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New Insights in the Study of Strawberry Fungal Pathogens

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ABSTRACT

Strawberry (*Fragaria ananassa*) is one of the world's most commercially important fruit crops, and is grown in many countries The commercial viability of the crop is continually subject to various risks, one of the most serious of which is the diseases caused by phytopathogenic organisms. More than 50 different genera of fungi can affect this cultivar, including *Botrytis* spp., *Colletotrichum* spp., *Verticillium* spp., and *Phytophthora* spp. The development of new molecular biology technologies, based on genomics, transcriptomics and proteomics approaches, is revealing new insights on the diverse pathogenicity factors causing fungal invasion, degradation and destruction of the fruit (*in planta* and during storage and transport). Researchers have focused attention on the plant's own defence mechanisms against these pathogens. In this review, advances in the study and detection of fungal plant pathogens, new biocontrol methods, and proteomic approaches are described and the natural defence mechanisms recently discovered are reported.

Keywords: biocontrol, elicitor, molecular tools, proteomics, real-time PCR

Abbreviations: AFLP, amplified fragment length polymorphism; **APS**, America phytopathological society; **CECT**, Spanish type culture collection; **CTAB**, cetyl trimethyl ammonium bromide; **CUE**, critical use exemption; **DGGE**, denaturing gradient gel electrophoresis; **ELISA**, enzyme-linked immunosorbent assay; **EPPO**, the European and Mediterranean Plant Protection Organization; **FAO**, the Food and Agriculture Organization of the United Nations; **GADPH**, glyceraldehyde-3-phosphate dehydrogenase; **IGS**, interGenic spacer; **ITS**, internal transcribed spacer; **LUX**, light upon extension; **MALDI-TOF**, matrix-assisted laser desorption/ionization-time of flight; **MDH**, malate dehydrogenase; **MeBr**, methyl bromide; **PCR**, polymerase chain reaction; **PIC**, chloropicrin; **PMF**, peptide mass fingerprinting; **RAPD**, random amplified polymorphic DNA; **RFLP**, restriction fragment length polymorphism; **SCAR**, sequence characterized amplified region; **QPS**, quarantine and preshipment; **U.S.**, United States

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INTRODUCTION

The genus *Fragaria* is a member of the *Rosaceae* family, *Rosoideae* subfamily, which comprises twenty eight species and several subspecies. *Fragaria x ananassa* Duch. is the most important species commercially, being the predominant species cultivated for strawberry production globally (FAO 2000); it is, however, of only recent historical origin. Prior to the relatively recent development of *F. x ananassa*, other species, such as *F. chiloensis*, *F. virginiana*, *F. vesca* and *F. moschata*, had been cultivated in Europe, America and Asia for centuries (JA 2004). Over the last two hundred and 50 years, these species were largely superseded by cultivation of *F. x ananassa*. In the middle of the 18th century, the North American *F. virginiana* Duchesne (male) and the South American *F. chiloensis* Duchesne (female) were crossed in France, leading to production of hybrid seedlings that came to be known as Pineapple or Pine strawberries (Maas 1998). These hybrids are the progenitors of the modern cultivated strawberry *F. x ananassa* Duch. In the following years, after further hybridizations, *F. x ananassa* developed a larger, more fragrant and tastier red berry than its progenitors and, in the middle years of the 1800's, this new species was introduced into America from Europe. Cultivation of the other older species has progressively declined and they have been superseded by *F. x ananassa*; the other species are only occasionally grown domestically or in small isolated regions around the world (Bertelson 2010).

Many different cultivars or varieties of *F. x ananassa* are found around the world. Since this species has been distributed and cultivated very widely geographically, new cultivars are constantly appearing because the strawberry plant is strongly influenced by photoperiod, temperature, and other environmental conditions of various regions. Although there are about a dozen cultivars constituting the most common varieties used for world strawberry production, it is very complicated to discover the exact number and names of actual cultivars of strawberry existing in the

Received: 24 March, 2010. Accepted: 21 August, 2010.

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world. Strawberry cultivars are often grouped or classified based on environmental control or habit of flowering. There are three main types of cultivars: "short-day", "long-day" and "day-neutral", in reference to the sensitivity of the plant to day length, and the type of photoperiod which induces flower formation. In spite of the different classifications, any particular strawberry cultivar will differ greatly in its adaptation to regional and environmental conditions; a cultivar may grow satisfactorily in one area, and present resistance to several species of strawberry pathogens, but the same cultivar may not thrive in another region with different environmental conditions. A cultivar may be resistant to a particular pathogen in one region yet be very susceptible to it elsewhere because pathogens present different strains or pathotypes (Garrido *et al.* 2008, 2009b).

The Food and Agriculture Organization of the United Nations estimates that the annual world production of strawberry exceeds 3,800,000 tons. The fruit is produced in seventy-three countries all around the world, and the total area cultivated is approximately 215,000 ha (FAO 2007). The European Union is a major producer of strawberry (accounting for 47% of world output), grown on 165,000 ha. The United States produces approximately a 29% of world output) (JA 2004; FAO 2007). In the USA strawberry production is concentrated in three states, California, Florida and Oregon, in order of importance. The growers of California account for 80% of the U.S. strawberry production (FAO 2000; Martin *et al.* 2002). In Europe, Spain is the main producer country, with annual output of approximately 333,000 and 264,000 tons in 2006 and 2007, respectively. This production is concentrated in the provinces of Huelva and Cádiz, in southwest Spain, on the Atlantic coast and near the mouths of the Guadalquivir and Guadiana rivers, where weather conditions are particularly suitable for the intensive cultivation of this fruit. This area has more than 5,000 commercial horticultural operations dedicated to the cultivation of strawberry and other crops (JA 2004).

In line with the financial and economic importance represented by the cultivation and marketing of this crop, serious financial losses can be incurred due to health problems affecting the strawberry plant. These plants, like other commercial crops, can be damaged by environmental, genetic and biological factors, either directly or through interaction between these factors. Diseases produced by biological factors require a pathogen, a susceptible host, and environmental conditions that favour the growth of the pathogen. However, the cultivated strawberry plant presents considerable genetic differences between various clones and cultivars, and this implies differences in their reactions to many pathogens. The strawberry plant can be affected by arthropods, nematodes, and many types of fungus, bacteria and virus, among other pests. The group of phytopathogenic fungi that attack this crop is especially extensive in terms of the number of genera and species that can produce diseases, and hence in terms of the considerable financial losses, running into hundreds of millions of dollars/euros per year, that this agrarian sector may suffer (FAO 2000; JA 2004). More than 50 different genera can affect *F. x ananassa* cultivars, but not all of them have the same commercial importance. The genera *Botrytis*, *Colletotrichum*, *Verticillium*, and *Phytophthora* are included among the most significant. These pathogens are capable of infecting not only the strawberry crop but more than one hundred other crops cultivated around the world.

At the global level, there are several agencies working in cooperation with governments to control trade in this crop between countries. The United States has no federal certification program for strawberry plant production, but some states and countries, such as the United Kingdom, do have such programs (Maas 1998). The EPPO is an intergovernmental organization, with 50 member countries, responsible for cooperation in plant protection in Europe and the Mediterranean region (www.eppo.org). Since the 1970's, this organization has published and maintained two quarantine pest lists for this large region. The A1 list includes pests not present in the EPPO region, and the A2 list includes pests that are locally present in the region. In response to the lists and reports published by this organization, the member countries adopt various different phytosanitary measures and plant protection programs. Although in recent years several genera of strawberry fungal pathogens, such as *Colletotrichum acutatum*, *Botrytis cinerea*, *Phytophthora* spp. have been included in the EPPO A2 list (EPPO 2004), currently only *Phytophthora fragariae* (specific for strawberry), and *Verticillium dahliae* and *Verticillium albo-atrum* are in the updated A2 list (EPPO 2009). This limited listing means that the other genera or species of fungi are likely to be either currently absent or else only present in small pockets, scattered widely across the EPPO region. Therefore the main pest control efforts are likely to be focused on preventing *Verticillium* spp. from becoming definitively established in the EPPO region, while other action may be taken to identify the other pathogens, and to control and fight them where they may be found.

Currently, the detection and identification of pathogenic fungi have traditionally been performed using classical mycological methods involving isolation from host material, by plating plant parts, soil or soil extracts onto selective media, followed by morphological, biochemical, chemical and immunological analyses (Singleton *et al.* 1992; Lievens *et al.* 2005). However, these methods are often time-consuming, laborious, and require extensive knowledge of classical taxonomy. Furthermore, quantification, based on these culture-plating techniques, is considered relatively inaccurate and unreliable (McCartney *et al.* 2003; Lievens *et al.* 2005; Garrido *et al.* 2008). As has been reported by Garrido *et al.* (2008, 2009b), since 1991 several molecular methods for more accurate characterization and differentiation of phytopathogenic fungi have been developed and are being implemented widely. These methods include iso-enzyme comparisons, restriction fragment length polymorphism (RFLP) analyses of mitochondrial DNA, AT-rich analyses, random amplified polymorphic DNA (RAPD), genus and species-specific polymerase chain reactions (PCR) and enzyme-linked immunosorbent assay (ELISA).

