Biological Control 63 (2012) 210–221

Contents lists available at SciVerse ScienceDirect

Biological Control

journal homepage: www.elsevier.com/locate/ybcon

Effects of fluorescent *Pseudomonas* spp. isolated from mushroom cultures on *Lecanicillium fungicola*

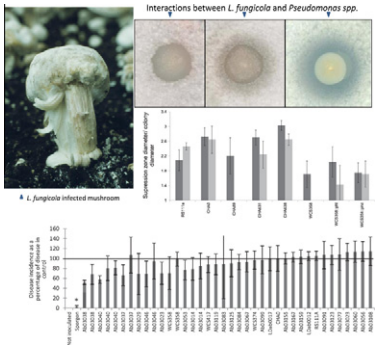
Roeland L. Berendsen [a,*], Stefanie I.C. Kalkhove [b], Luis G. Lugones [b], Johan J.P. Baars [c], Han A.B. Wösten [b], Peter A.H.M. Bakker [a]

[a] *Plant–Microbe Interactions, Department of Biology, Utrecht University, Padualaan 8, 3584 CH Utrecht, The Netherlands*
[b] *Molecular Microbiology, Department of Biology, Utrecht University, Padualaan 8, 3584 CH Utrecht, The Netherlands*
[c] *Plant Breeding, Plant Research International, Droevendaalsesteeg 1 , 6708 PB Wageningen, The Netherlands*

HIGHLIGHTS

- Bacterial strains were isolated from mushroom cultures.
- *Pseudomonas* spp. were dominant and screened for *in vitro* antagonism of *L. fungicola*.
- *In vitro*, *L. fungicola* is sensitive to competition for iron and antibiosis.
- None of the antagonistic strains effectively controlled dry bubble disease.
- The insensitivity of dry bubble disease to biological antagonism is discussed.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 14 May 2012
Accepted 30 July 2012
Available online 7 August 2012

Keywords:
Lecanicillium fungicola
Agaricus bisporus
Mushroom pathogen
Antagonistic bacteria
Pseudomonas
Dry bubble disease

ABSTRACT

Dry bubble disease, caused by *Lecanicillium fungicola,* is a serious economic problem in the cultivation of the white button mushroom. Biological control of the disease would meet the mushroom industry's efforts to minimize the use of chemicals. A total of 160 bacterial strains were isolated from colonized casing and screened for *in vitro* antagonism of *L. fungicola*. Fifty-three isolates inhibited *L. fungicola in vitro*. Using BOX-PCR, the 53 antagonistic isolates were grouped in 18 unique genotypes. Further characterization based on the 16S rDNA identified all isolates as *Pseudomonas* spp. Using previously characterized Pseudomonas isolates and their mutants it was determined that *L. fungicola* is sensitive to both siderophore-mediated competition for iron and production of antibiotics. However, when tested for disease suppression, none of the *Pseudomonas* spp. strains isolated from colonized casing effectively controlled dry bubble disease. The insensitivity of dry bubble disease to direct biological antagonism and the implications for biological control of mushroom diseases are discussed.

1. Introduction

Lecanicillium fungicola (Preuss) Zare and Gams (synonyms: *Verticillium fungicola* (Preuss) Hassebrauk, *Verticillium malthousei* (Preuss) Ware) causes dry bubble disease and is a serious and economically important problem in commercial production of the white button mushroom, *Agaricus bisporus.* Depending on the time point of infection, disease symptoms can vary. Small necrotic lesions on the caps of the fruiting bodies develop when mushrooms are infected late in development. Infections at earlier time points lead to partially deformed fruiting bodies called stipe blow-out or totally deformed and undifferentiated masses of mushroom tissue, the so called dry bubble (North and Wuest, 1993). Annual costs of *L. fungicola* infections for mushroom growers are estimated at 2–4% of the total revenue. Control of

* Corresponding author. Fax: +31 30 253 2837.
E-mail address: r.l.berendsen@uu.nl (R.L. Berendsen).

1049-9644/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.biocontrol.2012.07.012

L. fungicola relies on strict hygiene and the use of fungicides. Only few chemicals can be used for control of dry bubble as the fungal host is also negatively affected by many fungicides. The few fungicides that have been used are either no longer available or are becoming increasingly ineffective because of the development of fungicide resistance in the pathogen (Wuest et al., 1974; Bollen and van Zaayen, 1975; Fletcher and Yarham, 1976; Gea et al., 2005). Currently, the control of dry bubble disease relies heavily on the use of prochloraz-manganese (i.e. Sporgon), but reduced sensitivity of the pathogen to this fungicide has been reported (Gea et al., 2005; Grogan, 2008). Therefore, new ways to combat dry bubble disease are urgently needed.

A. bisporus is generally cultivated on a composted mixture of horse and chicken manure, but will not form fruiting bodies unless the compost is covered with a casing layer. In the casing layer vegetative hyphae of *A. bisporus* aggregate and eventually form mushrooms. Different materials have been used for the casing layer, but mostly peat is the main ingredient (Noble et al., 2003). Mushrooms are not formed on axenic casing, and so it was recognized that the casing microflora is important for the initiation of mushroom formation by *A. bisporus* (Visscher, 1988). Addition of activated carbon can reinstate mushroom formation in axenic casing. Mushroom formation can also be restored by thorough ventilation of axenic casing (Noble et al., 2009). It is therefore assumed that the casing microflora consumes volatile self-inhibiting compound(s) produced by *A. bisporus*. Removal of such compounds by the microflora seems at least partially responsible for the on-set of mushroom formation. Bacterial isolates that stimulated mushroom formation have been identified as *Pseudomonas putida* or close relatives thereof (Hayes et al., 1969; Rainey et al., 1990). However, these single isolates were never as effective in stimulating mushroom formation as the natural microflora.

As the vegetative mycelium of *A. bisporus* is not affected by *L. fungicola*, it is assumed that infection by *L. fungicola* takes place in the casing after the hyphae start aggregating (Cross and Jacobs, 1968; Calonje et al., 2000; Bernardo et al., 2004) . Germination of *L. fungicola spores* is effectively inhibited in microbially active casing (Cross and Jacobs, 1968). Thus, spore germination is postponed until the casing is colonized by *A. bisporus,* resulting in germination of *L. fungicola* just at the right moment, that is when the host is present in a susceptible stage (Berendsen et al., 2012). The sensitivity of *L. fungicola* to microbial antagonism may be exploited for control of the pathogen. Antagonistic microorganisms in soils have been exploited for effective control of fungal plant pathogens and this has resulted in a number of commercial products (Fravel, 2005; Weller et al., 2002). Mechanisms that are involved in plant disease suppression by bacteria, fluorescent *Pseudomonas* spp. in particular, have been studied in detail over the last decades and include siderophore-mediated competition for iron (Duijff et al., 1999), consumption of pathogen stimulatory compounds (Van Dijk and Nelson, 2000), production of antifungal compounds (Chin-A-Woeng et al., 2003; Haas and Defago, 2005) or lytic enzymes (Shapira et al., 1989), and induction of systemic resistance (Van Loon et al., 1998; Bakker et al., 2007). All these modes of action may also be effective to control *L. fungicola* infections.

Biocontrol bacteria that are introduced into the environment have to compete with the indigenous microflora. Often, the abundance of the introduced biocontrol bacterium decreases in time, resulting in reduced protection against the pathogen (Mazzola et al., 1992; Raaijmakers et al., 1995; Lugtenberg and Dekkers, 1999). Thus the selection of possible biocontrol agents for the dry bubble disease should focus on microorganisms that naturally occur in the casing soil in high densities, and preferably associated with *A. bisporus*. In the compost and the casing soil bacteria are in close contact with the *Agaricus* mycelium (Masaphy et al., 1987; Miller et al., 1995). Fluorescent *Pseudomonas* spp. form a dominant group within the bacterial casing microflora and population densities of these pseudomonads increase when the casing is colonized by *A. bisporus* (Doores et al., 1986; Samson, 1986; Miller et al., 1995; Fermor et al., 2000; Pardo et al., 2002). The increase in populations of *Pseudomonas* spp. is comparable to the superior ability of fluorescent pseudomonads to colonize the rhizosphere (Lugtenberg and Dekkers, 1999) and may be explained by the fact that *A. bisporus* secretes sugars, amino acids and volatiles (Grewal and Rainey, 1991; Noble et al., 2009) that stimulate growth and activity of these bacteria. Interestingly, *Pseudomonas* spp. are the most extensively studied group of biocontrol bacteria (Chin-A-Woeng et al., 2003; Bakker et al., 2007; Weller, 2007; Höfte and Altier, 2010) because they have many traits that make them effective biocontrol agents of plant pathogens. They can grow on a wide variety of substrates, are abundant in nature, have a high growth rate, grow at relatively low temperatures and have a variety of mechanisms to suppress growth of other microorganisms (Weller, 2007). These bacterial species are thus also interesting candidates for biocontrol of *L. fungicola*.

Some attempts to identify biological control agents effective against *L. fungicola* have been reported (de Trogoff and Ricard, 1976; Bhatt and Singh, 2000; Singh et al., 2000; Bhat et al., 2010), but serious follow up has not been published. Moreover, culture tests are necessary to confirm the efficacy of the bacteria in controlling the dry bubble disease (Largeteau and Savoie, 2010). This study aimed at identifying bacteria in the mycosphere of *A. bisporus* that are antagonistic to *L. fungicola* and that can effectively suppress dry bubble disease. Known biocontrol agents of plant pathogens were used to identify possible mechanisms for biocontrol of dry bubble disease. It was found that, *in vitro, L. fungicola* was sensitive to competition for iron and to antibiosis. *In vitro* antagonistic isolates that were isolated from the colonized casing and compost were further characterized and screened for their ability to control the dry bubble disease in mushroom cultures. The effectiveness of the putative biocontrol agents was compared to that of prochloraz-manganese.

2. Materials and methods

2.1. Fungal cultures

Spore suspensions (10^6 spores/ml) of *L. fungicola* strain V9503 (Fungal strain collection of PRI Plant Breeding) were stored in phosphate buffer (0.21 M NaH_2PO_4, pH 7.2) with 10% glycerol at −80 °C. *L. fungicola* strain V9503 was cultured on potato dextrose agar (PDA) (Difco, Lawrence, USA) by plating 100 µl of spore suspension and incubating for 5 days at 24 °C. Spore suspensions were prepared from these cultures by adding 10 ml of demineralized water (DEMI) to each Petri dish and filtering over sterile glass wool to remove mycelial fragments. Densities of the suspensions were determined using a haemocytometer.

