$	$0,7 \pm 0,1^{a}$	$0,4 \pm 0,1^{a}$
N3	$1,8 \pm 0,1^{b}$	$0,9 \pm 0,2^{a}$	$0,0^{b}$
Th11	$0,4 \pm 0,1^{c}$	$0,5 \pm 0,1^{b}$	$0,0^{b}$
Th12	$0,5 \pm 0,2^{c}$	$0,5 \pm 0,1^{b}$	$0,0^{b}$
Th16	$0,6 \pm 0,2^{c}$	$0,4 \pm 0,1^{b}$	$0,4 \pm 0,1^{a}$

[1] *R. solani* was incubated for 48 hours; *F. solani* and *P. lycopersici* were incubated for 96 hours. [a,b,c] Different letters indicate significant differences.

Table 2. Number of isoenzymes of chitinase (Ch), β -1,3-glucanase (Gl) and protease (Pr) expressed by each isolate of *Trichoderma harzianum*: analysis in basic and acid gels.

a) Analysis in basic gels

	Number of isoenzymes expressed in culture media containing							
Isolate	Crab chitin		*R. solani* walls		*F. solani* walls		*P. lycopersici* walls	
	Ch	Gl	Ch	Gl	Ch	Gl	Ch	Gl
N3	1	1	0	1	1	0	0	1
Th11	3	1	3	1	2	0	4	4
Th12	1	1	2	2	2	1	3	4
Th16	2	1	1	2	1	0	1	1

b) Analysis in acid gels

	Number of isoenzymes expressed in culture media containing											
Isolate	Crab chitin			*R. solani* walls			*F. solani* walls			*P.lycopersici* walls		
	Ch	Gl	Pr[a]	Ch	Gl	Pr[a]	Ch	Gl	Pr[a]	Ch	Gl	Pr[a]
N3	0	4	1	0	2	1	0	3	3	2	0	1
Th11	5	4	2	0	2	2	2	5	2	5	2	2
Th12	1	1	2	1	1	1	1	2	1	1	2	1
Th16	1	3	1	0	2	2	1	1	1	0	3	1

[a]Proteases were analyzed in a hemoglobin containing gel in native conditions. Electrophoresis was run at a pH = pI of hemoglobin. Proteases were visualized as white bands on a blue background after Coomassie blue R-250 staining of proteins.

pathogens. From all four isolates, Th11 and Th12 were the best control strains which agrees with results obtained from dual cultures.

Secretion of enzymes

The number of isoenzymes of chitinases, β -1,3-glucanases and proteases also depended on the isolate and fluctuated from zero to five different isoenzymes, depending on the culture medium (Table 2).

Isolates Th11 and Th12 expressed the largest numbers of isoenzymes of chitinases and glucanases in the presence of cell walls of the different pathogens, suggesting that the number of isoenzymes could be relevant in accomplishing effective control. Similar results were obtained by Grondona *et al.* (1997).

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Biological Control of Fungal and Bacterial Plant Pathogens
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Studies on the mode of action of *Rahnella aquatilis* Ra39 against *Erwinia amylovora*

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Abstract: The interaction between populations of the fire blight pathogen *Erwinia amylovora* strain Ea 7/74 and the antagonistic epiphytic bacterium *Rahnella aquatilis* strain Ra 39 was studied *in vitro*. In mixed culture of both strains an inhibitory effect of Ra 39 on Ea 7/74 could be noted only on the fourth and fifth day after inoculation. As another aspect of the mode of action, resistance induction was checked. Here the presence of the superoxide anion was determined after infiltration of apple and begonia leaves with cell suspensions of Ra 39, Ea 7/74, *Xanthomonas campestris* pv. *begoniae* strain Xcb 525 and with purified cell wall fragments of Ra 39. Cell suspension and purified cell wall fragments of Ra 39 caused the induction of superoxide in leaves of apple and begonia. However, cell suspensions of Ea 7/74 and Xcb 525 showed this effect only after infiltration into leaves of the non host plant, begonia and apple, respectively. This result indicates, that induced resistance may play a role in antagonism of Ra 39 against fire blight.

Key words: *Erwinia amylovora*, *Rahnella aquatilis*, antagonism, mode of action, induced resistance

Introduction

The epiphytic bacterial strain *Rahnella aquatilis* Ra 39, which has shown a significant reduction of fire blight symptoms in field trials during 1998, 1999 and 2000 (Wesche, 1998; Laux *et al.*, 1999; Laux & Zeller, 2000) was investigated with regard to its mechanism against *Erwinia amylovora*, the causal agent of the disease. No information on the mode of action of *Rahnella aquatilis* against fire blight has been available until now.