When the pathogens are detected in the field, or to prevent their appearance between crop seasons, disease control has been carried out using chemical treatments. Since 1950's, Californian growers have produced "back-to-back" crops of strawberry on the same soil fumigated with MeBr and Pic (Martin *et al.* 2002), and the use of these chemical treatments has spread around the world since that date. Although such treatments are still widely used to control diseases caused by plant pathogens, the emergence of fungicide-resistant strains, de-registration of fungicides, and public concerns regarding the health and environmental impacts of agrochemicals may all act to limit their application in the future (Massart *et al.* 2007). Alternative approaches to disease control, such as the use of biological, cultural and physical methods, must be integrated to reduce the amount of fumigant used for soil and plant treatments. Biological control of fungal plant pathogens with microorganisms has been studied for more than 60 years (Howell *et al.* 2003). This form of biocontrol appears to be an attractive and realistic approach, and numerous microorganisms have been identified as potential biocontrol agents. Nevertheless, using microbiological, microscopic, and biochemical techniques over many years, researchers have tried to understand the mechanisms of action of fungal biocontrol agents without yet fully elucidating them.

The development of molecular techniques has yielded innovative alternative tools for understanding and demonstrating the mechanisms underlying the properties of biocontrol agents. To date, many studies have been published describing the use of molecular techniques for this purpose (Martin *et al.* 2002; Fernández-Acero *et al.* 2007; Massart *et al.* 2007). Proteomics studies for the description of biological, metabolic, physiological processes have been undergoing rapid development in the last few years. Advances in proteomic techniques have yielded extensive and definitive

Table 1 Strawberry fungal pathogens causing leaf diseases.

Fungal pathogen	Phylum	Strawberry disease	Authors[a]
Alternaria alternata	Ascomycota	black leaf spot	Miyamoto *et al.* 2009
Cercospora fragariae	Ascomycota	cercospora leaf spots	Maas *et al.* 1998
Cercospora vexans	Ascomycota	cercospora leaf spots	Maas *et al.* 1998
Colletotrichum acutatum	Ascomycota	anthracnose leaf spot	Garrido *et al.* 2009
Colletotrichum gloeosporioides	Ascomycota	anthracnose leaf spot	Chung *et al.* 2010
Colletotrichum fragariae	Ascomycota	anthracnose leaf spot	Ortega-Morales *et al.* 2009
Diplocarpon earlianum	Ascomycota	leaf scorch	Whitaker *et al.* 2009
Gnomonia comari	Ascomycota	leaf blotch	Morocko *et al.* 2007
Macrophomina phaseolina	Ascomycota	macrophomina leaf blight	Maas *et al.* 1998
Mycosphaerella fragariae	Ascomycota	purple leaf spot	Ehsani-Moghaddam *et al.* 2006
Mycosphaerella louisianae	Ascomycota	purple leaf spot	Maas *et al.* 1998
Phoma lycopersici	Ascomycota	leaf stalk rot	Maas *et al.* 1998
Phomopsis obscurans	Ascomycota	phomopsis leaf blight	Nita *et al.* 2003
Rhizoctonia solani	Basidiomycota	rhizoctonia leaf blight	Chiba *et al.* 2009
Sclerotium rolfsii	Basidiomycota	sclerotium rot	Errakhi *et al.* 2009
Septoria fragariae	Ascomycota	septoria leaf spot	Maas *et al.* 1998
Septoria aciculosa	Ascomycota	septoria leaf spot	Maas *et al.* 1998
Septoria fragariaecola	Ascomycota	septoria leaf spot	Maas *et al.* 1998
Sphaerotheca macularis	Ascomycota	powdery mildew	Davik *et al.* 2005

[a] Recent and relevant publication working with the pathogen in the plant pathology field

biological information, describing the link between gene and phenotypical features, thus bridging the gap between genotype and phenotype. The application of these techniques to phytopathogenic fungi is helping us to understand the highly complex life cycle of fungal plant pathogens (Fernández-Acero *et al.* 2006, 2007a, 2007b).

In this review, we will consider the most recent advances in the study of these phytopathogenic fungi that cause diseases in strawberry crops. The continuous development of molecular methods, resulting from research projects carried out by laboratories and research centres on the world, is providing more and better knowledge about these pathogens, and is equipping the scientific community, official laboratories of governments and strawberry growers in many countries with more and better techniques and tools for fighting these harmful fungi.

MAIN STRAWBERRY FUNGAL DISEASES

The cultivation of *F. x ananassa* is now widely spread around the world, and progressively more countries are adopting cultivars of this genus due to the good commercial results obtained from this crop. Currently, numerous different cultivars of *F. x ananassa* exist, and this number is continually increasing, because researchers and growers are looking for the "*perfect strawberry plant*". This ideal plant should produce a large, fragrant, tasty red berry, with a high production yield per plant and per year; it should be well adapted to the particular environmental factors of particular regions, and of course, it must be resistant to all pathogens. To date, a strawberry plant with all these characteristics has not been developed. Among these requirements, one of the most difficult characteristics to obtain is general resistance to the main diseases.

The initiation and evolution of a disease in plants requires a susceptible host plant, a pathogen, and favourable environmental conditions (Maas 2004). Most of the fungal pathogen species affecting strawberry can be found in all the crop regions around the world, but the damage caused in the strawberry crop and the commercial losses suffered by the growers due to these species are of a different scale in each region. This is because several strawberry pathogens also exist as races or pathotypes which, although indistinguishable at the species level, differ genetically in their abilities to cause disease. The races are not evenly distributed around the world, and they are often found only in one specific strawberry crop region (Maas 2004).

Since 1978, the American Phytopathological Society (APS) has had a standing a committee charged with maintaining listings of plant pathogens and the diseases to which they give rise. These lists are continually updated in the APS databases (www.apsnet.org/online/common/toc.asp). Pathogenic organisms are classified into bacteria, viruses, fungi and nematodes. In the case of strawberry, the list is alphabetically arranged by common names of plant diseases. The APS describes a total of 57 species of fungal pathogens. This list includes thirty nine species of ascomycota, seven of oomycetes, four of basidiomycota, and five basal fungal lineages (**Tables 1-3**). Sometimes, the name of a disease covers several species of pathogens: for example, anthracnose fruit rot can be caused by *Colletotrichum acutatum*, *C. fragariae* and *C. gloeosporioides*. In other cases, one particular disease can even be caused by more than one genus: for example, black leaf spot is caused by *Alternaria alternata* or by *Colletotrichum gloeosporioides*. When a pathogen is very damaging, or when several species of one genus produce similar symptoms, the disease takes the name of the pathogen genus; for example, phytophthora crown rot and root rot are caused by *Phytophthora cactorum*, *P. citricola*, *P. citrophthora*, *P. megasperma* or *P. nicotinae*.

Most of the species listed in the tables (**Tables 1-3**) are fungal pathogens that are not exclusive to strawberry. They can cause disease in many other crops; this is the case with *Botrytis cinerea*, *Phytophthora citricola* and *Colletotrichum gloeosporioides*. Hence, because these fungi can affect several different genera of commercially important plants, governments, researchers and growers take more interest in them. The greater the potential commercial losses, the more important it is to study them and acquire more knowledge about pathogenic cycles, diagnostic methods, control measures and practical means to combat them.

One of the most common ways to classify strawberry fugal pathogens is to arrange them by reference to the part of plant in which they produce the damage. In this section, we have compiled the information published by other authors in reports, articles and chapters of books, describing the main symptoms caused by the fungal diseases in leaves, fruits, crowns and roots.

Leaf diseases

The leaf of a plant is the principal site of the conversion of light energy to metabolic energy by photosynthesis. Thus, the number of leaves and the total leaf area of strawberry plants in the fall or autumn are directly correlated with fruit production. Individual strawberry leaves usually have a life of 1-3 months. The morphology of leaves varies between cultivar of strawberry, but a common characteristic is the large number of stomata that they have on the surface (Maas 1998). Leaf diseases commonly appear on straw-

Table 2 Strawberry fungal pathogens causing fruit diseases.