2.2. Isolation of bacteria

Bacteria were isolated from aggregated hyphae of *A. bisporus* taken from colonized casing. Colonized casing was sampled from mushroom production cultures of *A. bisporus* when the first pins appeared on the casing. Aggregated hyphae were isolated from the casing using tweezers and they were incubated for 20 min in 10 mM $MgSO_4$ to remove loosely-adhering bacteria. Firmly attached bacteria were brought in suspension through sonication as described by Cho et al., 2003. Suspensions were plated on King's medium B agar (KB agar (King et al., 1954)), 10% tryptic soy agar (TSA; per liter: 3 g tryptic soy broth (Difco), 15 g granulated agar (Difco)), Casing extract agar (Rainey, 1991) and *A. bisporus* mycelial

exudate agar (mycelial exudates as described by Grewal and Rainey (1991)), 15 g granulated agar (Difco) per liter) and incubated at 28 °C for 48 h. From each of these media, 40 colonies were selected randomly and streaked to confirm purity.

To exclude *Pseudomonas tolaasii* from the bacterial collection the so called white-line test, that is indicative for this bacterial mushroom pathogen (Wong and Preece, 1979), was used. Bacteria were stored in KB with 20% glycerol at −80 °C.

2.3. Screening for in vitro antagonism to L. fungicola

The bacterial isolates were point inoculated on Pseudomonas agar F (Difco) and incubated for 2 days at 24 °C. Subsequently, a *L. fungicola* spore suspension (10^6 conidia/ml) was atomized over the plates using a test tube atomizer (Desaga GmbH, Wiesloch, Germany). Plates were sealed with Parafilm and incubated at 24 °C. The bacterial colony diameter and the diameter of the zone in which growth of *L. fungicola* was inhibited were determined after 3 days. The effect of siderophore and antibiotic production on *L. fungicola* was determined with well-studied biocontrol strains and mutant derivatives (Table 1).

2.4. BOX-PCR analysis of antagonistic isolates

Isolates were cultured on KBA at 28 °C for 1 day. Single colonies were transferred to 20 µl sterile lysis buffer (0.05 M NaOH, 0.25% SDS) and heated to 95 °C for 20 min. Lysates were quickly cooled on ice and 200 µl cold sterile water was added. Debris was pelleted by brief centrifugation (12,000×*g*) and lysates were stored at −20 °C.

BOX-PCR was performed with primer BOX A1R (5′-CTA CGG CAA GGC GAC GCT GAC G) (Versalovic et al., 1994). 0.5 µl of the colony lysate was added to a total volume of 25 µl BOX-PCR-reaction mix (1× Stoffel buffer, Applied Biosystems, Foster City, CA, USA), 3.75 mM $MgCl_2$, 600 µM of each dNTP, 1 µM primer BOX A1R and 2.5 U AmpliTaq Stoffel fragment DNA polymerase (Applied Biosystems, Foster City, CA, USA). PCR conditions used in the thermocycler (Hybaid, Ashford, UK) were 7 min at 95 °C, followed by 30 cycles of 30 s at 90 °C, 1 min at 95 °C, 1 min at 52 °C and 8 min at 65 °C, and a final extension of 16 min at 65 °C. Amplified PCR fragments were analyzed by electrophoresis on 1.5% agarose gels in 1× TAE buffer (40 mM Tris–acetate/1 mM EDTA, pH 8).

2.5. Identification of antagonistic isolates

The V6-V8 region of the *16S rRNA* gene was amplified with primers 968f_GC and 1401R (Nübel et al., 1996). 1 µl of the colony lysate (see above) was added to a total volume of 50 µl PCR reaction mixture (1× PCR buffer 2, Roch Diagnostics, Mannheim, Germany), 250 µM of dNTPs, 200 nM of each primer, and 2.5 U Expand Long Template enzyme (Roch Diagnostics). PCR conditions used in the thermocycler (Hybaid, Ashford, UK) were 5 min at 94 °C, followed by 35–40 cycles of 1 min at 94 °C, 1 min at 66 °C and 3 min 72 °C, and a final extension of 10 min at 72 °C. Presence of PCR product was checked by electrophoresis on 1.5% agarose gels in 1x TAE buffer (40 mM Tris–acetate/1 mM EDTA, pH 8). PCR-products were sequenced by Baseclear (Leiden, the Netherlands). Ribosomal database project (RDP) Classifier was used to assign taxonomic lineage to the genus level (http://rdp.cme.msu.edu).

2.6. Bioassay for suppression of dry bubble disease

Isolates were grown on KBA overnight at 28 °C. Cells were harvested in 10 ml $MgSO_4$ and washed twice by centrifugation (10 min, 4500 rpm) The pellet was resuspended in sterile 10 mM $MgSO_4$ and cells were added to casing soil (CNC, Milsbeek, The Netherlands) at a density of 10^6 colony forming units (cfu)/g of casing. Mushrooms were cultured in Economic premium climate chambers (Snijders scientific, Tilburg, the Netherlands, dimensions, 99 × 63 × 134 cm). For each isolate, three plastic containers (20 × 30 × 22.5 cm) were filled with 3.5 kg of compost colonized by *A. bisporus* strain A15 (CNC, Milsbeek, the Netherlands) and

Table 1
Biocontrol strains and their mutant derivatives that were used in this study.

Strain	Relevant characteristics	References
P. fluorescens CHA0	Isolated from tobacco rhizosphere; produces DAPG, PLT, PRN and HCN; produces iron regulated PSB, PCH and SA	Laville et al. (1992)
CHA89	*gacA* mutant of CHA0; lacks production of DAPG, PLT, PRN and HCN ; produces PSB, PCH and SA	Laville et al. (1992)
CHA631	*phlA* mutant of CHA0, does not produce DAPG	Schnider-Keel et al. (2000)
CHA638	*phlF* mutant of CHA0, constitutively produces DAPG	Schnider-Keel et al. (2000)
P. putida WCS358r	Spontaneous rifampicin-resistant mutant of WCS358. Wild type isolated from potato rhizosphere, produces PSB	Geels and Schippers (1983) and Glandorf et al. (1992)
WCS358-JM213	Tn*5* mutant of WCS358r that does not produce PSB	Marugg et al. (1985))
WCS358::phl	Transformant of WCS358 constitutively producing DAPG	Bakker et al. (2002b)
WCS358::phz	Transformant of WCS358r constitutively producing PCA	Bakker et al. (2002b)
P. fluorescens WCS374r	Spontaneous rifampicin-resistant mutant of WCS374. Wild type isolated from potato rhizosphere; produces PSB, (PSM) SA	Geels and Schippers (1983)
WCS374-02	Psb^-, Psm^+, SA^+, Tn*5* transposon mutant of WCS374	Weisbeek et al. (1986)
WCS374-4A1	Psb^+, Psm^-, SA^+, *pmsA* mutant of WCS374r obtained by site-directed mutagenesis	Djavaheri (2007) and De Vleesschauwer et al., 2008
WCS374-AT12	Psb^-, Psm^-, SA^+, Tn*5* transposon mutant of 4A1	Djavaheri (2007) and De Vleesschauwer et al. (2008)
WCS374-4B1	Psb^+, Psm^-, SA^-, *pmsB* mutant of WCS374r obtained by site-directed mutagenesis	Djavaheri (2007) and De Vleesschauwer et al. (2008)
WCS374-BT1	Psb^-, Psm^-, SA^-, Tn*5* transposon mutant of 4B1	Djavaheri (2007) and De Vleesschauwer et al. (2008)
P. fluorescens WCS417r	Spontaneous rifampicin-resistant mutant of WCS417. Wild type was isolated from wheat rhizosphere	Lamers et al. (1988) and Van Wees et al. (1997)
WCS417-M634	Tn*5* mutant of WCS417 that does not produce PSB	Duijff et al. (1993)
RS111-a	Unspecified antifungal compound	de Boer et al. (1999)and Bakker et al. (2002a)

DAPG, 2,4-diacetylphloroglucinol; PLT, pyoluteorin; PRN, pyrrolnitrin; PSB, pseudobactin; PCH, pyochelin; SA, salicylic acid, PCA, phenazine-1-carboxylic acid; PSM, pseudomonine.

covered with 1 kg of casing mix (CNC, Milsbeek, the Netherlands). Five isolates were tested in each experiment and three control containers were taken along in which casing soil was treated with sterile 10 mM $MgSO_4$.

Conditions were set to 24 °C and 95% relative humidity(RH) for the first 8 days and subsequently set to 20 °C and 88% RH until the end of the experiment. On day 8, casing was inoculated with *L. fungicola* V9503 by spreading 50 ml of a conidial suspension (6.10^4 conidia/ ml) on the casing of each box, resulting in an inoculum density of 10^6 conidia/ m^2 of casing. As a positive control, containers were treated with 200 ml 0.1% Sporgon (BASF, Arnhem, the Netherlands) immediately after inoculation with *L. fungicola*. The first two flushes were harvested and scored in four categories: healthy, spotted cap, stipe blow-out and dry bubble (Berendsen et al., 2010). Weight and number of mushrooms in each category was determined.

Isolates *Ab03038* and *Ab3040* (Table 2) were tested as described before but with 18 replicates per treatment evenly distributed over three climate chambers. Six replicates per treatment were used in experiments to compare effects of *P. fluorescens* CHA638 and CHA631, mutant derivatives of *P. fluorescens* CHA0 that either overproduce (CHA638) or no longer express (CHA631) DAPG, with those of their wild type CHA0.

2.7. Survival of bacteria in the casing and attachment to mushrooms

The survival of the rifampicin-resistant *P. fluorescens* WCS417r and WCS374r, and *P. putida* WCS358r was tested during the bioassay for suppression of dry bubble disease. Additionally, bacterial survival was investigated by incubating casing containing the bacteria (added as described above) in Petri dishes. Plates were sealed with Parafilm and incubated at 24 °C. Approximately 1 g of casing was taken from the mushroom cultures and non-colonized casings 0, 7, 14 and 22 days after addition of bacteria. Casing samples of mushroom cultures and non-colonized casings were suspended in 10 ml 10 mM $MgSO_4$. Dilution series of the suspensions were plated on 10% TSA for quantification of the total culturable bacteria and on KBA supplemented with 150 μg rifampicine and 100 μg Delvocid (DSM Food Specialties, Delft, the Netherlands) per ml for quantification of the rifampicin-resistant pseudomonads.