Mechanisms of bacterial antagonists against the fire blight pathogen include antibiosis (Vanneste *et al.*, 1992), competition for nutrients and sites (Wilson & Lindow, 1993; Wright & Beer, 1997) and induced resistance. Antagonism against fire blight has been described mainly for strains of the species *Pantoea agglomerans, Pseudomonas fluorescens* and *Bacillus subtilis*. While for the majority of *P. agglomerans* strains antibiosis has been assumed as the principle mode of action against fire blight (Vanneste *et al.*, 1992). This was not found for *P. fluorescens* which was suggested to inhibit *E. amylovora* by colonization of the nectaries (Wilson & Lindow, 1993). Also for *B. subtilis*, which produces bacteriocines activite against a wide range of bacteria (Klaenhammer, 1988), no bactericide activity against *Erwinia amylovora* has been described until now. We report here the involvement of induced resistance in the activity of *R. aquatilis* strain Ra 39 against *E. amylovora* strain Ea 7/74.

Materials and methods

Bacterial strains and culture media

Bacteria (Table 1) were cultured in glucose-asparagine (GA)-medium (g/l: K_2HPO_4: 11.5; KH_2PO_4: 4.5; $MgSO_4$: 0.12; glucose: 20.0; L-asparagine: 0.30; nicotinic acid: 0.05) at 24°C.

Liquid cultures were agitated constantly at 250 rpm. Plate counts of mixed cultures of Ea 7/74 and Ra 39 were conducted on solid Miller Schroth (MS)-medium (Miller & Schroth, 1972).

Table 1. Bacterial strains.

Strain	Isolated from	Source or reference
Erwinia amylovora Ea7/74	*Cotoneaster* sp.	Zeller & Meyer, 1975
Rahnella aquatilis Ra 39	*Malus domestica*	Steinbrenner, 1991
Xanthomonas campestris pv. begoniae Xcb 525	*Begoniae* sp.	Collection of phytopathogenic bacteria Gütingen (GSPB)

Purification of cell wall fragments was done according to the following steps:
Growth of Ra 39 in 500 ml NS-liquid culture to an optical density of 1.2 at 660 nm; Washing the pellet 2 times in 500 ml saline containing 5 mM EDTA; Suspension of the pellet in ethanol (5 mg/ml), centrifugation (8000g, 10 min); Suspension in acetone (5ml/g), sonification (10 min), centrifugation (8000g, 10 min); Suspension in diethylether (2 mg/ml), centrifugation (8000g, 10 min), overnight drying; Suspension in water (10 ml/g), digestion with DNAse and RNAse (each 0,3 mg/g); Digestion with proteinase K (0,3 mg/g dry weight, 37 U/mg; Sigma) overnight; Dialysis against demineralised water (12000 Da; Serva) for three days, lyophilisation.

Inoculation of plants
For inoculation experiments two-year-old apple trees (variety Golden Delicious) grown in pots of 20 cm diameter and one-year-old begonia plants (variety Oslo) grown in pots of 12 cm diameter, were used. All plants were placed in a greenhouse at 25 °C and 5000 Lux for 48 h before starting the experiments. Purified cell wall fragments were applied at a concentration of 5 mg per ml. Suspensions of bacteria were adjusted to a concentration of $1x10^8$ CFU per ml. Leaves of begonia were inoculated by using a syringe with a fine needle. The intercellular space of one intercostal field was inoculated per preparation; the control was infiltrated with de-mineralised water. Leaves of apple were pressure inoculated (2 bar) by using a laboratory sprayer. The intercellular space of four leaves was inoculated per preparation. After 24 hours treated leaves were checked for the presence of the superoxide anion by staining with nitro-tetrazolium-blue (Doke, 1983).

Results

Multiplication of Ea 7/74 and Ra39 in pure and mixed culture
Ra39 and Ea7/74 were first cultivated separately in GA-medium. Maximum bacterial concentrations of $1.4x10^{10}$ (Ea7/74) and $1.2x10^{10}$ (Ra39) CFU/ml were observed after 2 and 3 days, respectively. When Ra39 was grown together with Ea7/74 in GA-medium, the maximal numbers of CFU/ml of $1,4x10^{10}$ (Ea 7/74) and $1.6x10^{10}$ (Ra39) were recorded after 2 and 3 days, correspondingly to the results with pure cultures. From the 4th day after inoculation the number of CFU per ml of Ea 7/74 was significantly lower than in the pure culture (Fig. 1).