Fungal pathogen	Phylum	Strawberry disease	Authors[a]
Alternaria tenuissima	Ascomycota	alternaria fruit rot	Shafique *et al.* 2009
Aspergillus niger	Ascomycota	aspergillus fruit rot	Chiotta *et al.* 2009
Botrytis cinerea	Ascomycota	botrytis fruit rot; gray mold	Fernández-Acero *et al.* 2009
Cladosporium spp.	Ascomycota	cladosporium fruit rot	Ruiz-Moyano *et al.* 2009
Colletotrichum acutatum	Ascomycota	anthracnose fruit rot	Garrido *et al.* 2009
Colletotrichum gloeosporioides	Ascomycota	anthracnose fruit rot	Chung *et al.* 2010
Colletotrichum fragariae	Ascomycota	anthracnose fruit rot	Ortega-Morales *et al.* 2009
Gnomonia comari	Ascomycota	stem end rot	Morocko *et al.* 2007
Mucor hiemalis	*Basal fungal lineages*	mucor fruit rot	Hauke *et al.* 2004
Mucor mucedo	*Basal fungal lineages*	mucor fruit rot	Hauke *et al.* 2004
Mucor piriformis	*Basal fungal lineages*	mucor fruit rot	Hauke *et al.* 2004
Mycosphaerella fragariae	Ascomycota	black seed disease	Ehsani-Moghaddam *et al.* 2006
Pestalotia longisetula	Ascomycota	pestalotia fruit rot	Maas *et al.* 1998
Penicillium cyclopium	Ascomycota	penicillium fruit rot	Gutierrez *et al.* 2009
Penicillium expansum	Ascomycota	penicillium fruit rot	Liu *et al.* 2007
Penicillium frequentans	Ascomycota	penicillium fruit rot	Redondo *et al.* 2009
Penicillium purpurogenum	Ascomycota	fruit rot; fruit blotch	Redondo *et al.* 2009
Peronospora potentillae	Oomycete	downy mildew; fruit blotch	Choi *et al.* 2009
Phytophthora cactorum	Oomycete	leather rot	Nicastro *et al.* 2009
Phytophthora citrophthora	Oomycete	leather rot; phytophthora crown and root rot	Kong *et al.* 2009
Phytophthora nicotianae	Oomycete	leather rot; phytophthora crown and root rot	Böszörményi *et al.* 2009
Rhizoctonia fragariae	Basidiomycota	anther and pistil blight	Lamondia *et al.* 2005
Rhizopus solonifer	*Basal fungal lineages*	rhizopus rot	Maas *et al.* 1998
Rhizopus sexualis	*Basal fungal lineages*	rhizopus rot	Maas *et al.* 1998
Rhizoctonia solani	Basidiomycota	hard brown rot	Liu *et al.* 2009
Schizoparme straminea	Ascomycota	fruit blotch	
Sclerotinia sclerotiorum	Ascomycota	sclerotinia fruit rot	Ren *et al.* 2010
Stagonospora fragariae	Ascomycota	stagonospora hard rot	Maas *et al.* 1998
Sclerotium rolfsii	Basidiomycota	southern blight; fruit blotch	Errakhi *et al.* 2009
Septoria fragariae	Ascomycota	septoria hard rot and leaf spot	**
Sphaeropsis malorum	Ascomycota	fruit blotch	**
Sphaerotheca macularis	Ascomycota	powdery mildew	Davik *et al.* 2005

[a] Recent and relevant publication working with the pathogen in the plant pathology field.

Table 3 Strawberry fungal pathogens causing crown and root diseases.

Fungal pathogen	Phylum	Strawberry disease	Authors[a]
Armillaria mellea	Basidiomycota	armilla crown rot and root rot	Prodorutti *et al.* 2009
Colletotrichum acutatum	Ascomycota	anthracnose fruit rot, crown rot and black spot	Garrido *et al.* 2009
Colletotrichum gloeosporioides	Ascomycota	anthracnose fruit rot, crown rot and black leaf spot	Chung *et al.* 2010
Colletotrichum fragariae	Ascomycota	anthracnose fruit rot, crown rot and black spot	Ortega-Morales *et al.* 2009
Cylindrocarpon destructans	Ascomycota	root rot	Martin *et al.* 2002
Fusarium oxysporum	Ascomycota	fusarium wilt	Avis *et al.* 2009
Idriella lunata	Ascomycota	idriella root rot	Maas *et al.* 1998
Macrophomina phaseolina	Ascomycota	macrophomina root rot	Javaid *et al.* 2009
Phytophthora cactorum	Oomycete*	phytophthora crown and root rot	Nicastro *et al.* 2009
Phytophthora citricola	Oomycete*	phytophthora crown and root rot	Haesler *et al.* 2008
Phytophthora fragariae	Oomycete*	phytophthora crown and root rot	Nicastro *et al.* 2009
Pythium ultimum	Oomycete*	black root rot	Triky-Dotan *et al.* 2009
Rhizoctonia fragariae	Basidiomycota	black root rot	Lamondia *et al.* 2005
Rhizoctonia solani	Basidiomycota	hard brown rot	Liu *et al.* 2009
Sclerotinia sclerotiorum	Ascomycota	sclerotinia crown and fruit rot	Ren *et al.* 2010
Verticillium albo-atrum	Ascomycota	verticillium wilt	Larsen *et al.* 2007
Verticillium dahliae	Ascomycota	verticillium wilt	Costa *et al.* 2007

[a] Recent and relevant publication working with the pathogen in the plant pathology field.

berry plants. Stomata also are often sites of infection by pathogens that cause leaf diseases, although some fungi can penetrate the leaf cuticle directly. Although many species of fungi can occasionally cause damage on leaves, there are at least nineteen species that cause damage on susceptible cultivars if environmental conditions are conducive to their development (**Table 1**). When enough leaf tissue has been destroyed by disease, the plant is weakened and, in such cases, the plant is more subject to winter injury. Additionally, the pathogens that cause these diseases on leaves can infect berries, causing quality problems or even loss of fruits.

Taking into account the amount of commercial damage caused by disease in leaves and the worldwide spread of the corresponding pathogens, attention must be focussed on eight species of fungal pathogens from **Table 1**. These are, in alphabetical order, *Alternaria alternata* (alternaria black leaf spot); *Colletotrichum acutatum*, *Colletotrichum gloeosporioides* and *Colletotrichum fragariae* (anthracnose leaf spot and irregular leaf spot); *Diplocarpon earlianum* (leaf scorch); *Mycospharella fragariae* (leaf spot); *Phomopsis obscurans* (phomopsis leaf blight); *Rhizoctonia solani* (rhizoctonia leaf blight) and *Sphaerotheca macularis* (powdery mildew) (**Fig. 1**). These diseases occur on strawberry plants in all areas of the world where they are grown, from temperate climates to subtropical and tropical regions. The major effect of these pathogens is the progressive destruction of the foliage, which may weaken plants and reduce yields. Some pathogens cause very distinctive leaf symptoms; for example, *Sphaerotecha macularis* forms white patches of mycelium on the abaxial surface of the leaf. Other species, however, cause similar, even identical, symptoms, from cir-

Fig. 1 *Sphaerotheca macularis* causing powdery mildew in strawberry leaves cv. 'Camarosa'.

Fig. 2 *Botrytis cinerea* affecting strawberry fruit.

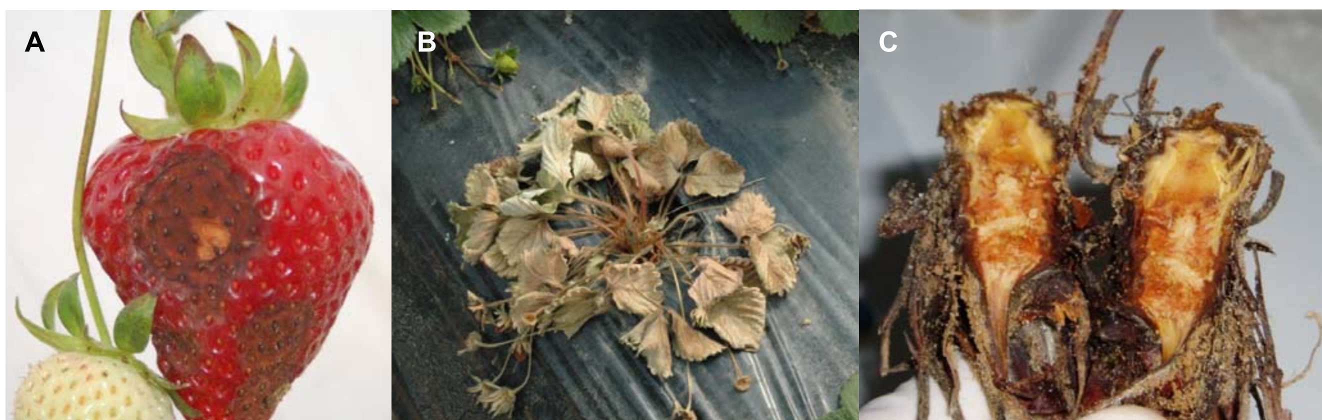

Fig. 3 *Colletotrichum acutatum* causing anthracnose in strawberry. (**A**) Fruit, (**B**) plant dead, (**C**) lesions inside the crown.

cular spots with grey centres and dark margins to irregular purplish red or brown areas with dark reddish purple margins. It is usual in the literature to find incorrect identifications and many citations confirm that symptoms are often confused between pathogens; for example, leaf scorch, caused by *Diplocarpon earlianum* is often confused with symptoms of leaf spot, caused by *Mycosphaerella fragariae*; and the symptoms produced by the latter are also wrongly identified as phomopsis leaf blight caused by *Phomopsis obscurans* (Maas 1998). Two anthracnose diseases affect strawberry leaves: anthracnose leaf spot, caused by *Colletotrichum fragariae* and *C. gloeosporioides*, and irregular leaf spot, caused by *C. acutatum*. These three species can be found in any part of the plant and produce similar symptoms, including circular lesions similar to those described above caused by other fungi (Garrido *et al*. 2009a). This confusion or incorrect observation is a serious matter because the correct identification of the disease, and therefore of the causal pathogen, is necessary before applying the correct control treatment, whether chemical products, biocontrol agents, or even changing to cultivars that are resistant to the pathogen identified.