To determine population densities of the introduced bacteria on the mushroom caps, healthy mushrooms were harvested at day 23, and bacteria were suspended in 20 ml 10 mM $MgSO_4$ by shaking for 2 h at 4 °C and sonication for two times 30 s in a sonication bath (Julabo). Dilution series of the suspensions were plated as described previously.

2.8. Assay for antagonism on mushroom caps

Fresh mushrooms of strain Sylvan A15 (Hooijmans Champignons, Kerkdriel, the Netherlands) were harvested and the stipe was removed. Bacterial cell suspensions of selected isolates (10^9 cfu/ml) and a conidial suspension of *L. fungicola* (10^6 conidia/ml) were prepared as describe above. The suspensions of *L. fungicola* conidia were mixed with each bacterial cell suspension in a 1:1 (v:v) ratio. Fifteen mushrooms were inoculated for each isolate, by placing 2 μl of the mixture on the surface of a mushroom cap, halfway in between the center of the mushroom cap and its edge. As a control, 15 mushroom caps were inoculated with a suspension of *L. fungicola* mixed with 0.1% Sporgon (BASF, Arnhem, the Netherlands). Moreover, each mushroom cap was inoculated at the opposite side with a suspension containing *L. fungicola* conidia only. The inoculated mushrooms were incubated in an Economic premium climate chamber (Snijders scientific, Tilburg, the Netherlands) at 24 °C and 95% relative humidity. Diameters of the lesions were determined after 4 days of incubation.

Antagonism against *L. fungicola* was tested for two isolates on mushrooms that were not harvested. Mushrooms of strain Sylvan A15 were cultivated as described above. Mixtures of bacterial cells and *L. fungicola* conidia were prepared for the isolates Ab03128 and Ab03038 (Table 2) as described above. Eight mushrooms of 0.5–1 cm in diameter were inoculated with a mixture of bacterial cells and *L. fungicola* conidia on one side of the cap and with conidia of *L. fungicola* on the other side. Lesion diameters were scored after 4 days.

2.9. Statistics

Statistical analysis was performed with SPSS 16.0. Significant differences in the bioassay for suppression of dry bubble disease were analyzed by ANOVA of the treatments in one climate chamber. The bioassay of isolates Ab03038 and Ab3040 with 18 replicates was performed in a randomized block design with each climate chamber as a block. Lesion size of *L. fungicola* infections on a mushroom cap was compared to control lesions with a paired Students *T*-test. Bacterial plate counts were 10log transformed and analyzed by ANOVA.

Table 2
Overview of the *Pseudomonas* spp. selected for *in vitro* antagonism against *L. fungicola*.

BOX-type	Frequency	Type of *in vitro* antagonism (Fig. 1)	Selected isolate	Taxonomy as assigned by RDP classifier
I	1	B	Ab03023	Pseudomonas
II	1	B	Ab03037	Pseudomonas
III	12	B	Ab03040	Pseudomonas
IV	1	C	Ab03046	Pseudomonas
V	1	C	Ab03053	Pseudomonas
VI	14	B	Ab03038	Pseudomonas
VII	12	C	Ab03029	Pseudomonas
VIII	1	B	Ab03056	Pseudomonas
IX	1	C	Ab03067	Pseudomonas
X	1	B	Ab03083	Pseudomonas
XI	1	B	Ab03084	Pseudomonas
XII	1	B	Ab03091	Pseudomonas
XIII	1	C	Ab03108	Pseudomonas
XIV	1	B	Ab03119	Pseudomonas
XV	1	B	Ab03125	Pseudomonas
XVI	1	B	Ab03155	Pseudomonas
XVII	1	B	LDab0012	Pseudomonas
XVIII	1	B	LDab0017	Pseudomonas

3. Results

3.1. Isolation of bacteria and screening for in vitro antagonism

In total 160 bacterial strains were isolated from casing colonized by *A. bisporus*. The isolates were screened for their abilities to inhibit spore germination and hyphal growth of *L. fungicola* on KBA. The majority of the isolates (107) did not affect germination and growth of *L. fungicola* (Fig. 1a). The remaining 53 isolates did inhibit *L. fungicola* in the zone surrounding their colony. Growth of *L. fungicola* was slightly reduced by 37 of these isolates (Fig. 1b), whereas 16 isolates produced clear zones of inhibition (Fig. 1c).

The genotypic diversity of the antagonistic isolates was determined using BOX-PCR (Table 2). Fifteen out of the 53 box-fingerprints were unique. The remaining 38 BOX-fingerprints comprised three different BOX-types, consisting of 12, 12 and 14 isolates, respectively. 16S rDNA was amplified for at least one isolate of each BOX-type and sequenced (Baseclear, Leiden, the Netherlands). RDP Classifier was used to assign taxonomic lineage to genus level. All sequences were assigned to the genus *Pseudomonas* with an estimated reliability of 100%.

3.2. In vitro effects of bacterial antibiotic and siderophore production on L. fungicola

In vitro antagonism of known biocontrol agents against *L fungicola* was investigated on KBA and KBA with 200 μM $FeCl_3$. Sensitivity of *L. fungicola* to antibiosis was investigated using strains of fluorescent pseudomonads that are known producers of antifungal compounds. *P. fluorescens* RS111a, CHA0, CHA89, CHA631 and CHA638 and *P. putida* WCS358, WCS358::phl and WCS358::phz all inhibited the growth of *L. fungicola* on KBA (Fig. 2a). Addition of $FeCl_3$ abolished the inhibition by CHA89 and WCS358. Both strains do not produce antifungal compounds. CHA89 is *gacA* knock-out of CHA0 impaired in the production of antibiotics. The inhibition zone produced by CHA0 was not affected by the iron concentration of the medium. Mutants of WCS358, that were generated to constitutively produce either PCA or DAPG, produced a bigger inhibition zone under iron-rich conditions than the wild type. It can be concluded that on iron-rich medium, *L. fungicola* inhibition is mediated by antibiotics.

Mutants defective in the production of siderophores have been generated for *P. fluorescens* strains WCS374r and WCS417r, and *P. putida* strain WCS358r. The three wild-type strains all produce pseudobactin. Analysis of the pseudobactins of WCS358 and WCS374 revealed that their structures are different (Fuchs et al., 2001; Djavaheri, 2007). In addition to pseudobactin, WCS374r also produces the siderophores pseudomonine and salicylic acid (SA) (Mercado-Blanco et al., 2001). Siderophore knock-out mutants of these three strains were compared to the parental strains for their ability to inhibit *L. fungicola*. The wild type strains all inhibited *L. fungicola* on KBA, whereas the pseudobactin knock-out mutants were less (WCS374r) or not (WCS358r and WCS417r) inhibitory (Fig. 2b). WCS374r-4A1, in which the production of pseudomonine is knocked out and which consequently produces more pseudobactin (Djavaheri, 2007) showed more inhibition of *L. fungicola* than the wild-type. This additional inhibition is not observed for WCS374r-4B1, which only produces pseudobactin and not SA. WCS374r-AT12, in which production of both pseudobactin and pseudomonine is knocked out, did not inhibit *L. fungicola*. It can be concluded that on iron poor medium, *L fungicola* inhibition is mediated by siderophores and especially pseudobactin.

3.3. Effects of selected bacterial isolates on dry bubble disease

Unique BOX-PCR fingerprint representatives of the *Pseudomonas* spp. isolates that were antagonistic to *L. fungicola in vitro*, were tested for their ability to control dry bubble disease in mushroom cultures (Fig. 3). The bacteria were mixed through the casing at a density of 10^6 cfu/g casing before applying the casing layer to the compost. The pathogen was inoculated on the casing 8 days after applying the casing. Within 2–3 weeks after casing application a first flush of mushrooms appeared.

Sporgon reduced the dry bubble disease significantly. Addition of antagonistic bacteria to the casing of mushroom cultures never significantly reduced dry bubble disease compared to the untreated control. Disease incidence in cultures treated with bacteria ranged from 55% of the disease in the control of the same experiment for isolate Ab3038 to 114% of the disease in the control for Ab3108. Isolates Ab03038, Ab03040, Ab03037, Ab03046, Ab03023 and WCS358 were found to reduce disease incidence with more than 20% in the first experiments and were therefore tested again in subsequent experiments. Only Ab3038 and Ab3040 reduced disease consistently with more than 20%, but never statistically significant. However, this bioassay was performed with only three replicates.

To increase the power of the bioassay, it was therefore performed with 18 replicates for each of these strains. Compared to the control treatment, disease was slightly, but not significantly, reduced to 91% by Ab03038 and 97% by Ab03040 (Fig. 4). This experiment was repeated with similar results. *P. fluorescens* CHA0

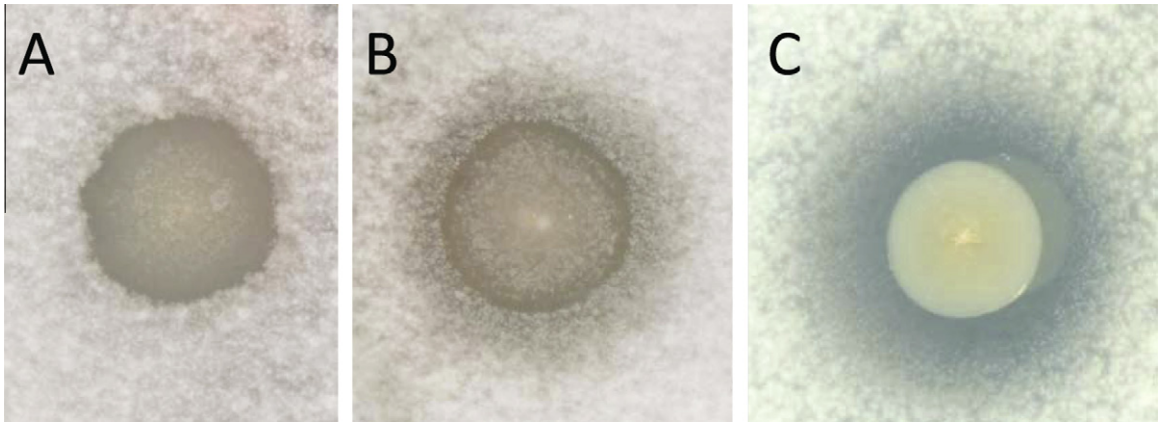


Fig. 1. *In vitro* antagonism of bacterial isolates to *L. fungicola* after 72 h. In a zone around their colony, bacteria: (A) did not affect growth of *L. fungicola*, (B) reduced growth of *L. fungicola*, (C) inhibited growth of *L. fungicola*.