***Induction of the superoxide anion* (Doke, 1983)**
After infiltration of leaves from apple and begonia with a cell suspension and cell fragments of Ra39 the appearance of a red coloured formazan, as an indication for induced resistance, was observed. The cell suspension of *X. campestris* pv. *begoniae*, the causal agent of the bacterial oil spot disease of begonia caused only a reaction in apple leaves. Similar results were observed using the cell suspension of Ea 7/74 in begonia leaves (Table 2).

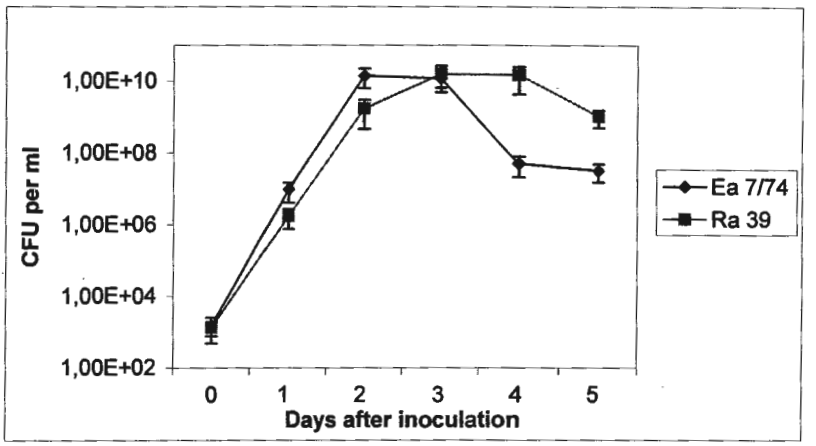

Figure 1. Growth of *Erwinia amylovora* Ea 7/74 and *Rahnella aquatilis* Ra 39 in mixed culture in glucose-asparagine (GA)-medium; bars represent standard errors.

Table 2. Staining of superoxide (Doke, 1983) in leaves of apple (Golden Delicious) and begonia (Oslo) after infiltration of cell suspensions of Ra 39 and Xcb 525 and cell fragments of Ra 39. + = red coloured, - = no reaction.

Preparation	Test plant	
	Apple (Golden Delicious)	Begonia (Oslo)
Ra39 (cell suspension)	+	+
Ra 39 (cell free extract)	+	+
Ea 7/74 (cell suspension)	-	+
Xcb 525 (cell suspension)	+	-

Discussion

A direct inhibition of Ea 7/74 by Ra 39 *in vitro* could only be observed after 4 days in GA-medium. For many *P. agglomerans*-strains the inhibition of bactericide activity by the presence of amino acids in the medium has been described (Wodzinski *et al.*, 1994). Therefore, amino acid composition of the plant tissue was suggested to affect the efficacy of

P. agglomerans strains in preventing fire blight symptoms. Also for Ra 39, it cannot be excluded that specific substrates, only available at the flower surface of fire blight host plants, are stimulating a direct inhibition of the pathogen. Nethertheless, an involvement of further mechanisms in antagonism of Ra 39 against *E. amylovora* can be considered as likely. Induced resistance has been described as mechanism of *P. agglomerans* against *P. syringae* pv. *syringae* on barley (Braun *et al.*, 1997). Several other epiphytic bacteria have been reported to induce resistance against plant diseases (Goodman, *et al.*, 1986). Furthermore, it is known that bacterial plant pathogens act as inducers of resistance in non-host plants. As a first step in the resistance reaction, formation of superoxide has been described (Doke, 1983). The fact that the cell suspension of Ra 39 induced formation of superoxide in apple and begonia leaves, similar to those of Xcb 525 and Ea 7/74, respectively, indicates that induced resistance may play also a role in antagonism of Ra 39 against Ea 7/74. Because purified cell fragments of Ra 39 showed the same resistance inducing activity as the cell suspension, an involvement of cell wall compounds in this process can be considered as likely and should be clarified in further studies.

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Biological Control of Fungal and Bacterial Plant Pathogens
IOBC wprs Bulletin Vol. 24 (3) 2001
p.363