Leaves of strawberry can be also attacked by other fungi than those discussed above, but their commercial importance is minor since they are limited to a few particular regions or countries and they occur only occasionally in strawberry. These other minor diseases of leaves include *Gnominia comari* (leaf blotch); *Mycophaerella louisianae* (purple leaf spot); *Septoria fragariae*, *S. aciculosa* and *S. fragariaecola* (septoria leaf spots); *Macrophomina phaseolina* (macrophomina leaf blight); *Cercospora fragariae* and *C. vexans* (cercospora leaf spots); *Sclerotium rolfsii* (sclerotium rot); and *Phoma lycopersici* (leaf stalk rot) (Maas 1998).

Fruit diseases

A number of diseases affect strawberry fruit. The fruit diseases are very important since they are the most direct and visible cause of commercial losses, not only while the plant is in the field but also during the storage and transport of the fruit to the market. *Botrytis cinerea* and *Colletotrichum* spp. are the pathogens responsible for the major strawberry fruit diseases around the world. They also cause the greatest losses of fruit; and they are responsible for the largest quantities of fungicides used in most nations for strawberry fruit production, and the consequent expenditure on these treatments (Maas 2004). The yearly cost of treatments to combat *B. cinerea* in all vulnerable crops, in all countries, has been estimated at up to €540 million, which constitutes about 10% of total world expenditure on fungicides; in Spain around €2 million are spent on fungicides for protecting strawberry crops (Fernández-Acero *et al*. 2007a).

Botrytis fruit rot, also called grey mould (caused by *B. cinerea*) appears in the fields before harvest, especially when the crop has remained persistently wet, but it chiefly develops in picked fruit. Losses can be severe (i.e. proportionately high) at harvest, during marketing and shipping, and after final sale. Lesions in green and white fruit develop slowly. The fruit becomes misshapen as it enlarges and may die before reaching maturity. The rot expands rapidly in the fruit as it nears maturity, often until the entire fruit is affected. A key diagnostic feature of botrytis fruit rot is the greyish mass of mycelium, conidiophores, and conidia of *B. cinerea* on the surface of rotted tissues (**Fig. 2**). Since the 1960's, recommendations for control of *B. cinerea* have emphasized applications of fungicides during blooming of flowers (Maas 2004). A degree of variation in susceptibility to botrytis rot exists among cultivars, but since genetic re-

sistance to *B. cinerea* infection in strawberry is apparently a multigene characteristic and has a very low general combining ability, there has been little success in breeding and selecting cultivars resistant to this disease (Maas 2004).

Anthracnose of strawberry, especially anthracnose fruit rot, is a very destructive disease caused by the same three species of *Colletotrichum* described above for the anthracnose leaf spot, but the predominant species is *C. acutatum* (**Fig. 3A**). Infected flowers and buds may become dry and withered. Fruit infection can be devastating during periods of favourable temperature and the presence of moisture, either in the form of rain or high relative humidity. Circular, firm, sunken lesions that typically become black may develop on ripening fruit (Garrido *et al*. 2008). Control of anthracnose can be difficult since few available fungicides are effective against anthracnose of strawberry; once an epidemic of anthracnose fruit rot begins in a susceptible cultivar, it is nearly impossible to control (Maas 2004). In the literature, several strawberry cultivars have been reported to be resistant to at least one anthracnose disease, and an informative list has been published by Maas (2004).

Leather rot is the third disease in importance for strawberry fruit, especially considering that it can occur worldwide, from the USA to Europe and Asia. The losses of fruit may be considerable; fruit with leather rot has a distinctly unpleasant odour and taste that may be imparted to processed fruit products. *Phytophthora cactorum* is the causal pathogen of the disease but this species is more important because it also causes a serious crown rot. Control of leather rot requires a program that integrates various cultivation practices and the use of effective fungicides. Captan is the most effective fungicide available at present (Maas 2004).

There are other 12 genera of fungi that cause considerable losses on strawberry when they appear, but it happens only sporadically in widely separated areas. This group of fungi includes the next genera: *Rhizoctonia fragariae* (anther and pistil blight), *Mycosphaerella fragariae* (black seed disease) and *Sclerotinia sclerotiorum* (sclerotinia fruit rot) vary widely in severity and no specific control measures have been developed for them, although preplant soil treatment and cultivation practices, such as mulching and removal of plant debris, may help to minimize the incidence of these diseases. *Gnominia comari* (stem-end rot), *Pestalotia longisetula* (pestalotia fruit rot), *Alternaria* spp. (alternaria rot) and *Cladosporium* spp. (cladosporium rot), *Aspergillus niger* (aspergillus rot), and *Stagonospora fragariae* (stagonospora hard rot) are of less commercial importance. Lastly, there is one group of pathogens that are especially important because they cause postharvest losses to strawberry in storage. This group includes *Rhizopus solonifer* and *R. sexualis* (rhizopus rot); *Mucor mucedo*, *M. piriformis* and *M. hiemalis* (mucor fruit rot); *Penicillium frequentans*, *P. cyclopium*, *P. expansum* and *P. purpugenum* (penicillium fruit rot). The importance of these pathogens causing postharvest rot has been substantially reduced by modern storage and shipping methods (Maas 1998).

Crown and root diseases

Strawberry roots and crowns are attacked by several fungi. *Phytophthora fragariae*, *P. cactorum*, *Colletotrichum acutatum*, and *Verticillium*, for example, are species that cause specific and important diseases. Other diseases, such as black root rot, may be caused by one or more of several fungal pathogens, with or without the interaction of other factors, such as nematodes, bacteria, winter damage, poor drainage, depletion of soil oxygen, soil fertility imbalance, and drought stress.

In the case of strawberry roots, their growth is influenced not only by the external environment of soil and air but also by the amount of food reserves stored by the plant. Drainage is a key factor to maintaining strawberry rootlet health, including the removal of standing water on the surface, especially during winter, as well as provisions for the percolation of subsurface water through the soil. In cold parts of the world, winter injury to strawberry roots, caused by alternate freezing and thawing of the soil, favour root infection by *Fusarium* spp., *Cylindrocarpon destructans*, and many other fungi, and thus contributes to the incidence of root diseases. Strawberry roots, perhaps more than roots of other plants, are closely associated with numerous species of fungi and bacteria, many of which may be pathogenic under certain conditions.

The genus *Colletotrichum*, including the same three species previously described *C. acutatum*, *C. gloeosporioides* and *C. fragariae*, attacks the crowns and roots of strawberry causing serious anthracnose crown rot disease. *Colletotrichum* spp. causes red-brown discoloration and necrosis within the crown tissue (Garrido *et al.* 2008; Smith *et al.* 2008). Other symptoms of crown rot are the wilting of infected plants during periods of moisture stress, such as early afternoon in the summer. Under environmental conditions that favour infection, this process may continue for several days until the crown infection is extensive and causes the entire plant to wilt and die (**Figs. 3B, 3C**). Fungicidal control of anthracnose has not been satisfactorily achieved in most areas. Currently only a few resistant cultivars are available. Resistant cultivars to one anthracnose pathogen are usually resistant to the other two; however, races of the fungi can occur, and therefore a cultivar resistant in one locality may not be resistant in another. Cultivars resistant to petiole and crown infections are not necessarily resistant to fruit infection.

Another important pathogen, that is favoured by cool climates, which is the most widespread strawberry root pathogen, is the genus *Pythium* spp., especially the species *P. ultimum*. This species has been shown to be a major cause of black root rot disease. *Pythium* spp. is not only a strawberry pathogen; it can attack many other crops, both annual and perennial. In strawberry, *Pythium* spp. destroys juvenile root tissue, such as feeder rootlets. Control of *Pythium* spp. is effective with the application of chloropicrin mixtures.

Phytophthora spp. is another important genus causing disease of crowns and roots. Crown or rhizome rot, whose causal pathogen is *P. cactorum*, has resulted in commercial losses in several European countries since about 1960. The disease, which is generally worse in warmer climates, has also been reported in the United States and other temperate to subtropical regions. The youngest leaves turn bluish green and often wilt suddenly; wilting quickly spreads to the entire plant, which collapses and dies, typically within a few days. Phytophthora citricola root rot, caused by *P. citricola*, usually develops in fields with a high incidence of dead plants, and is also caused by an unidentified species of *Phytophthora*. In California, *P. citricola* has been adequately controlled by preplant soil fumigation, applying mixtures of methyl bromide and chloropicrin. Red stele root, caused by *Phytophthora fragariae*, was first observed in Scotland and it is a major disease in areas with cool and moist climates. Plants with severe root rot are often stunted, and they may wilt in hot weather. The most characteristic root symptom is a reddish discoloration of the steles, which occurs when the soil is cool. Locally-adapted cultivars with resistance to several races of *P. fragariae* should be selected for planting, but none is known to be resistant to all races.