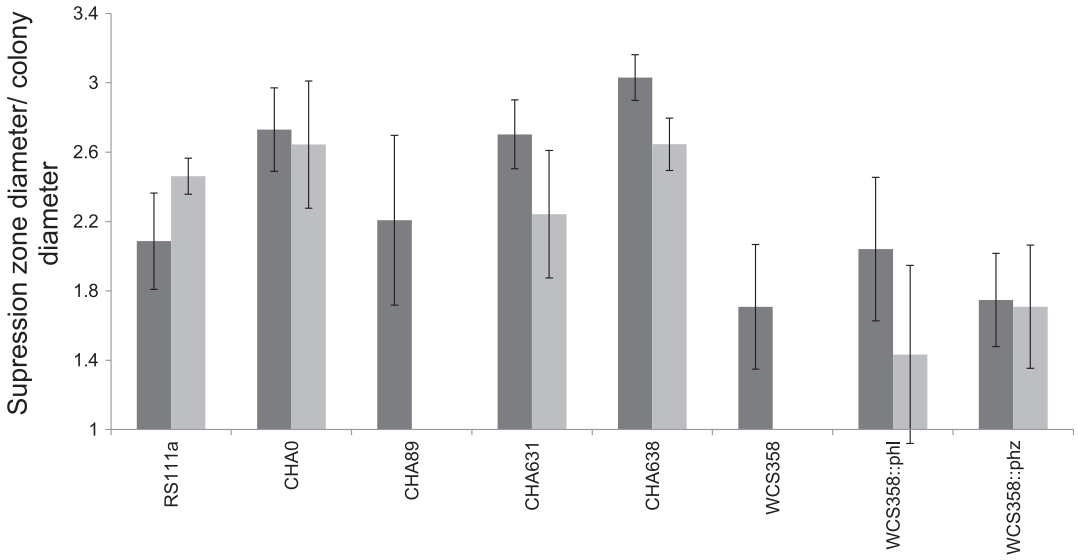


Fig. 2a. Quantification of *in vitro* antagonism of RS111a, CHA0, WCS358r and various mutant strains to *L. fungicola*. CHA0 produces four antibiotics (DAPG, PLT, PRN and HCN). Its mutant derivatives are affected in the production of antibiotics: CHA89 (Δ*gacA*) produces no antibiotics; CHA631 (Δ*phlA*) does not produce DAPG, whilst CHA638 (Δ*phlF*) overproduces DAPG. WCS358r produces no known antibiotics but its mutant derivatives constitutively produce DAPG (WCS358::phl) or PCA (WCS358::phz). Bars represent the average diameter of the inhibition zone around three bacterial colonies on KB (dark bars) or on KB amended with $FeCL_3$ (light bars). Error bars represent standard deviations.

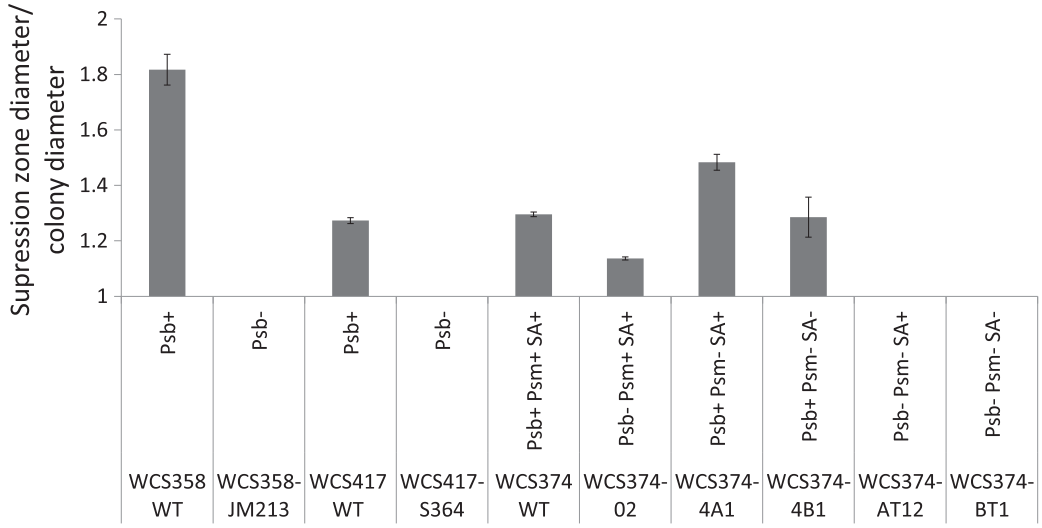


Fig. 2b. Quantification of *in vitro* antagonism of WCS358r, WCS417r, WCS374r and various mutant strains to *L. fungicola*. Mutants derived from WCS358 and WCS417 do not produce siderophores. Mutants derived from WCS374r have the following characteristics: 02 (Psb-, Psm+, SA+), 4A1 (Psb+, Psm-, SA+), AT12 (Psb-, Psm-, SA+), 4B1 (Psb+, Psm-, SA-), and BT1 (Psb-, Psm-,SA-), where Psb = pseudobactin and Psm = pseudomonine. Bars represent the average diameter of the inhibition zone around three bacterial colonies on KB. Error bars represent standard deviations.

was the most effective antagonist *in vitro*, but *in vivo* did not affect disease incidence (Fig. 3). To exclude the possibility that the effective antibiotic DAPG was not produced by CHA0 in the casing, a mutant that constitutively produces DAPG (CHA638, *phlF knock-out)* was included in the bioassay, and as a control mutant CHA631 (*phlA knock-out)* that does not produce DAPG. Both the wild type CHA0 and the mutants CHA638 or CHA631 (Fig. 5a and b) did not affect disease incidence significantly making it unlikely that bacterially produced DAPG can contribute to control of dry bubble disease.

3.4. Assay for antagonism on the mushroom caps

To study interactions between the bacterial isolates selected in this study and *L. fungicola* on the fruiting body of Agaricus, suspensions of the pathogen, in which the bacteria were also suspended were prepared. Development of lesions was measured after inoculation with a suspension of the pathogen, in which the bacteria were also suspended (treatment) or not (control). Two microliter droplets of these suspensions were inoculated on the caps of harvested mushroom. Lesions appeared within 2 days and their diameters were measured after 4 days (Fig. 6a). None of the bacterial isolates significantly affected lesion diameter. The treatment with Sporgon drastically reduced lesion diameter.

Because harvested mushrooms start decaying rapidly after they have been detached from the mycelium (Brennan et al., 2000) and thus may have increased levels of nutrients available to the pathogen that can interfere with bacterial antagonism (Garbeva et al., 2011), two isolates were also tested on mushrooms still attached to the mycelium. The effect of the isolates Ab03038 (most effective in bioassay) and Ab03128 (not antagonistic *in vitro*) and Sporgon was tested on growing mushrooms (Fig. 6b). Sporgon

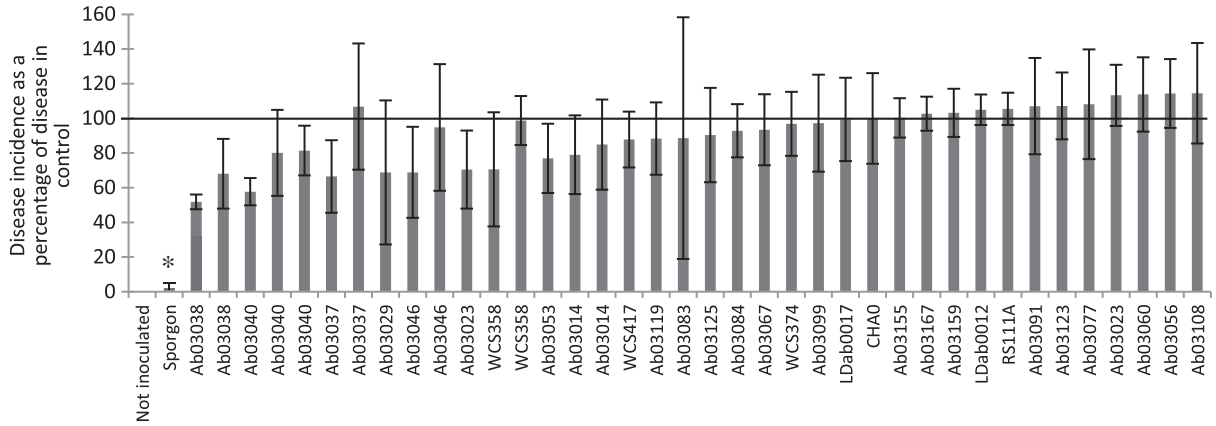


Fig. 3. Effect of selected *Pseudomonas* isolates on dry bubble disease. Bars represent the average percentage of disease in three treated mushroom cultures relative to disease in the control-treatment which was set to 100%. Error bars represent standard deviation. ∗ denotes significant difference from control treatment as determined through ANOVA.

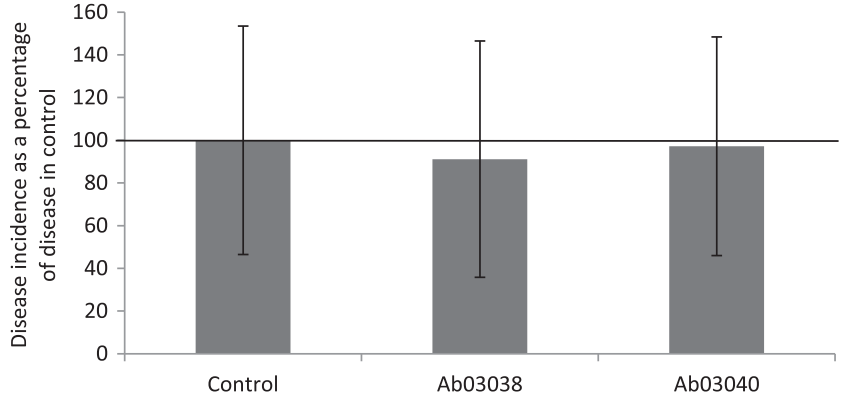


Fig. 4. Effect of *Pseudomonas* isolates Ab03038 and Ab03040 on dry bubble disease. Bars represent the average percentage of disease in 18 treated mushroom cultures relative to disease in the control-treatment, which was set to 100%. Error bars represent standard deviation.