Chitinase 33 gene expression in *Trichoderma harzianum* during mycoparasitism

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Chitinase Chit33 from *T. harzianum* CECT 2413 is encoded by the *chit33* gene, a major component of the fungus' chitinolytic enzyme system and important for biocontrol. Regulation of expression of *chit33* was investigated using a 1.4-kb fragment of the 5'-upstream sequences of *chit33*. This fragment was cloned and fused to the *A. niger goxA* (glucose oxidase encoding) gene and the *A. victoria* green fluorescent protein (GFP) gene as two different reporter systems. Different transformants of *T. harzianum*, bearing a mutant *gfp* under the control of the 1.4 kb *chit33* promoter, were used to study the expression in different carbon sources. These transformants were also used to carry out confrontation assays in dual culture using *R. solani* and *B. cinerea* as hosts. *Chit33* is expressed during growth on chitin, *R. solani* cell walls and low (0.1 %, w/v) concentrations of glucose or glycerol. Addition of N-acetylglucosamine transiently induced *chit33* expression in resting cells of the fungus. High concentrations (1%, w/v) of glucose or glycerol repressed the expression. Data obtained by Northern analysis, glucose oxidase activity and GFP expression were compatible, thus showing that the 1.4-kb fragment carries all the information necessary for *chit33* gene expression. Confrontation assays using *R. solani* and *B. cinerea* showed that *chit33* is expressed only during, but not before the stage of overgrowth. These data show that Chit33 is an enzyme involved in mycoparasitism and its formation is controlled by induction and carbon starvation.

Biological Control of Fungal and Bacterial Plant Pathogens
IOBC wprs Bulletin Vol. 24 (3) 2001
p.365

Increased hypersensitive response of transgenic tobacco plants expressing chitinases from *Trichoderma harzianum*

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In many plant-pathogen interactions a defense response against microbial infection is correlated with the appearance of a hypersensitive reaction (HR), characterized by the formation of small necrotic lesions at the invasion zone, and transcriptional activation of defense-related genes. This gene activation is triggered both locally, at the infection site where necrosis occurs, and systemically in plant tissues away from the infected area. Many plant defense-related genes have been identified and a potential defensive role has been attributed to the corresponding encoded products. Among these products, ß-1,3-glucanases and chitinases have shown *in vitro* antifungal activity.

Trichoderma harzianum is a filamentous fungus, known for its use as a biocontrol agent of phytopathogenic fungi. One of its antagonistic mechanisms is the production of hydrolytic enzymes responsible for the breakdown of the host cell walls.

It has been previously reported that transgenic tobacco plants expressing the chitinase 42 gene (*chit42*) from *T. harzianum* were highly tolerant to *Alternaria alternata*, *A. solani*, *Botrytis cinerea* and *Rhizoctonia solani*. We have extended the study of *chit42* in transgenic plants by testing its activity toward a pathogenic bacterium. Furthermore, we have included in this study transgenic tobacco plants expressing the chitinase 33 gene (*chit33*) from *T. harzianum*. Expression of these genes in tobacco plants had no visible effects on plant growth and development, however, healthy untreated transgenic plants showed notably higher peroxidase activity than control plants. After inoculation with *Pseudomonas syringae pv. tobacci*, a very rapid hypersensitive response was observed in transgenic plants. These results suggest that fungal chitinases may act as inducers of the plant defence system during pathogen attack.

Biological Control of Fungal and Bacterial Plant Pathogens
IOBC wprs Bulletin Vol. 24 (3) 2001
p.367

Homologous and heterologous overexpression of a β-1,6-glucanase (BGN16.3) from *Trichoderma harzianum*

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Strains of the mycoparasitic fungus *Trichoderma* are increasingly being applied to control phytopathogenic fungi due to their antagonistic abilities. Three mechanisms are involved in antagonism of *Trichoderma* strains against other filamentous fungi: i.- antibiosis (synthesis and secretion of toxic compounds) ii.- substrate competition and iii.- synthesis and secretion of hydrolitic enzymes, mostly cell-wall-degrading enzymes (CWDE). We have developed two different systems to improve the production of β -1,6-glucanase III, a CWDE from *T. harzianum*. The methylotrophic yeast *Pichia pastoris* was transformed with the cDNA coding for this protein ligated into the expression vector pPIC9 under the control of the alcohol oxidase gene promoter and terminator. Transformation was conducted by electroporation and the resultant colonies were selected on a histidine-deficient medium. A preliminary screening to select the best producer among all the colonies was done using plate assays in agar medium containing pustulan as a specific substrate for the enzyme. After one days growth, the non-degraded substrate was ethanol precipitated and the clones producing the enzyme showed haloes. The diameter of these haloes was considered to select clones with high production levels. The best producers were then cultured in liquid medium. The β -1,6-glucanase activity of culture filtrates was measured and SDS-PAGE was performed to detect the presence of BGN16.3.