The pathogens, *Verticillium albo-atrum* and *V. dahlia*e find a wide range of hosts among annual and perennial crops and weeds, and are capable of persisting for long periods in the soil. They cause verticillium wilt which occurs throughout the temperate zones of the world. The initial symptoms appear rapidly, when a sudden onset of high temperatures, high light intensity, or drought, interrupt mild conditions. Verticillium wilt tends to be most severe in plants that are already fruiting, and symptoms may continue throughout summer and autumn. Excellent long-range control of verticillium wilt has been obtained by preplant soil fumigation, with the application of mixtures of chloropicrin and methyl bromide, but new strategies of biocontrol will

be explained in later sections of this review.

Fusarium wilt and rhizoctonia root rot are two crown and root diseases that are favoured by high temperatures; when they appear severe damage, with losses as high as 50%, can result. *Fusarium oxysporum* produces distinct reddish brown discoloration on the crown, and the lower crown tissues may decay extensively as the disease advances. *Rhizoctonia solani* and *R. fragariae* kill structural roots as well as feeder rootlets of strawberries. Lesions on young roots are reddish brown at first, and darken with age. The infected crowns show internal brown discoloration of basal tissues and frequently collapse and die. Traditionally these diseases have been controlled by fumigating the soil prior to planting.

Crowns and roots of strawberry plants may be also attacked by other fungi, with less serious commercial consequences; these include *Idriella lunata* (idriella root rot); *Sclerotinia sclerotiorum* (sclerotinia crown rot); *Armillaria mellea* (armillaria root and crown rot); *Dematophora necatrix* (dematophora root and crown rot) and *Macrophomina phaseolina* (macrophomina root rot) (Agrios 1998).

BIOCONTROL OF DISEASES

Currently, the control of strawberry pathogens is still based on the use of fungicides and other biocides. World-wide, the most prevalent strawberry disease management practice is the application of chemical treatments, in conjunction with other practices for managing plant health, including, for example, the reduction of over-crowding and over-watering of plants, the rapid removal of diseased plants and plant debris, the constant monitoring of plants to ensure healthy production stocks, and more recently the progressive use of biocontrol agents in the field (Maas 2004). Soil fumigation has been of paramount importance for strawberry fruit and nursery-plant production in recent decades, and has enabled growers to develop systems for very high-value annual crop production.

Historically, one of the primary reasons for soil fumigation was to reduce the incidence of destructive pathogens such as *Verticillium dahliae* and several species of *Phytophthora* spp. (Martin *et al*. 2002). MeBr and chloropicrin, individually or in mixtures, are the classic chemical products used most widely as preplant treatments and for soil fumigation, to control a broad range of pathogens around the world. Preplant MeBr fumigation was first used to control verticillium wilt in California in 1956. Apart from being very effective for pathogen control, this compound has been demonstrated to increase growth and yield responses in strawberry crops when planted in soil that had first been fumigated with MeBr. The benefits include increased growth response (especially of the root system), reduction in N fertilizer requirements (by approximately 50% in some cases), improved nutrient uptake, and control of weed species (Wilhelm *et al*. 1980).

Although there are many advantages in using chemical products to control crop pathogens – mainly their proven effectiveness and other positive benefits for the crops – they nevertheless have very serious negative effects for the environment, and their use can cause fungicide-resistant strains of pathogens to evolve. The use of MeBr, for example, is currently regulated by United Nations under the Montreal protocol on substances that deplete the ozone layer (Montreal protocol) and the clean air act (CAA). The protocol calls for a 50% reduction per year of methyl bromide sold to users, although some exemptions from the phase-out date are allowed, including: i) the quarantine and preshipment (QPS) exemption, to eliminate quarantine pests; and ii) the critical use exemption (CUE), designed for agricultural users for whom no technically or economically feasible alternatives are available. Strawberry nurseries and strawberry fruit production are included in the list of critical uses of methyl bromide; therefore strawberry growers have been given additional and valuable time to adapt their control practices before the phase-out date for methyl bromide. The international community is increasingly committed to finding alternative disease control methods, including biological, cultural or other chemical measures, to integrate into their general crop production systems.

The biocontrol of fungal plant pathogens with microorganisms has been studied for more than 70 years (Massart *et al*. 2007). Today, biocontrol is becoming a feasible alternative but, although much knowledge has been accumulated from studies conducted in recent years, there is still a long way to go before a realistic and effective alternative to the use of chemical products is available. One of the first problems that the scientific community found was that, because MeBr and other biocides had been widely used for more than 50 years, little was known about the pathogens that need to be controlled, in their own environmental niches; understanding the ecology of these pathogens and how biocontrol agents exert their protective effects are prerequisites in managing these diseases and for the effective practical application of biocontrol agents (Martin *et al*. 2002). Microbiological, microscopic, biochemical studies and, more recently, advanced molecular techniques have all contributed to existing knowledge on the antagonistic mechanisms of biocontrol agents (Massart *et al*. 2007).

Microorganisms introduced to control disease must interact with the crop plants, with potential pathogens, with environmental variables, and with indigenous organisms under prevailing microclimate conditions (Sutton *et al*. 1994). In strawberry, most attention has been focussed on rhizosphere ecology, in which numerous populations of bacteria, yeasts and fungi have been identified and isolated for their ability to antagonize plant pathogens. The rhizosphere is the soil surrounding the root, the micro-environment where intense microbial activity and more numerous microbial populations occur, and is the site for interactions between plants, pathogenic microorganisms, antagonistic rhizobacteria and fungi (Berg *et al*. 2000). The biocontrol agent is assumed to be a natural colonizer of strawberry fruit, leaves, and roots, and to be either non-pathogenic or only weakly so, or else saprophytic, and to be capable of interacting successfully with the plant, microbiological and other environmental conditions, and cultivation systems (Maas 2004). From consulting the literature on strawberry diseases, two main groups of microorganisms, between others, can be found that previously have been studied as biocontrol agents against strawberry fungal pathogens: i) rhizobacteria, with the *Pseudomonas* and *Streptomyces* species being the main members of this group; and ii) the genus *Trichoderma*, which is the most studied and effective fungal biocontrol agent identified to date (Berg *et al*. 2000; Woo *et al*. 2006).

Rhizobacteria antagonism toward plant pathogenic fungi may result from various mechanisms, based mainly on competition for space and nutrients, mycoparasitism or antibiosis, or the elicitation of plant defences. The physiological and molecular characterization of potential rhizobacterial antagonisms is important in order to select the isolates for detailed study as candidate biological control agents. Berg *et al*. (2000) developed a screening strategy to investigate rhizobacterial populations isolated from strawberry species and to determine their antagonistic activity against *Verticillium dahliae*, *V. albo-atrum*, *Fusarium culmorum*, *Rhizoctonia solani*, *Pythium ultimum*, and *Phytophthora cactorum*. Twenty rhizobacterial isolates, out of more than three hundred, were isolated from strawberry cultivars because they presented a powerful antifungal activity. The species included *Pseudomonas fluorescens* and *P. chlororphis*, *Streptomyces albidoflavus*, *S. rimosus*, *S. diastatochromogenes*, and *S. exfoliates*. After *in vitro* assays, the authors concluded that all isolates inhibited the growth of *V. dahliae* and four isolates of *Streptomyces* species were active against all of the fungi tested. The rhizobacteria tested produced antibiotics and presented siderophore activity. *Streptomyces* isolates also presented chitinase activity, whereas none of the *Pseudomonas* did.

Berg *et al*. (2000) also studied the antagonistic activity

of these isolates against verticillium wilt in greenhouse and fields. The results showed that, in the greenhouse and in fields naturally infected by *Verticillium*, bacterial treatment resulted in the efficient suppression of the pathogen. Additionally, the authors observed an indirect effect on the yield, a phenomenon that often occurs with some antagonistic microorganisms through their activity in rhizosphere ecology. It was found that *Pseudomonas* treatment enhanced the yield of strawberry fruits by as much as 344% (Berg *et al*. 2004). The explanation of this effect lies in the interactions that take place between the host and the microorganisms; this association provides the plant with the nutrients and environmental conditions for enhanced development of its root systems. This effect will also be considered later in this section with respect to the fungus *Trichoderma* spp. Berg *et al.* (2000) obtained encouraging results because they isolated several good candidates for combating seven important species of strawberry fungal pathogens. In addition, it was possible to patent three biocontrol agents (one *P. fluorescens* isolate and two *Streptomyces diastatochromogenes* isolates). In recent years, studies of rhizobacteria populations and their antagonistic potential have been focussed on the molecular aspects of the community. With the advances made in molecular biology techniques, much more is now understood about the molecular mechanisms and genes involved in this antagonistic activity. Costa *et al.* (2007) studied the presence of *Pseudomonas* spp. communities in the rhizosphere using a novel PCR-DGGE system developed to characterize *gacA* types within a collection of *Pseudomonas* isolates antagonistic to *V. dahliae*. The gene *gacA* is required for the production of many secondary metabolites and exoenzymes in *Pseudomonas* spp. (Heeb *et al*. 2001). The study carried out by Costa *et al.* (2007) suggests that this family of genes, in conjunction with the PCR-DGGE system, might be a suitable strategy for the simultaneous analysis of *Pseudomonas* community structure and function in soil studies.