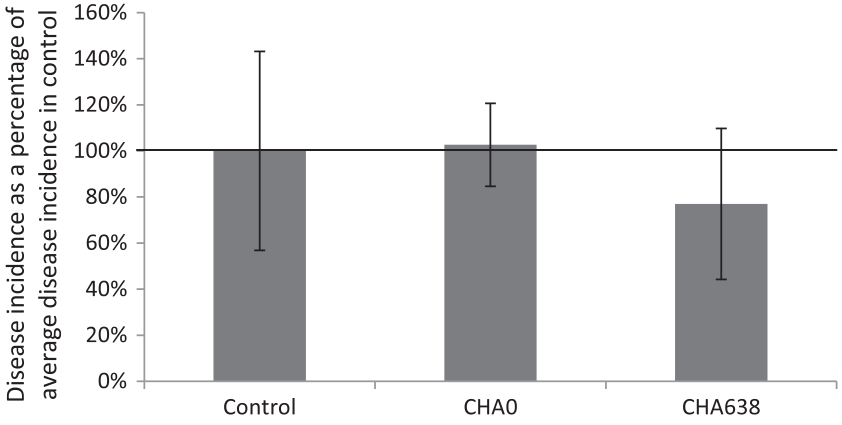


Fig. 5a. Effect of *P. fluorescens* CHA0 and CHA638 on dry bubble disease. Bars represent the average percentage of disease in six treated mushroom cultures relative to disease in the control-treatment, which was set to 100%. Error bars represent standard deviation.

completely inhibited the formation of lesions, while the size of the lesions was again not affected by the isolates.

3.5. Survival of bacteria in the casing

The ability of strains of fluorescent *Pseudomonas* spp. to maintain relatively high population densities is an important trait in relation to their abilities to control diseases. Therefore, the survival of rifampicin-resistant derivatives of *P. fluorescens* WCS374r and WCS417r, and *P. putida* WCS358r was tested in the casing in the bioassays (Fig. 7a). In the first week population densities of total bacteria, as determined on 1/10 TSA, increased 71-fold. In this week the casing was colonized by vegetative hyphae of *A. bisporus*. In the 2 weeks that followed, total number of bacteria decreased

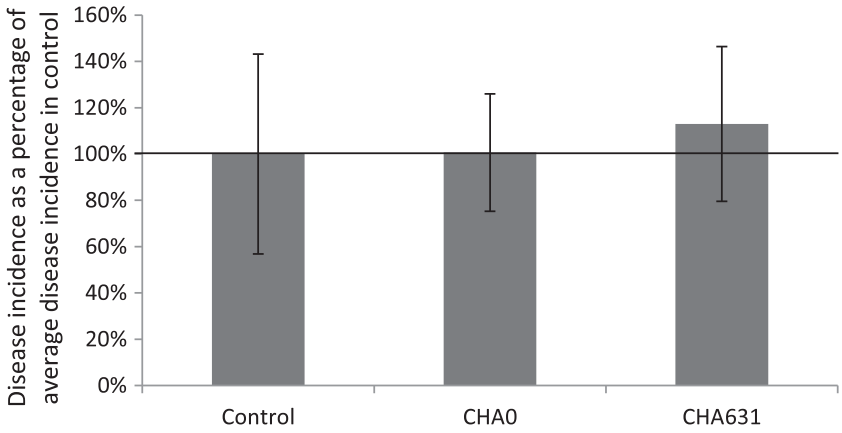


Fig. 5b. Effect of *P. fluorescens* CHA0 and CHA631 on dry bubble disease. Bars represent the average percentage of disease in six treated mushroom cultures relative to disease in the control-treatment, which was set to 100%. Error bars represent standard deviation.

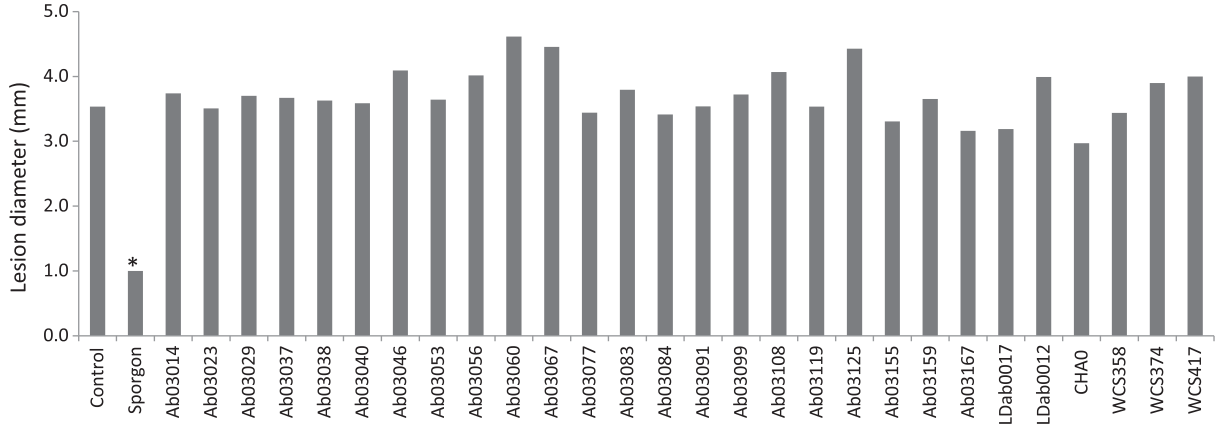


Fig. 6a. Lesion diameter on harvested mushroom caps 4 days after inoculation with a suspension of *L. fungicola* spores and selected bacterial strains. Asterisk denotes significantly smaller lesion diameter than the control treatment.

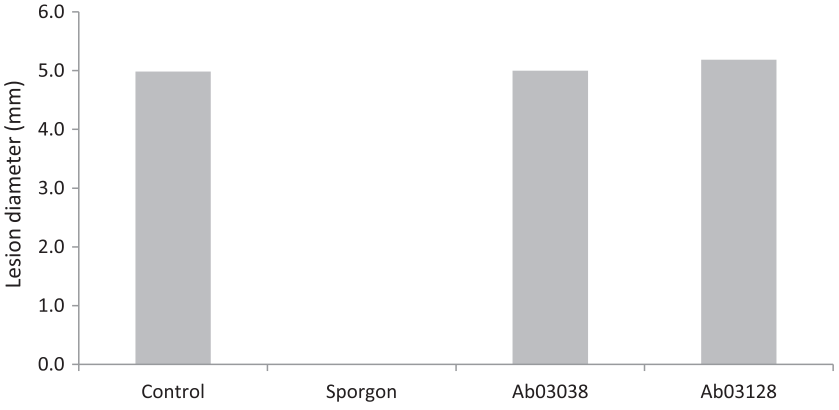


Fig. 6b. Lesion diameter on mushroom caps that were still attached to the mycelium, 4 days after inoculation with a suspension of *L. fungicola* spores and selected bacterial strains.

again. During the experiment, populations of WCS358r followed a pattern similar to those of the total culturable bacteria. Populations of WCS417r remained stable throughout the experiment, while the population of WCS374r decreased from 10^6 cfu/g casing at the start of the experiment to approximately 10^4 cfu/g casing at day 22. On day 23 of the bioassay, presence of bacteria on mushrooms of the second flush was investigated. For each treatment, the total number of culturable and the number of rifampicin-resistant bacteria on the caps of healthy mushrooms were determined (Fig. 7b). The total number of bacteria did not differ significantly between the three treatments and ranged between 10^5 and 10^6 cfu/g of mushroom cap. The numbers of WCS374r on mushrooms were sig-

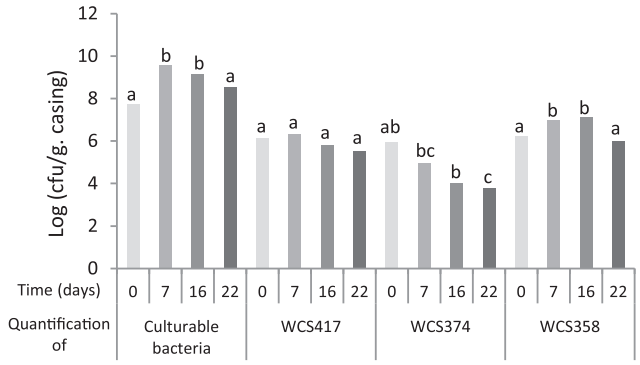


Fig. 7a. Numbers (log cfu/g casing) of total culturable bacteria and rifampicine-resistant strains in the casing during a bioassay. Letters indicate significant differences between time points. Bars represent the average number of three samples for the three rifampicine-resistant strains and of all nine samples for the culturable bacteria.

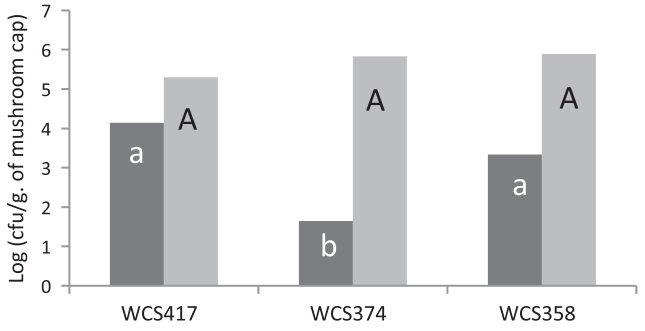


Fig. 7b. Numbers (log cfu/g casing) of total culturable bacteria (light bars) and rifampicine-resistant strains (dark bars) on mushroom caps during a bioassay. Letters indicate significant differences between treatments. Bars represent the average number of three samples.

nificantly lower than of WCS417r and WCS358r. The numbers of WCS358r and WCS417r on mushrooms did not differ significantly. The proportions of the selected bacteria on the mushrooms were comparable to the proportions in the casing on day 22 with less cfu of WCS374r than the other two bacteria.