In addition, *T. harzianum* was cotransformed with the same cDNA ligated into the expression vector pLMRS3 (under control of the pyruvate kinase gene promoter and cellobiohydrolase II gene terminator, both from *T. reesei*) and p3SR2 carrying the *amdS* gene from *Aspergillus nidulans*. The transformation was developed by the polyethylene glycol (PEG) method. The resultant colonies were alternatively cultured in selective and non-selective medium to obtain stable transformants, and those showing 100% mitotic stability were subjected to the plate screening assay described before for yeast transformants.
We have tested the antifungal activity of these two recombinant enzymes using antifungal assays in microtiter plates. The antagonistic ability of transformed *Trichoderma* strains has also been tested in confrontation assays in dual culture with phytopathogenic fungi (*Penicillium italicum* and *P. digitatum*).

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Purification and characterisation of a protease, Pra1, from *Trichoderma harzianum* with affinity for fungal cell walls

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The mycoparasitic fungus *Trichoderma harzianum* is used as a biocontrol agent against a large variety of phytopathogenic fungi. Lytic enzymes produced and secreted into the media are considered one of the main mechanisms involved in antagonistic activity of *Trichoderma* strains. Within the complex cocktail of lytic enzymes produced by *Trichoderma*, a principal role has been attributed to chitinases and glucanases. However, fungal proteases may also be significant in cell wall degradation, since fungal cell walls contain chitin and glucan polymers embedded in and covalently linked to a protein matrix. Recently, a new role for extracelular proteases in antagonism of *Trichoderma* has been proposed, acting as proteolytic inactivators of pathogen enzymes involved in the plant infection process.

Despite these facts, the *Trichoderma* proteolytic system involved in antagonism has not been dissected and only an alkaline protease (Prb1) related to mycoparasitism has been identified and characterised by Geremia *et al.* (1993).

Here, we report the purification and biochemical characterization of a novel protease from *T. harzianum* CECT 2413, which showed affinity for fungal cell walls. The fungus produced several extracellular proteases, detected after electrophoresis in casein-SDS-PA gels, when cultivated in MM medium supplemented with purified cell walls from the phytopathogen *Colletotrichum acutatum*, as a carbon source. One of these proteases showed affinity for cell walls from *C. acutatum* and *Botrytis cinerea,* and its purification was attempted. The protein was purified to electrophoretic homogeneity by $(NH_4)_2SO_4$ precipitation, followed by chromatofocussing and gel filtration. The molecular mass of this protease, named Pra1, is 28 kDa and its pI is acidic (4.7-4.9). Partial amino-acid sequences from the N-terminal and an internal peptide showed high homology with trypsin-like proteases. The nature of Pra1 was confirmed using a trypsin-specific synthetic substrate, N-acetil-Ile-Glu-Ala-Arg-pNA, which proved to be an excellent substrate for this protease, with an estimated Km of 0.22 mM. Pra1 showed no activity towards synthetic substrates specific for chymotrypsin and elastase. The inhibition profile confirmed that this enzyme belongs to the serin-type peptidase group. Pra1 has its maximum activity at alkaline pH (7.5), although it is more stable at acidic pH. The optimum temperature for the enzymatic activity is between 35-40°C at pH 7.5, and is not affected by incubation temperatures up to 40°C.

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Biological Control of Fungal and Bacterial Plant Pathogens
IOBC wprs Bulletin Vol. 24 (3) 2001
pp.371 - 374

Enzyme production by a biocontrol strain of *Trichoderma atroviride*

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Abstract: *Trichoderma* species have been described as antagonistic agents against various phytopathogenic fungi. This ability makes *Trichoderma* a healthier, safer and environmentally more friendly biological choice, compared with polluting chemical pesticides, usually employed in agriculture. Mycoparasitism is one of the mechanisms involved in antagonistic processes, based on activity of extracellular lytic enzymes produced by *Trichoderma*. The chosen strain of *T. atroviride* has been proven as a good biological agent in our laboratory, in previous studies. In the present work, we have studied different inducing conditions related to mycoparasitism, and we have selected the ones that induced higher lytic activities, including β-1,3-glucanase, β-1,6-glucanase, cellulase, chitinase and protease. For this purpose, various carbon sources as well as different saline media were assayed. Some of the carbon sources used were obtained from food industry wastes, allowing production of fungal biomass at low cost. Once the ideal conditions were found, molecular characterization of lytic isoenzymes was achieved by electrophoretic separation in renaturalizing conditions. The present study allowed us to determine the ideal inducing conditions, as well as correlate between enzyme activity and antifungal effect.