Other groups of antagonistic microorganisms that are important for biocontrol of strawberry fungal pathogens are several genera of filamentous fungi. The first of such microorganisms to be considered here is *Glicocladium roseum*. This ascomycete has been shown to be effective in reducing the incidence in fruit of grey mould caused by *Botrytis cinerea*. It has been proved that *Glicocladium roseum* acts by reducing the inocula from infected leaves and protecting the flower parts from attack by *B. cinerea* (Maas 2004). In one curious case it was found that non-pathogenic isolates of *Fusarium oxysporum* can be used to control fusarium wilt in a field test when plants were inoculated with the non-pathogenic isolates prior to planting (Okayama 1991; Tezuka *et al*. 1991).

Although we have already presented some attractive and interesting results of biocontrol agents, the genus *Trichoderma* is the most studied and most effective fungal biocontrol agent found to date. Fungal species belonging to this genus occur all around the world and are easily isolated from soil, decaying wood and other forms of plant organic matter (Howell 2003). Since the 1930s, it has been known that fungi of the *Trichoderma* species have the potential to act as biocontrol agents of plant diseases. *Trichoderma* spp. have developed an astonishing ability to interact, both parasitically and symbiotically, with different substrates and living organisms, including plants and other microbes (Woo *et al*. 2006). Over the past several decades, the literature has contained many publications reporting the results obtained with these fungi as biocontrol agents, but even today we do not have a complete understanding of the complex mechanisms that the fungi use to carry out their antagonistic activity. In two reviews Howell (2003) and Woo *et al*. (2006) tried to summarize the main features of the molecular boilogy of the interactions between *Trichoderma* spp., plants and phytopathogenic fungi, and the main aspects of this research area and the conclusions that can be drawn will be discussed next.

Trichoderma spp. can utilize a variety of nutrient sources; the fungi are among the most resistant microorganisms to natural and man-made chemicals and toxins; and they are capable of effectively degrading some of them, including hydrocarbons, chlorophenolic compounds, polysaccharides and xenobiotic pesticides (Harman *et al*. 2004). It is important to understand the mechanisms employed by *Trichoderma* spp. to act in the biological control of plant diseases. Advances in molecular biology have permitted the genetic modification of these fungi in a very precise manner, and this has led to the improvement of their ability to secrete desired enzymes, to kill plant pathogens, and to stimulate plant growth and resistance to diseases. Intensive research into biocontrol using *T. harzianum* has been carried out under commercial conditions, and there have been some significant achievements in greenhouse crops and in vineyards (Freeman *et al*. 2003). *Trichoderma* spp. are now widely used in agriculture and industry; the first biocontrol agent to be commercialized, registered and used was isolate T-39 of *T. harzianum* (TRICHODEX), which is effective for the control of *B. cinerea*, *S. sclerotiorum*, *C. fluvum* and *C. acutatum*. Today, more than fifty different *Trichoderma*-based agricultural products have been registered in the world, and are sold and applied to protect and improve yields of vegetables, ornamental plants and fruit trees (Woo *et al*. 2006).

During the last few decades, the scientific community has been learning more and more about the mechanisms involved in the biocontrol activities of *Trichoderma* spp. This fungus is very versatile in adapting many different environmental situations and has a large arsenal of molecular weapons to use against the particular microbe that they are confronting. One of the most salient characteristics of the genus *Trichoderma* is its ability to parasitize other fungi. What has been called the "*mycoparasitism*" of *Rhizoctonia solani* hyphae by the hyphae of this biocontrol agent has been described, and its actions include coiling around the hyphae of the pathogen, penetrating and subsequently dissolving the cytoplasm of the host (Howell 2003). The fungus also excretes several kinds of "*toxins*" which are toxic for *R. solani*, *Sclerotinia americana*, and even strongly inhibitory for *Pythium ultimum* and *Phytophthora* species; two of the most important toxins excreted by *Trichoderma virens* are gliotoxin and gliovirin (Howell 2003). In subsequent studies, a mutant of *T. virens* deficient for both mycoparasitism and toxins biosynthesis still retained biocontrol efficacy; these results indicate that neither mycoparasitism nor toxins are essential for biocontrol of these pathogens (Liu *et al*. 2009).

Competition through rhizosphere competence is another control mechanism that has been proposed because *Trichoderma* grows rapidly and easily colonizes the root systems of plants. Rhizosphere competence is important because biocontrol agents can work by competing successfully for space and nutrients. However one problem for this theory is that some species of *Trichoderma* (excellent roots colonizers) exhibit little or no biocontrol activity against *R. solani* in cotton seedlings (Howell 2003). In addition, this genus is characterized by its capacity to secrete a large number of compounds, and even to generate complex interactions between plants and pathogens; therefore a control mechanism based on physical competition does not seem to be very likely or feasible. During its interactions with host plants *Trichoderma* spp. exhibits other characteristics that may contribute to disease resistance or tolerance by the plants. This mechanism is similar to that employed by *Pseudomonas* spp., explained above, in that it induces plant root and shoot growth, resistance to biotic and abiotic stresses, and improvements in the nutritional status of the plant. Harman *et al*. (2000) showed that, after treatment of seed with *T. harzianum* (T-22), corn planted in low nitrogen soil produced plants that were greener and larger in the early part of the growing season. Harman *et al.* (2000) reported a strong interaction between T-22 and the nitrogen-fixing bacterium *Bradyrhizobium japonicum*. Theoretically, the combination of a nitrogen-fixing bacterium and a fungus

that enables the plant to utilize nitrogen more efficiently is directly related to a general beneficial growth effect on the root system, which thereby gives the plant more resistance against pathogens (Harman *et al*. 2000).

Research data accumulated in the past few years have led to a completely novel understanding of the ways in which these fungi interact not only with other microbes but also with plants and soil components (Woo *et al.* 2006). The production of enzymes and the induction of defence responses in plants by *Trichoderma* spp. have been discussed. The use of relatively novel tools to investigate these complex processes, in particular proteomic analysis, has demonstrated that a molecular "cross-talk" or intercommunication is established between *Trichoderma* spp., the plants and the pathogens.

DIAGNOSIS AND MONITORING OF DISEASES

The ability to detect, identify and quantify plant pathogens accurately is the cornerstone of plant pathology. The reliable identification of the organism(s) responsible for a crop disease is an essential prerequisite to the implementation of disease management strategies. Therefore, in the diagnosis of strawberry fungal infections, it is essential to devise and carry out a correct preventive disease management program. Because many fungal pathogens of strawberry (e.g. *Diplocarpon earlianum*, *Mycosphaerella fragariae* and *Phomopsis obscurans*) produce similar symptoms, as described in the preceding section, it is important to be able to distinguish between different species. Decisions must be made, for example, on the extent to which the disease is likely to damage the crop, and what are the most appropriate control measures to take. Fungus identification needs to take into account not only those pathogens included in the current EPPO quarantine lists and discussed in this review (e.g. *Colletotrichum acutatum* and *Verticillium dahliae*), but also those that appear only on previous lists. Many pathogens are subjected to special regulation through quarantine programs agreed among producer countries. For all these reasons, accurate and rapid methods of detection and identification are essential (Debode *et al*. 2009; Garrido *et al.* 2009b). More generally, pathogen identification is crucial to all aspects of fungal diagnostics and epidemiology in the field of plant pathology, medical science, environmental studies and biological control (McCartney *et al.* 2003; Atkins *et al*. 2004).

Classic methods have been used for many years in the detection and identification of pathogenic fungi, including interpreting visual symptoms of the plant or the morphological characteristics of fungal structures after growing in several media. Biochemical, chemical and immunological analyses have also been used until recently. These traditional methods can contribute a great deal of information increasing the biological knowledge of the fungal species, but they have many disadvantages related with the accuracy and reliability. They are often time-consuming and laborious methods and the organism itself must be capable of being cultured. This is a significant handicap because less than 1% of the microorganisms present in an environmental sample can be cultured (Lievens *et al*. 2005). Such analyses also require experienced and skilled laboratory staffs, which needs to have extensive knowledge of taxonomy (McCartney *et al*. 2003).