Survival of WCS417r, WCS374r and WCS358r was also determined in casing that was not colonized by mycelium of *A. bisporus* (Fig. 8). None of the bacteria were able to proliferate in this casing soil, and the population densities of all strains declined significantly in time. Again, population densities of WCS374r were lower than those of WCS417r and WCS358r.

4. Discussion

Biological control of fungal pathogens using antagonistic bacteria has proven its merits in the management of plant diseases (Penyalver et al., 2000; Raaijmakers et al., 2009; Bailey et al., 2010). We therefore investigated the potential of antagonistic bacteria to control *L. fungicola,* the causal agent of dry bubble disease of the white button mushroom.

One hundred and sixty bacterial isolates were obtained from casing colonized by *A. bisporus*. We identified 53 isolates that can inhibit *in vitro* growth of *L. fungicola.* These isolates comprised 18 genotypes as determined with BOX-PCR, all of which belonged to the genus *Pseudomonas*. This is not a surprising result because this genus is a dominant part of the microbial community of casing, especially after colonization of *A. bisporus* (Doores et al., 1986; Samson, 1986; Miller et al., 1995; Fermor et al., 2000; Pardo et al., 2002). The potential of *Pseudomonas* spp. to control plant diseases has been widely recognized (Weller, 2007) and this has led to the development and registration of commercial products (Fravel, 2005; Haas and Defago, 2005; Stockwell and Stack, 2007; Höfte and Altier, 2010).

Most of the antagonistic isolates that inhibited *L. fungicola* on KBA produced a yellow fluorescent pigment, a pseudobactin/pyoverdine type siderophore (Fuchs et al., 2001). These siderophores have been implicated to play a role in disease suppression by effective competition for iron with the pathogen (Duijff et al., 1993; Duijff et al., 1999; Loper and Henkels, 1999). It appears that *L. fungicola* is sensitive to siderophore-mediated competition for iron since well characterized siderophore negative mutants of *P. fluorescens* WCS374r and WCS417r, and *P. putida* WCS358r did not inhibit the pathogen or inhibited to a lesser extent compared to the parental strain (Fig. 2b). Under iron rich conditions, strains WCS417r, WCS358r and WCS374r no longer or to a lesser extent inhibited *L. fungicola.* However, the pathogen is also sensitive to other metabolites produced by fluorescent pseudomonads since *P. fluorescens* RS111a and CHA0 also inhibited *L. fungicola* when iron was in abundance (Fig. 2a). CHA0 produces the antifungal metabolites 2,4-diacetylphloroglucinol, pyoluteorin, pyrrolnitrin, and cyanide, under the control of the *gacA/gacS* regulatory system (Laville et al., 1992; Dubuis and Haas, 2007). Whereas CHA0 inhibited growth of *L. fungicola* both under iron deficient and iron rich conditions, mutant CHA89, a *gacA* knock-out derivative of CHA0 that does not produce these antibiotics, only inhibited *L. fungicola* when iron was deficient (Fig. 2a), suggesting that the fungus is sensitive to at least one of the antifungal metabolites of CHA0. Further evidence for the sensitivity of *L. fungicola* to 2,4-diacetylphloroglucinol was obtained using derivatives of CHA0 and *P. putida* WCS358 that constitutively produce this compound and using a similar approach it was found that the fungus is sensitive

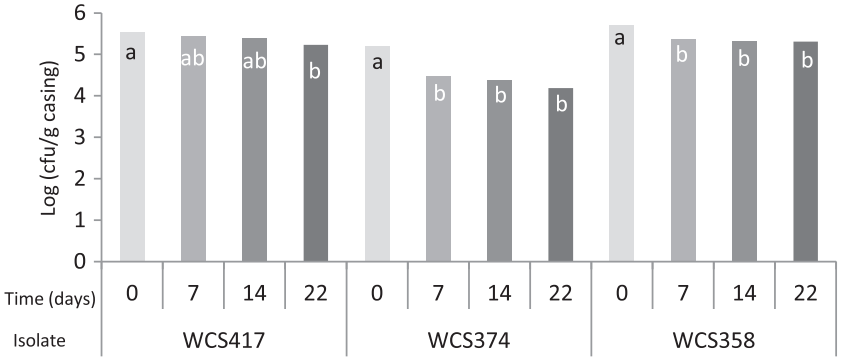


Fig. 8. Numbers (log cfu/g casing) of rifampicine-resistant strains in the casing without hyphae of *A. bisporus.* Letters indicate significant differences between time points within each treatment. Bars represent the average number of three samples for the three rifampicine-resistant strains and of all nine samples for the culturable bacteria.

to phenazine-1-carboxylic acid (Fig. 2a). Thus, *L. fungicola* is sensitive to siderophore-mediated competition for iron and to antibiosis by pseudomonads, suggesting that application of strains that produce effective siderophores and antibiotics can potentially control the dry bubble disease caused by this fungal pathogen.

The isolates that were antagonistic to *L. fungicola in vitro,* were tested in a bioassay for their ability to suppress dry bubble disease *in vivo* (Fig. 3). In this bioassay, treatment with Sporgon (active ingredient: Prochloraz-manganese), the only effective chemical available to Dutch mushroom growers, significantly reduced dry bubble disease. Whereas dry bubble disease incidence in the treatments with antagonistic bacteria was most often lower than in the control treatment, differences were never statistically significant. The power of the bioassay to detect differences was increased by testing the most promising bacterial strains (Ab03038 and Ab03040) using 18 replicates, compared to three replicates in the initial screening. In two independent experiments both Ab03038 and Ab03040 did not control the disease. Therefore we conclude that the antagonistic bacteria that were isolated in this study are not able to reduce dry bubble disease, certainly not to the level of control of Sporgon. To study interactions between the bacterial isolates and *L. fungicola* on the fruiting body of *Agaricus*, development of lesions was measured after inoculation with the pathogen on the cap, either with or without the *Pseudomonas* isolates (fig. 6). Also under these conditions none of the bacterial isolates affected the development of *L. fungicola*, neither on harvested mushrooms nor on mushrooms that were still attached to the mushroom mycelium.

For effective biological control of plant pathogens by *Pseudomonas* spp., effective colonization of the plant roots by these bacteria is a prerequisite (Raaijmakers et al., 1995). The lack of effectiveness of the bacterial isolates to control dry bubble disease in this study thus may have been due to too low population densities of the introduced pseudomonads. We investigated colonization of the casing by three *Pseudomonas* strains that are well studied in plant-soil systems. When introduced into casing, the population densities of strains WCS358r, WCS417r and WCS374r all declined over a three week period. However, when the casing was colonized by *A. bisporus* population densities of strains WCS358r and WCS417r were much higher (10- to 100-fold) compared to non-colonized casing. Strain WCS374r behaved differently and decreased during the two weeks *A. bisporus* needed to colonize the casing and form two flushes of mushrooms. This differential behavior of the WCS strains confirms observations on their abilities to colonize the rhizosphere of *Arabidopsis thaliana* (Doornbos et al., 2009, 2012). The total culturable fraction of bacteria in the casing increased during the first week, in which the casing was colonized by *A. bisporus* vegetative hyphae, and decreased slowly to pre-colonization levels during the next two weeks, in which the vegetative hyphae aggregated and formed fruiting bodies. We assume that during colonization of the casing by *A. bisporus* exudates from the hyphae stimulate growth of bacteria in the casing (Grewal and Rainey, 1991). Apparently, also WCS358r and to a slightly lesser extent WCS417r are stimulated by these exudates. The effectiveness of a biocontrol agent can be correlated with its population size (Bull et al., 1991). For control of fungal plant pathogens, it was found that a minimum density of 10^5 bacteria/g root is required (Raaijmakers et al., 1995; Raaijmakers et al., 1999). Population densities of both WCS417r and WCS358r remained above this threshold level. However, these strains were originally isolated from plant roots (Geels and Schippers, 1983), and they may be less adapted to survival in the casing compared to the strains isolated in this study. However, none of the strains isolated from colonized casing significantly reduced dry bubble disease, despite their effective *in vitro* antagonism. *In vitro*, we found that *L. fungicola* is sensitive to siderophores and antibiotics produced by strains of fluorescent *Pseudomonas* spp. For the control of plant pathogens by fluorescent pseudomonads, both siderophore and antibiotic production can fully explain the disease suppressive abilities of selected strains of these bacteria (Laville et al., 1992; Duijff et al., 1993). The lack of control of dry bubble disease observed in this study may have been due to the possibility that the selected antagonists do not produce the *L. fungicola* inhibitory compounds *in vivo.* However, the constitutive DAPG producer *P. fluorescens* CHA638 did also not reduce dry bubble disease significantly. Apparently, dry bubble disease is not susceptible to the action of antagonistic bacteria in the casing layer. Although germination of *L. fungicola* spores is inhibited in uncolonized casing, spores germinate freely in casing colonized by *A. bisporus* (Cross and Jacobs, 1968; Berendsen et al., 2012). It was proposed that inhibition of spore germination in uncolonized casing is likely due to production of fungistatic compounds and that the spores are desensitized by nutrients leaking from *A. bisporus* hyphae when they colonize the casing. Possibly, colonized casing is such a nutrient-rich environment that addition of antagonistic bacteria cannot prevent *L. fungicola* spores from germinating.

Gilligan (1985) introduced the concept of the pathozone. For a fungal pathogen, it is the zone around an organ of its host in which a propagule of the pathogen must be if it is to have a chance at infecting its host. Probabilities of successful infection, i.e. (infection efficacy) of a pathogen propagule decline with increasing distance from its host. Around plants, it overlaps and mostly exceeds the rhizophere and it is limited by the saprophytic capabilities of a pathogen in a soil. Addition of antagonistic bacteria would aim to reduce the distance a pathogen can grow and so reduce the pathozone. In this respect, we observed that *A. bisporus* colonizes the casing totally and very densely. Contrasting to when plants and soil-borne pathogens interact, there are no large parts of the casing left uncolonized by the mushroom mycelium and the pathozone consequently covers all of the casing. It can therefore be argued that, although the antagonistic bacteria in our assay might reduce the distance *L. fungicola* can grow saprophytically, a suitable host is always nearby and infection efficacy is not significantly affected. Besides reducing saprophytic growth capabilities of a pathogen, biocontrol agents of plant pathogens can also interfere with the infection process by eliciting induced systemic resistance (Bakker et al., 2007; Van Wees et al., 2008). Induced systemic defense responses are commonly found in both plant and animals and but have not been reported in fungi (Berendsen et al., *submitted).*The inability of antagonistic bacteria to induce resistance in mushrooms might further explain their ineffectiveness against dry bubble disease.