Key words: *Trichoderma*, biocontrol, mycoparasitism, lytic activities, enzyme production

Introduction

Biological control of plant diseases with fungal agents has received increasing attention. Biocontrol agents provide a healthier, safer and environmentally more friendly choice, compared with polluting chemical pesticides, usually employed in agriculture. We have focused our study on a strain of *Trichoderma*, belonging to a genus, described as an effective biocontrol agent against fungal plant pathogens such as *Botrytis cinerea* (Elad & Zimand, 1992) and other pathogens causing damping-off (Grondona, 1994). The mechanisms that allow *Trichoderma* to control phytopathogens are recognized as mycoparasitism, antibiosis and competition. The antagonistic process is based on mycoparasitism, that is, *Trichoderma* produces extracellular enzymes which hydrolyze the cellular walls of phytopathogenic fungi. The main enzymes employed by *Trichoderma* in its mycoparasitic action are: β-1,6-glucanases, β-1,3-glucanases, cellulases, chitinases and proteases. In this context, the objectives of this study are to search for the optimal conditions to produce lytic enzymes by *T. atroviride* NBT1.1 and to identify those isoenzymes with cell-wall-degrading activities.

Material and methods

Fungal material

Trichoderma atroviride NBT1.1 was used in this study. Potato dextrose agar (PDA, Sigma Chemical) was used for growth of the culture.

Enzyme production

Two minimal salt media (MM1 and MM2) supplemented with an appropriate carbon source (glucose, purified chitin, or natural chitin) were assayed. The MM1 medium included: KH_2PO_4, 15 g/l; glucose, 5 g/l; $FeSO_4 \cdot 7H_2O$, 5 mg/l; $MnSO_4 \cdot H_2O$, 1.6 mg/l; $ZnSO_4 \cdot 7H_2O$, 1.4 mg/l; $CoCl_2 \cdot 6H_2O$, 3.7 mg/l; $(NH_4)_2SO_4$, 5 g/l; $MgSO_4 \cdot 7H_2O$, 0.59 g/l and $CaCl_2$, 0.6 g/l; pH=5.5. The MM2 medium included: KH_2PO_4, 9.5 g/l; glucose, 0.36 g/l; lactose, 1.91 g/l; sacarose, 0.24 g/l; KNO_3, 5 g/l; $CuSO_4 \cdot 5H_2O$, 1.25 µg/l; $FeSO_4 \cdot 7H_2O$, 2.78 mg/l; $MnSO_4 \cdot H_2O$, 10 mg/l; $ZnSO_4 \cdot 7H_2O$, 23 mg/l; $CoCl_2 \cdot 6H_2O$, 5.2 mg/l; $(NH_4)_2SO_4$, 2.64 g/l; $MgSO_4 \cdot 7\ H_2O$, 1.72 g/l and $CaCl_2$, 0.29 g/l; pH=5.5. Crude preparations were obtained by ammonium sulphate precipitation (90% saturation) of culture filtrates after growing the fungi during 4 days. Culture filtrates were concentrated approximately 35-fold.

Enzyme assay

The β-1,3, β-1,4 and β-1,6-glucanase activities were determinated by measuring the amount of the reducing sugars released from laminarin, carboxymethylcellulose and pustulan, respectively, as previously described (de la Cruz *et al.*, 1995; Mateos *et al.*, 1992; and Soler *et al.*, 1999). Chitinase activiy was determined by measuring the GlcNAc liberated from colloidal chitin (Reissig *et al.*, 1955; Boller *et al.*, 1983). Protease activity was assayed according to Howerda and Rogers (1992).

Gel electrophoresis

Electrophoresis under denaturing conditions (SDS-PAGE) was performed by the method of Laemmli (1970) with 4% acrylamide in the stacking gel and 12% acrylamide in the separating gel. Low molecular mass standard proteins were used for molecular mass determination.

Enzyme detection in SDS-PAGE gels

The proteins were renatured in the renaturalization buffer of the SDS-PAGE after electrophoresis (McGrew and Green, 1990). β-1,3, β-1,4 and β-1,6 glucanase, protease and chitinase isozymes were detected according to Pan et al. (1989), Mateos *et al.* (1992), Soler *et al.* (1999), García-Carreño *et al.* (1993) and Inbar *et al.* (1995), respectively.

Results and discussion

Lytic activities

Table 1 shows specific enzymatic activities found in extracts of *T. atroviride* NBT1.1 inoculated in the different assayed induction media. When glucose was used as a carbon source, enzyme production was negligible. The addition of chitin as a carbon source induced high lytic enzymatic activities. The medium MM2 supplemented with natural chitin (1%) provided higher lytic activities (β-1,3-glucanase, β-1,4-glucanase, β-1,6-glucanase, chitinase and protease) than MM1 supplemented with the same carbon source. The use of MM2 with natural chitin as an induction medium has the following advantages: requires a lower concentration of carbon source (1%) compared with MM1 (2%), and is based on a cheap waste product of the food industry.