Since the 1990s, new methods based on molecular biology have provided new tools for more accurate and reliable detection, identification and quantification of plant pathogens. These methods are based on immunological and DNA/RNA study strategies, including, amongst others: RFLP analyses of mitochondrial DNA (Sreenivasaprasad *et al.* 1992; Garrido *et al.* 2008), AFLP, AT-rich analyses (Freeman *et al.* 2000), RAPD-DNA (Whitelaw-Weckert *et al.* 2007), genus-specific and species-specific PCR primers (Mills *et al*. 1992; Sreenivasaprasad *et al*. 1996; Martinez-Culebras *et al*. 2003; Garrido *et al*. 2008), real-time PCR studies (Garrido *et al.* 2009b), and ELISA assays (Hughes *et al*. 1997). These methods are faster and can be used by personnel with little experience in plant pathology (McCartney *et al*. 2003). Additionally, non-cultivable microorganisms can also be detected and quantified, samples can be tested directly, and isolates do not require culturing because minute quantities of fungal DNA can be detected from environmental samples, even before symptoms occur. Diagnosis time can be reduced from a period of weeks, typically experienced with culture plating, to only a few days, thus allowing the appropriate control methods to implemented much sooner and more effectively (Atkins *et al*. 2004; Lievens *et al*. 2005). These methods can show also some disadvantages because non-viable or dead propagules are also detected, but this can lead only to overestimate the possibilities of risk in a very low percentage. In comparison with the advantages that these methods provide, they are a potential alternative to conventional diagnostic methods (Garrido *et al*. 2009b).

Advances in polymerase chain reaction technology have opened alternative approaches to the detection and identification of strawberry fungal pathogens. The development of PCR technology relies on three fundamental steps: i) the selection of a specific target region of DNA/RNA to identify the fungus; ii) extraction of total community DNA/RNA from the environmental sample; iii) a method for identifying the presence of the target DNA/RNA region in the sample (Atkins *et al*. 2004). In recent years, many research studies have been published reporting improvements to each of the three fundamental steps described above, and working on some of the most serious fungal pathogens of strawberry, such as *Botrytis cinerea* (Suárez *et al*. 2005), *Colletotrichum acutatum* (Debode *et al*. 2009; Garrido *et al*. 2009b), *Colletotrichum gloeosporioides*, *Colletotrichum* spp. (Garrido *et al*. 2009b), *Fusarium oxysporum* (Lievens *et al*. 2003), *Verticillium albo-atrum* (Larsen *et al*. 2007), and *Verticillium dahliae* (Atallah *et al.* 2007). We will next describe the main results obtained in the study of these pathogens.

There are two main ways to select the target DNA: i) using specific sequence information available in databases; and ii) cloning and sequencing arbitrary parts of the fungal genome (Atkins *et al*. 2004). The most commonly-used DNA region targeted to design primers for PCR-based identification and detection of fungal plant pathogens are the ribosomal RNA genes, because of the highly variable sequences of the internal transcribed spacers ITS1 and ITS2, which separate the 18S/5,8S and 5,8S/28S ribosomal RNA genes, respectively (Garrido *et al*. 2009b). These regions have been used by Debode *et al*. (2009) and Garrido *et al*. (2009b) to develop protocols of identification for the genus *Colletotrichum,* and to distinguish between the three species causing anthracnose in strawberry: *Colletotrichum acutatum*, *C. gloeosporioides* and *C. fragariae*. Another highly variable region of the ribosomal RNA genes is the IGS region which separates the 28S/18S genes. This region has been less used than ITSs, but Suárez *et al*. (2005) used it successfully to design primers in the polymorphic regions for the specific identification of *B. cinerea*. Although the ITS and IGS regions are the main targets, other genes are becoming more widely studied, in particular the β-tubulin gene (Atkins *et al*. 2004). Sequences of the β-tubulin gene were also analyzed by Suárez *et al*. (2005), Atallah *et al.* (2007) and Debode *et al*. (2009).

In some cases, where the data obtained using specific sequences from databases are inadequate because a specific primer cannot be designed, screening arbitrary regions of the genome is often the next step. This strategy consists of an initial screening of random amplified polymorphism DNA (RAPD), and subsequent analyses of the products with the object of developing a SCAR marker. This protocol was used by Larsen *et al*. (2007) to develop SCAR markers for quantifying *Verticillium albo-atrum* DNA. Rigotti *et al.* (2002), studying *B. cinerea* isolates, identified SCAR markers for the specific identification of this pathogen in *Fragaria* x *ananassa*. Three years later, Suárez *et al*. (2005)

compared their primers designed in the IGS regions and proved that this new assay was more sensitive than all the previous assays for *B. cinerea*.

The sensitivity of PCR-based protocols depends mainly on the instrumentation and technique used (i.e. conventional PCR *vs.* real-time PCR), but in a high proportion of cases this sensitivity depends on the quality of the total community DNA/RNA extracted from the environmental samples. There are many methods that can be used to extract DNA directly from fungal colonies removed from an agar plate. When working with environmental samples (mainly with plant material), a very common problem encountered is that chemical products are extracted in conjunction with total DNA, and these act as PCR-inhibitors; as a result, the sensitivity of the method decreases and can even lead to false negative results. One strategy for solving the problem is to dilute the samples, but in that case, the quantity of the target DNA and the sensitivity of detection are also reduced. A range of different commercial kits are available for extraction of fungal DNA from environmental samples such as plant tissue or soil, supplied by several companies (Dynal's DNA direct, the Soil DNA isolation kit from Mo Bio Laboratories, Inc., and the Qiagen DNeasy range are examples). These kits are characterized by their simplicity of use; however they can represent a high cost per sample analysed and are not always totally reliable in not co-extracting PCR inhibitors. Garrido *et al*. (2009b) optimized a DNA extraction protocol that can be used for samples of strawberry plant material, directly, or from fungal colonies removed from an agar plate. This method uses sample material physically ground using a grinding machine, in the presence of CTAB lysis buffer. The lysated samples are washed in various chemical products (chloroform, isopropanol, ethanol, etc.) and then the final step involves using Magnesil® beads and GITC lysis buffer (guanidinium thiocyanate buffer) in a Kingfisher robotic processor (Kingfisher ML, Thermo Scientific). Garrido *et al*. (2009b) demonstrated that this method is very reliable for extracting DNA from any strawberry plant material. It was tested with roots, crowns, petioles, leaves and fruits and the extraction methods always showed very high yields of DNA in both quantity and quality. PCR-inhibitors were not co-extracted from any samples, and therefore, a dilution step is not necessary prior to using the sample with a PCR test. The sensitivity of the entire detection protocol is thus improved, using this protocol.

The third difficulty that an effective detection protocol must overcome is the detection stage. Conventional PCR has been a fundamental part of fungal molecular diagnostics, but due to its limitations: gel-based methods, possibility of quantification, sensitivity, etc. The development of real-time PCR has been a valuable response to these limitations; it is more sensitive, more accurate and less time-consuming than conventional end-point quantitative PCR (Lievens *et al*. 2005). A specific target organism is both detected and quantified by measuring the intensity of amplification-specific fluorescence generated during each cycle in a closed tube format using an integrated cycler/fluorimeter. Real-time PCR can be performed using different chemistries, such as TaqMan®, SYBR® Green I dye, or a new approach, developed by Invitrogen, called LUX. The TaqMan® system consists of a fluorogenic probe specific to the DNA target, which anneals to the target between the PCR primers; The TaqMan® system tends to be the most sensitive of the methods but the new approach, LUX, is achieving the same level of sensitivity while further simplifying the techniques, as well as reducing the cost. This new system uses two primers, one of which has a hairpin loop structure with a fluorophore (Suárez *et al*. 2005). Although this method is replacing TaqMan® probes in real-time PCR technology, with the object of providing cheaper, reliable methods with the specificity of TaqMan® probes without some of the constraints, to date TaqMan® technology is continuing to be used in the majority of research projects working with strawberry fungal pathogens.

The level of specificity and sensitivity of real-time PCR-based methods with some of the main strawberry fungal pathogens reported in several publications vouch for the results offered by this technology. Suárez *et al*. (2005) designed three TaqMan® probe/primers sets based on the ribosomal IGS spacer, the β-tubulin gene, and the SCAR marker of *B. cinerea* published by Rigotti *et al*. (2002). The specificity of the assays was tested by investigating cross-reactivity with several species of *Botrytis* very closely related to *B. cinerea* (i.e. *B. fabae*, *B. elliptica*, *B. tulipae* and *B. narcissicola*). The assays described were specific for *B. cinerea* even when they were tested with DNA from other genera of fungal pathogens, in particular *S. sclerotiorum*, which is closely related to the *Botrytis* genus. When the sensitivity of the assays was investigated, the IGS assay proved to be more sensitive than all the other assays, with a limit of detection of approximately 0.2 pg of fungal DNA. Detection and quantification of *B. cinerea* was also tested using *Pelargonium* sp. leaf discs inoculated with a range of spores from *B. cinerea* (10^1 – 10^4). All the assays, apart from that based on β-tubulin, were shown to be sensitive for the total quantity of spores.