Pseudomonas spp. have often been found effective in controlling plant disease, are efficient colonizers of *A. bisporus* and can inhibit *L. fungicola in vitro,* but were in this study unable to effectively control dry bubble disease. We attribute this ineffectiveness to characteristics of both *Lecanicillium* and *Agaricus* and believe that biological control of dry bubble disease with antagonistic bacteria will be very difficult. Future efforts to control dry bubble disease should therefore focus on other methods.

Acknowledgments

This work was supported by the Dutch Technology Foundation (STW), Applied Science Division of NWO and the Technology Program of the Ministry of Economic Affairs. We would like to thank Merlin van Strien for his excellent technical assistance and Ed Hendrix for his mushroom cultivation tips. We are grateful to CNC for kindly supplying us with mushroom compost and casing soil and to Cristoph Keel for contributing *P. fluorescens* strains CHA0, CHA638 and CHA631.

References

Bailey, K.L., Boyetchko, S.M., Langle, T., 2010. Social and economic drivers shaping the future of biological control: A Canadian perspective on the factors affecting the development and use of microbial biopesticides. Biological Control 52, 221–229.

Bakker, P.A.H.M., De Boer, M., Zhang, K.,Van der Sluis, I., Van Loon, L.C., 2002a. Dose response relationships for control of fusarium wilts by *Pseudomonas fluorescens* RS111. IOBC/WPRS Bulletin 25, 107–110.

Bakker, P.A.H.M., Glandorf, D.C.M., Viebahn, M., Ouwens, T., Smit, E., Leeflang, P., Wernars, K., Thomashow, L.S., Thomas-Oates, J., Van Loon, L.C., 2002b. Effects of *Pseudomonas putida* modified to produce phenazine-1-carboxylic acid and 2,4-diacetylphloroglucinol on the microflora of field grown wheat. Antonie van Leeuwenhoek Journal of Microbiology 81, 617–624.

Bakker, P.A.H.M., Pieterse, C.M.J., Van Loon, L.C., 2007. Induced systemic resistance by fluorescent Pseudomonas spp.. Phytopathology 97, 239–243.

Berendsen, R.L., Baars, J.P.P., Kalkhove, S.I.C., Lugones, L.G., Wösten, H.A.B., Bakker, P.A.H.M., 2010. *Lecanicillium fungicola*: causal agent of dry bubble disease in white-button mushroom. Molecular Plant Pathology 11, 585–595.

Berendsen, R.L., Kalkhove, S.I.C., Lugones, L.G., Wösten, H.A.B., Bakker, P.A.H.M., 2012. Germination of *Lecanicillium fungicola* in the mycosphere of *Agaricus bisporus*. Environmental Microbiology Report 4, 227–233.

Bernardo, D., Cabo, A.P., Novaes-Ledieu, M., Mendoza, C.G., 2004. Verticillium disease or "dry bubble" of cultivated mushrooms: the *Agaricus bisporus* lectin recognizes and binds the *Verticillium fungicola* cell wall glucogalactomannan. Canadian Journal of Microbiology 50, 729–735.

Bhat, M.A., Simon, S, Munshi, N.A., Bhat, Z.A., 2010. In vitro efficacy of casing and compost isolated bacterial inoculants against *Verticillium fungicola* (Preuss) Hassebrauk and *Agaricus bisporus* (Lange) Imbach. Indian Journal of Biological Control, 24, 137–141.

Bhatt, N., Singh, R.P., 2000. Chemical and biological management of major fungal pathogens of *Agaricus bisporus* (Lange) Imbach. Mushroom Science 15, 587–593.

Bollen, G.J., van Zaayen, A., 1975. Resistance to benzimidazole fungicides in pathogenic strains of *Verticillium fungicola*. Netherlands Journal of Plant Pathology 81, 157–167.

Brennan, M., Le Port, G., Gormley, R., 2000. Post-harvest treatment with citric acid or hydrogen peroxide to extend the shelf life of fresh sliced mushrooms. Lebensmittel-Wissenschaft & Technologie 33, 285–289.

Bull, C.T., Weller, D.M., Thomashow, L.S., 1991. Relationship between root colonization and suppression of *Gaeumannomyces graminis* var. tritici by *Pseudomonas fluorescens* strain 2–79. Phytopathology 81, 954–959.

Calonje, M., Mendoza, C.G., Cabo, A.P., Bernardo, D., Novaes-Ledieu, M., 2000. Interaction between the mycoparasite *Verticillium fungicola* and the vegetative mycelial phase of *Agaricus bisporus*. Mycological Research 104, 988–992.

Chin-A-Woeng, T.F.C., Bloemberg, G.V., Lugtenberg, B.J.J., 2003. Phenazines and their role in biocontrol by *Pseudomonas bacteria*. 157, 503–523.

Cho, Y.-S., Kim, J.-S., Crowley, D.E., Cho, B.-G., 2003. Growth promotion of the edible fungus *Pleurotus ostreatus* by fluorescent pseudomonads. FEMS Microbiology Letters 218, 271–276.

Cross, M.J., Jacobs, L., 1968. Some observations on the biology of spores of *Verticillium malthousei*. Mushroom Science 7, 239–244.

de Boer, M., van der Sluis, I., van Loon, L.C., Bakker, P.A.H.M., 1999. Combining fluorescent *Pseudomonas* spp. strains to enhance suppression of fusarium wilt of radish. European Journal of Plant Pathology 105, 201–210.

de Trogoff, H., Ricard, J.L., 1976. Biological control of *Verticillium malthousei* by *Trichoderma viride* spray on casing soil in commercial mushroom production. Plant Disease Reporter 60, 677–680.

De Vleesschauwer, D., Djavaheri, M., Bakker, P.A.H.M., Hofte, M., 2008. *Pseudomonas fluorescens* WCS374r-induced systemic resistance in rice against *Magnaporthe oryzae* is based on Pseudobactin-mediated priming for a salicylic acid-repressible multifaceted defense response. Plant Physiology 148, 1996–2012.

Djavaheri, M., 2007. Iron-regulated metabolites of plant growth-promoting Pseudomonas fluorescens WCS374: Their role in induced systemic resistance, Ph.D. thesis. Utrecht University, Utrecht.

Doores, S., Kramer, M., Beefman, R. 1986. Evaluation and bacterial populations associated with fresh mushrooms (*Agaricus bisporus*). In: The Proceedings of the International Symposium on Technical Aspects of Cultivating Edible Fungi Pennsylvania, Elsevier Science Publishers.

Doornbos, R. F., Van Loon, L.C., Bakker, P.A.H.M., 2009. Beneficial Pseudomonas spp. have altered root colonization on Arabidopsis thaliana mutants affected in the expression of induced systemic resistance. 43, 307–310.r

Doornbos, R.F., Van Loon, L.C., Bakker, P.A.H.M., 2012. Impact of root exudates and plant defense signaling on bacterial communities in the rhizosphere. Agronomy for Sustainable Development 32, 227–243.

Dubuis, C., Haas, D., 2007. Cross-species GacA-controlled induction of antibiosis in pseudomonads. Applied and Environment Microbiology 73, 650–654.

Duijff, B.J., Meijer, J.W., Bakker, P.A.H.M., Schippers, B., 1993. Siderophore-mediated competition for iron and induced resistance in the suppression of fusarium-wilt of carnation by fluorescent *Pseudomonas* spp.. Netherlands Journal of Plant Pathology 99, 277–289.

Duijff, B.J., Recorbet, G., Bakker, P.A.H.M., Loper, J.E., Lemanceau, P., 1999. Microbial antagonism at the root level is involved in the suppression of Fusarium wilt by the combination of nonpathogenic *Fusarium oxysporum* Fo47 and *Pseudomonas putida* WCS358. Phytopathology 89, 1073–1079.

Fermor, T., Lincoln, S., Noble, R., Dobrovin-Pennington, A., Colauto, N., 2000. Microbiological properties of casing. Mushroom Science 15, 447–454.

Fletcher, J.T., Yarham, D.J., 1976. The incidence of benomyl tolerance in *Verticillium fungicola*, *Mycogone perniciosa* and *Hypomyces rosellus* in mushroom crops. Annals of Applied Biology 83, 4898–4903.

Fravel, D.R., 2005. Commercialization and implementation of biocontrol. Annual Review of Phytopathology 43, 337–359.

Fuchs, R., Schafer, M., Geoffroy, V., Meyer, J., 2001. Siderotyping a powerful tool for the characterization of Pyoverdines. Current Topics in Medicinal Chemistry 1, 31–57.

Garbeva, P., Hol, W.H.G., Termorshuizen, A.J., Kowalchuk, G.A., de Boer, W., 2011. Fungistasis and general soil biostasis – a new synthesis. 43, 469–477.

Gea, F.J., Navarro, M.J., Tello, J.C., 2005. Reduced sensitivity of the mushroom pathogen *Verticillium fungicola* to prochloraz-manganese in vitro. 109, 741–745.

Geels, F.P., Schippers, B., 1983. Selection of antagonistic fluorescent *Pseudomonas* spp. and their root colonization and persistence following treatment of seed potatoes. Phytopathologische Zeitschrift 108, 193–206.

Gilligan, C.A., 1985. Probability-models for host infection by soilborne fungi. Phytopathology 75, 61–67.

Glandorf, D.C.M., Brand, I., Bakker, P.A.H.M., Schippers, B., 1992. Stability of rifampicin resistance as a marker for root colonization studies of *Pseudomonas putida* in the field. Plant and Soil 147, 135–142.

Grewal, S.I.S., Rainey, P.B., 1991. Phenotypic variation of *Pseudomonas putida* and *P. tolaasii* affects the chemotactic response to *Agaricus bisporus* mycelial exudate. Journal of General Microbiology 137, 2761–2768.