Isozyme profiles

Isozyme profiles were obtained from NBT1.1 extract produced in MM2 supplemented with natural chitin (1%), which induced the higher lytic activities in this study. The β-1,3, β-1,4

and β-1,6 glucanase, chitinase and protease isozymes in NBT1.1 extract were detected and represented in Figure 1. The lytic isozyme variability was important in the case of β-1,3-glucanase. Some of the isozymes detected have not been described in the literature, such as the protease isozyme and part of the β-1,3-glucanase isozymes. No chitinolytic isozymes were detected with the fluorescent substrate used. This result could imply that the chitinases act through an endo-chitinolytic mechanism.

Table 1. Specific enzymatic activities of *Trichoderma atroviride* NBT1.1 inoculated in two minimum media (MM1 or MM2) supplemented with glucose or chitin.

Induction media	Specific enzymatic activities (U/mg)				
	ß-1,3-glucanase	ß-1,4-glucanase	ß-1,6-glucanase	Chitinase	Protease
MM1-G[1]	1,53±0,26	0,52±0,02	0,61±0,12	0,05±0,01	0,22±0,01
MM1-PC[2]	104,71±30,47	0,11±0,02	19,34±7,89	0,21±0,03	2,98±2,80
MM1-NC[3]	41,59±28,48	0,12±0,05	5,52±2,53	0,09±0,05	9,06±2,99
MM2-G[1]	0,31± 0,02	0,14±0,01	3,30±0,04	0,01±0,02	0,02±0,04
MM2-PC[4]	116,31± 8,43	0,26±0,01	24,66±2,34	0,16±0,12	1,70±0,21
MM2-NC[5]	121,76±56,79	0,24±0,15	11,46±0,68	0,22±0,13	12,32±2,28

[1] G: glucose (1%); [2] PC: purified chitin (2%); [3] NC: natural chitin (2%); [4] PC: purified chitin (1%); [5] NC: natural chitin (1%).

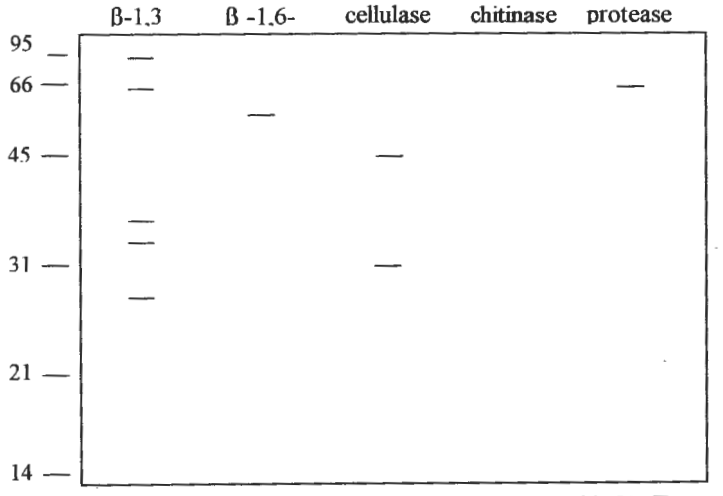

Figure 1. Diagram of the main extracellular lytic isozymes detected in NBT1.1 extract.

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Biological Control of Fungal and Bacterial Plant Pathogens
IOBC wprs Bulletin Vol. 24 (3) 2001
pp.375 - 378

Control of cucumber powdery mildew (*Sphaerotheca fuliginea*) with bacterial and fungal antagonists

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Abstract: Ninety *Bacillus* isolates were screened for antagonism against cucumber powdery mildew (*Sphaerotheca fuliginea*) on cucumber cotyledons. Twenty-seven selected isolates were further tested on young cucumber plants, at the cotyledon stage in a walk-in growth chamber. The best two, together with the known hyperparasite *Acremonium alternatum* and an unidentified hyperparasite (code CK1), were tested in a large-scale greenhouse experiment, in which the bacterial growth medium, tryptone soy broth (TSB), water control and pyrazophos were also included. Results obtained indicate that all biocontrol agents were effective against *S. fuliginea* compared to the water control, but they were less effective than pyrazophos. Bacteria and TSB were more effective than fungal hyperparasites at the final stages of the epidemic. The effectiveness of TSB and bacteria is being further investigated.

Keywords: Biological control, cucumber, *Acremonium alternatum, Sphaerotheca fuliginea,* powdery mildew, Bacillus antagonists, fungal antagonists, hyperparasites

Introduction

The damage caused by cucumber powdery mildew (Sphaerotheca fuliginea) and the increasing predominance of strains of the pathogen resistant to fungicides, call for alternative control methods. So far, several biological control agents, mostly hyperparasites, have been tested with moderate effectiveness (Bélanger *et al.*, 1998; Elad *et al.*, 1996; Malathrakis, 1997). On the other hand bacterial antagonists, often tested effectively against other pathogens (Seddon *et al.*, 1996), rarely have been tested against powdery mildews. The aim of the present work was to screen a large number of *Bacillus* isolates against cucumber powdery mildew and evaluate the field efficacy of the best of these together with fungal antagonists and a reference fungicide.

Materials and methods

Bioassay on detached cotyledons

Ninety Bacillus isolates, collected from plant surfaces, soil and composts, were tested on cotyledons of cucumber plants in Petri dishes containing the culture medium MSA (20g mannitol, 10g sucrose, 8g agar/L of tap water) against powdery mildew of cucumber. *Bacillus* isolates were grown in tryptone soy broth (TSB) on an orbital incubator at 37°C. Cotyledons were sterilised using 10% sodium hypochloride and then instantly dipped in liquid culture (10^8 spores/ml) of the *Bacillus* isolates amended with tween 80 (0,125%). Cotyledons were then inoculated by taking fresh conidia of *S. fuliginea* with a sterile Pasteur pipette and blowing on to the cotyledon surface. Four cotyledons treated with each *Bacillus* isolate were

placed on MSA medium in Petri dishes and incubated in a growth chamber maintained at 22°C with a 12h photoperiod. Infection was recorded seven days later on the basis of a 0-5 disease scale (0=healthy cotyledon, 1=start of infection, 2=1/4 of cotyledon infected 3=2/4 of cotyledon infected, 4=3/4 of cotyledon infected, 5=complete cotyledon infected).

Tests on young cucumber plants

Twenty-five of the most effective isolates were further tested on cucumber plants at the cotyledon stage. Seven plants were sprayed with liquid culture of each isolate (10^8 spores/ml). Inoculation was carried out by dusting cucumber leaves, heavily infected by powdery mildew, on to the surface of the experimental plants. Inoculated plants were incubated in a growth chamber at 22°C, 85% r.h., 12h photoperiod. Infection was recorded 10 days later by assessing the % of infected leaf area.

Greenhouse experiment

The following treatments were tested in an experiment carried out in a plastic greenhouse from early May till late June: water control (tap water); TSB 30g/L; pyrazophos 0.1ml/L; *Bacillus* isolate SB2 (1) 10^8 spores/ml; *Bacillus* isolate D4A2 10^8 spores/ml; *Acremonium alternatum* 10^7 spores/ml; CK1 (an unidentified hyperparasite) 10^6 spores/ml. They were arranged in a randomised block design with four replicates. Ten plants of the cultivar Pepinex 69 F1 were planted in each plot, in two rows. *Bacillus* isolates, TSB and pyrazophos were applied at 10-day intervals, with water control and hyperparasites at 7-day intervals. Tween 80 (0.125%) was added in all applications. Records were taken at 4-day intervals on a 0% to100% rating scale of leaf infection from the 8th till the 22nd of June.

Results

Most of the *Bacillus* isolates, tested on detached cucumber cotyledons, had a low to moderate effectiveness against *S. fuliginea*. Only a few of them were highly effective. Some isolates appeared to be phytotoxic (yellowing, brown spots etc.). Thirteen isolates, which reduced infection by more than 50% compared to TSB, appear in Fig. 1. Five of these reduced infection by 75% compared to the TSB control. Eight isolates with effectiveness higher than that of TSB on young cucumber plants are shown in Fig. 2.

Progress of infection in the plots of each treatment in the greenhouse trial is presented in Fig. 3. Results obtained indicate that all biocontrol agents were effective against *S. fuliginea* compared to water control throughout the experiment, but were significantly less effective than pyrazophos. *Bacillus* isolates and TSB were equally effective. Effectiveness of pyrazophos ranged from 75% to 95%, that of bacteria and TSB from 25% to 85% and fungal antagonists from 12% to 85%. Differences in efficacy between the biocontrol agents and pyrazophos were low at the initial stages of infection (<20%) but increased rapidly as soon as the infection exceeded 45%.

Discussion

Data obtained from the test on detached cotyledons and on young cucumber plants show that although a rather small number of *Bacillus* isolates were screened, several were effective against cucumber powdery mildew. This indicates that *Bacillus* spp. are potentially effective against powdery mildew. Screening *Bacillus* isolates on detached cucumber cotyledons placed in Petri dishes or on cucumber plants at the cotyledon stage gave similar results. Nearly all *Bacillus* isolates that were found to be effective against cucumber powdery mildew by the first method were found effective by the second method as well. Hence, both tests seem to be

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