Grogan, H.M., 2008. Challenges facing mushroom disease control in the 21st century. In: The Proceedings of the Sixth International Conference on Mushroom Biology and Mushroom Products Bonn, Germany, Höntges quickdruck.

Haas, D., Defago, G., 2005. Biological control of soil-borne pathogens by fluorescent pseudomonads. Nature Reviews Microbiology 3, 307–319.

Hayes, W.A., Randle, P.E., Last, F.T., 1969. The nature of the microbial stimulus affecting sporophore formation in *Agaricus bisporus* (Lange) Sing. Annals of Applied Biology 64, 177–187.

Höfte, M., Altier, N., 2010. Fluorescent pseudomonads as biocontrol agents for sustainable agricultural systems. Research in Microbiology 161, 464–471.

King, E.O., Ward, M.K., Raney, D.E., 1954. Two simple media for the demonstration of phycocyanin and fluorescin. Journal of Laboratory and Clinical Medicine 44, 301–307.

Lamers, J.G., Schippers, B., Geels, F.P., 1988. Soil-borne diseases of wheat in the Netherlands and results of seed bacterization with pseudomonads against Gaeumannomyces graminis var. tritici, associateed with disease resistance. In: M.L. Jorna and L.A.J. Slootmaker (eds.), Cereal Breeding Related to Integrated Cereal Production. Pudoc, Wageningen, pp. 134–139.

Largeteau, M., Savoie, J.-M., 2010. Microbially induced diseases of *Agaricus bisporus*: biochemical mechanisms and impact on commercial mushroom production. Applied Microbiology and Biotechnology 86, 63–73.

Laville, J., Voisard, C., Keel, C., Maurhofer, M., Défago, G., Haas, D., 1992. Global control in *Pseudomonas fluorescens* mediating antibiotic synthesis and suppression of black root rot of tobacco. Proceedings of the National Academy of Sciences of the USA 89, 1562–1566.

Loper, J.E., Henkels, M.D., 1999. Utilization of heterologous Siderophores enhances levels of iron available to *Pseudomonas putida* in the Rhizosphere. 65, 5357–5363.

Lugtenberg, B.J., Dekkers, L.C., 1999. What makes Pseudomonas bacteria rhizosphere competent? Environmental Microbiology 1, 9–13.

Marugg, J.D., van Spanje, M., Hoekstra, W.P., Schippers, B., Weisbeek, P.J., 1985. Isolation and analysis of genes involved in siderophore biosynthesis in plant-growth-stimulating *Pseudomonas putida* WCS358. Journal of Bacteriology 164, 563–570.

Masaphy, S., Levanon, D., Tchelet, R., Henis, Y., 1987. Scanning electron microscope studies of interactions between *Agaricus bisporus* (Lang) Sing Hyphae and bacteria in casing soil. Applied and Environment Microbiology 53, 1132–1137.

Mazzola, M., Cook, R.J., Thomashow, L.S., Weller, D.M., Pierson, L.S., 1992. Contribution of phenazine antibiotic biosynthesis to the ecological competence of fluorescent Pseudomonads in soil habitats. Applied and Environment Microbiology 58, 2616–2624.

Mercado-Blanco, J., van der Drift, K.M.G.M., Olsson, P.E., Thomas-Oates, J.E., van Loon, L.C., Bakker, P.A.H.M., 2001. Analysis of the pmsCEAB gene cluster involved in biosynthesis of salicylic acid and the siderophore pseudomonine in the biocontrol strain *Pseudomonas fluorescens* WCS374. Journal of Bacteriology 183, 1909–1920.

Miller, N., Gillespie, J.B., Doyle, O.P.E., 1995. The involvement of microbiological components of peat based casing materials in fructification of *Agaricus bisporus*. Mushroom Science 14, 313–321.

Noble, R., Dobrovin-Pennington, A., Hobbs, P.J., Pederby, J., Rodger, A., 2009. Volatile C8 compounds and pseudomonads influence primordium formation of *Agaricus bisporus*. Mycologia 101, 583–591.

Noble, R., Fermor, T.R., Lincoln, S., Dobrovin-Pennington, A., Evered, C., Mead, A., Li, R., 2003. Primordia initiation of mushroom (*Agaricus bisporus*) strains on axenic casing materials. Mycologia 95, 620–629.

North, L.H., Wuest, P.J., 1993. The infection process and symptom expression of Verticillium disease of *Agaricus bisporus*. Canadian Journal of Plant Pathology 15, 74–80.

Nübel, U., Engelen, B., Felske, A., Snaidr, J., Wieshuber, A., Amann, R.I., Ludwig, W., Backhaus, H., 1996. Sequence heterogeneities of genes encoding 16S rRNAs in

Paenibacillus polymyxa detected by temperature gradient gel electrophoresis. Journal of Bacteriology 178, 5636–5643.
Pardo, A., De Juan, J.A., Pardo, J.E., 2002. Bacterial activity in different types of casing during mushroom cultivation (*Agaricus bisporus* (Lange) Imbach). Acta Alimentaria Hungaricae 31, 327–342.
Penyalver, R., Vicedo, B., Lopez, M.M., 2000. Use of the genetically engineered *Agrobacterium* strain K1026 for biological control of crown gall. European Journal of Plant Pathology 106, 801–810.
Raaijmakers, J.M., Bonsall, R.E., Weller, D.M., 1999. Effect of population density of *Pseudomonas fluorescens* on production of 2,4-diacetylphloroglucinol in the rhizosphere of wheat. Phytopathology 89, 470–475.
Raaijmakers, J.M., Leeman, M., Van Oorschot, M.M.P., Van der sluis, I., Schippers, B., Bakker, P.A.H.M., 1995. Dose–response relationships in biological-control of Fusarium-wilt of radish by *Pseudomonas* spp. Phytopathology, 85, 1075–1081.
Raaijmakers, J.M., Paulitz, T.C., Steinberg, C., Alabouvette, C., Moenne-Loccoz, Y., 2009. The rhizosphere: a playground and battlefield for soilborne pathogens and beneficial microorganisms. Plant and Soil 321, 341–361.
Rainey, P.B., 1991. Phenotypic variation of *Pseudomonas putida* and *P. tolaasii* affects attachment to *Agaricus bisporus* mycelium. Journal of General Microbiology 137, 2769–2779.
Rainey, P.B., Cole, A.L.J., Fermor, T.R., Wood, D.A., 1990. A model system for examining involvement of bacteria in basidiome initiation of *Agaricus bisporus*. Mycological Research 94, 191–195.
Samson, R., 1986. Variability of fluorescent Pseudomonas populations in composts and casing soils used for mushroom cultures. In: The Proceedings of the International Symposium on Technical Aspects of Cultivating Edible Fungi Pennsylvania, Elsevier Science Publishers.
Schnider-Keel, U., Seematter, A., Maurhofer, M., Blumer, C., Duffy, B., Gigot-Bonnefoy, C., Reimmann, C., Notz, R., Defago, G., Haas, D., Keel, C., 2000. Autoinduction of 2,4-diacetylphloroglucinol biosynthesis in the biocontrol agent *Pseudomonas fluorescens* CHA0 and repression by the bacterial metabolites Salicylate and Pyoluteorin. Journal of Bacteriology 182, 1215–1225.
Shapira, R., Ordentlich, A., Chet, I., Oppenheim, A.B., 1989. Control of plant-diseases by chitinase expressed from cloned DNA in *Escherichia coli*. Phytopathology 79, 1246–1249.
Singh, M., Singhe, R.P., Chaube, H.S., 2000. Siderophore producing bacteria as potential biocontrol agents of mushroom diseases. Mushroom Science 15, 577–585.
Stockwell, V.O., Stack, J.P., 2007. Using *Pseudomonas* spp. for integrated biological control. Phytopathology 97, 244–249.
Van Dijk, K., Nelson, E.B., 2000. Fatty acid competition as a mechanism by which *Enterobacter cloacae* suppresses *Pythium ultimum* sporangium germination and damping-off. Applied and Environment Microbiology 66, 5340–5347.
Van Loon, L.C., Bakker, P.A.H.M., Pieterse, C.M.J., 1998. Systemic resistance induced by rhizosphere bacteria. Annual review of Phytopathology 36, 453–483.
Van Wees, S.C.M., Pieterse, C.M.J., Trijssenaar, A., Van 't Westende, Y.A.M., Hartog, F., Van Loon, L.C., 1997. Differential induction of systemic resistance in arabidopsis by biocontrol bacteria. Molecular Plant–Microbe Interactions 10, 716–724.
Van Wees, S.C.M., Van der Ent, S., Pieterse, C.M.J., 2008. Plant immune responses triggered by beneficial microbes. Current Opinion in Plant Biology 11, 443–448.
Versalovic, J., Schneider, M., de Bruijn, F.J., Lupski, J., 1994. Genomic fingerprinting of bacteria using repetitive sequence-based polymerase chain reaction. Methods in Molecular and Cellular Biology 5, 25–40.
Visscher, H.R., 1988. Casing soil. In: L.J.L.D. van Griensven (ed.). The Cultivation of Mushrooms. Waalwijk, Grafidrukkerij Waalwijk.
Weisbeek, P.J., Van der Hofstad, G.A.J.M., Schippers, B., Marugg, J.D., 1986. Genetic analysis of the iron-uptake system of two plant growth-promoting Pseudomonas strains. In: T.R. Swinburne (ed.), Iron, Siderophores and Plant Diseases, vol. 3. New York, Plenum Press, pp. 299–313.
Weller, D.M., 2007. Pseudomonas biocontrol agents of soilborne pathogens: looking back over 30 years. Phytopathology 97, 250–256.
Weller, D.M., Raaijmakers, J.M., Gardener, B.B.M., Thomashow, L.S., 2002. Microbial populations responsible for specific soil suppressiveness to plant pathogens. Annual Review of Phytopathology 40, 309.
Wong, W.C., Preece, T.F., 1979. Identification of *Pseudomonas tolaasi*: the white line in agar and mushroom tissue block rapid pitting tests. Journal of Applied Microbiology 47, 401–407.
Wuest, P.J., Cole, H., Sanders, P.L., 1974. Tolerance of *Verticillium malthousei* to Benomyl. Phytopathology 64, 331–334.