In 2007, Larsen *et al*. proposed a new assay for quantifying *Verticillium albo-atrum* DNA from plant samples. This is currently one of the quarantine pathogens of strawberry. They designed a TaqMan® set of probe/primers located inside the sequence of a SCAR product produced by RAPD primer Op87. Using that real-time PCR assay, DNA of *V. albo-atrum* could be detected in linear assay within a range of DNA quantities from 0.001 to 50 ng, and the assay did not amplify DNA from other pathogens evaluated by the authors, thus showing it to be highly specific for *V. albo-atrum*. Atallah *et al*. (2007) published also in this year other study to detect and quantify *Verticillium dahlia* using Q-PCR. The study was designed using the β-tubulin gene and achieving efficiency greater than 95%, and sensitivity as few as 148 fg of *V. dahliae* DNA, which is equivalent to five nuclei. QPCR detected *V. dahliae* in naturally infected potato stems and fresh stems of inoculated plants (Atallah *et al.* 2007).

In 2009, Garrido *et al*. published sets of new protocols for the detection of *Colletotrichum* spp., *C. acutatum* and *C. gloeosporioides*, and for monitoring strawberry anthracnose using real-time PCR. Several months later, Debode *et al*. (2009) proposed an additional set of probe/primers for *Colletotrichum acutatum* in strawberry. Both groups of authors used mainly the ITS regions to design the sets of probe/primers. Garrido *et al*. (2009b) tested the specificity of all assays using DNA from isolates of six species of *Colletotrichum* and from DNA of another nine fungal species commonly found associated with strawberry material. Additionally, they checked that samples did not contain PCR inhibitors co-extracted during the DNA extractions using one universal pair of primers for the 5,8S ribosomal gene by PCR SYBR®Green amplifications. All the new assays were highly specific for *Colletotrichum* spp., *C. acutatum* and *C. gloeosporioides*, no cross-reactions were observed with either related plant pathogens or healthy strawberry plant material. The sensitivity of the new real-time PCR assays was compared with that of previously published conventional PCR assays; they were confirmed to be 100 times more sensitive than the latter. The *C. acutatum*-specific real-time PCR assay was also compared with an existing ELISA assay for the diagnosis of this pathogen. Real-time PCR permitted the detection of the pathogen in samples that gave negative results for *C. acutatum* using ELISA. The real-time PCR assay detected the equivalent of 7.2 conidia per plant inoculated with a serial dilution of *C. acutatum* spores, demonstrating the high degree of sensitivity of the method (Garrido *et al*. 2009b).

Although we have shown the advantages and promising results achieved with these real-time PCR assays, there are other assay formats that have not yet been applied to plant pathogens, such as multiplex PCR, which enables the simultaneous detection and quantification of different pathogens

in one single reaction. The total number of PCR reactions that it is possible to detect in a single tube is severely limited by the number of different fluorescent dyes available, and the common use of a monochromatic energizing light source in the most common real-time PCR instruments (Lievens *et al*. 2003, 2005). To overcome these limitations, another technology based on DNA arrays is becoming available: this technology may lead to unlimited multiplexing, that is, the detection and identification of numerous plant pathogens per assay. DNA arrays were originally developed as a technique for screening for human genetic disorders in the 1990's but recently several authors have shown the utility of DNA arrays for multiplex detection and identification of plant pathogens from complex environmental samples (Lievens *et al*. 2003).

This technology combines nucleic acid amplification with the unlimited screening capability of DNA arrays, resulting in high degrees of sensitivity, specificity and throughput capacity. With this technology, oligonucleotide detectors are covalently bound to a solid support, such as a nylon membrane. The target DNA segment to be tested is simultaneously amplified and labelled, and subsequently hybridized to the membrane under stringent conditions. Lieven *et al*. (2005) developed a DNA array for the specific detection and identification within twenty four hours of the strawberry fungal pathogen *Fusarium oxysporium* and the quarantine pathogens *Verticillium albo-atrum* and *Verticillium dahliae*. This assay detects at the species level, based on the fungal ITS region, from several different sample matrices, including plant material, soils and soil extracts. This assay has proved to be highly sensitive, detecting 2.5 pg of DNA for *V. dahliae*, 0.35 pg DNA for *V. albo-atrum*, and 0.5 pg DNA for *F. oxysporium*. DNA arrays may be used for the detection and identification of microorganisms that are important for agricultural and horticultural practice; however a major limitation of the current technology is the reliable quantification of pathogen presence. Further investigations carried out by Lieven *et al*. (2005) demonstrated that a DNA macro-array could be used for accurate pathogen quantification, at concentration ranges typically encountered in horticultural practice. They developed an initial approach including several controls. The hybridization results can be standardized and accurately quantified, enabling pathogen biomass to be quantitatively estimated. This DNA array for diagnostic purposes in plant pathology requires PCR amplification; therefore the sensitivity of the results depends to a large degree on how this step is performed. Lievens *et al*. (2005) demonstrated that the detection limit is determined by the total population of microorganisms whose DNA is amplified by the same primer pair that amplifies the DNA of the target organisms. The lowest amount of fungal target DNA detected by the authors was 0.25 pg, but it could not be detected when the amount of non-target fungal DNA exceeded the target DNA by more than 1000 times.

In conclusion, DNA array technology has proved to be another practical alternative for the detection and identification of an unlimited number of microorganisms in a single assay. This array-based detection procedure for plant pathogens has been proved to be relatively cost-effective, and may lead to a comprehensive pathogen assessment method for detecting and quantifying all known pathogens (including fungi, bacteria, and nematodes, as well as viruses); it could also be used for the description of microbial communities in soil.

PROTEOMICS APPROACHES AS A TOOL TO STUDY STRAWBERRY FUNGAL PATHOGENS

Proteomics studies for the description of biological, metabolic, physiological and pathological processes have made considerable progress in recent years. Advances in proteomic techniques have been useful for obtaining extensive and decisive biological information about the life cycles of many organisms. Given the achievements made using these techniques in several fields, including plant pathology, some authors have started to describe the current period as the "post-genomic era" (Fernández-Acero *et al.* 2007a). Until recently, few advances had been made regarding the proteome of phytopathogenic and non-phytopathogenic fungi. However in the last few years, dozens of teams have focussed their research activities on the proteomic analysis of these filamentous fungi, and they are making significant advances in the knowledge of the biology, mechanisms of infection, and even the antagonistic activity of some important fungi, including *B. cinerea* (Fernández-Acero *et al.* 2006a, 2009a, 2009b) and *Trichoderma* spp. (Grinyer *et al.* 2004, 2005; Woo *et al.* 2006).

The increasing interest of the scientific community in proteomic analysis has been due to improvements in the proteomics technologies available, such as protein separation by two-dimensional gel electrophoresis (2-DE), and peptide analysis by mass spectrometry (MS), becoming a powerful strategy for gaining a better understanding of the complex molecular cross-talk that is known to take place between filamentous fungi and their environments, including other populations of microorganisms and plant hosts (Fernández-Acero *et al*. 2006b, 2007b). Proteomic analyses of filamentous fungi, both pathogenic and non-pathogenic, have raised serious limitations or handicaps. One of these limitations is the need to have an effective protein extraction method for obtaining a good resolution in the 2-DE gels, because various contaminants such as nucleic acids, salts, lipids, pigments, cell wall polysaccharides, etc., can be dragged along with the protein during the extraction process (Fernández-Acero *et al*. 2007a).

Another major handicap is the protein identification. Protein spots are often identified by MALDI-TOF using PMF. This technique compares the fragmentation masses obtained by MALDI with existing masses in the databases, but the information in those databases about the masses of filamentous fungi is not yet sufficient for successful identification. Fernández-Acero *et al*. (2007) deal with this handicap by identifying the protein using PMF followed by "*de novo*" peptide sequencing and sequence alignment. For more information regarding the experimental design of proteomic studies, protocols for obtaining fungal proteomic extracts, the identification of protein spots, and applications of proteomic advances to phytopathogenic fungi, reference to the specific review by Fernández-Acero *et al*. (2007a) is strongly recommended.

Considering again the biocontrol agents *Trichoderma* spp., during the last few years many authors have developed various approaches based on 2-DE studies and considerable advance have been made in understanding the mechanisms by which *Trichoderma* spp. is capable of controlling the diseases produced by other phytopathogenic fungi. Summarized below are some of the main achievements obtained with *Trichoderma* spp. In addition to the biocontrol mechanisms of this genus explained in the preceding section, it has also been demonstrated that *Trichoderma* spp. provides an abundance of biotechnologically-valuable proteins and secondary metabolites. Grinyer *et al.* (2004) reported the first data on the proteome of *T. harzianum* and *T. atroviride* grown on liquid substrates enriched with various different carbon sources, including pathogen cell walls. Subsequently, Woo *et al*. (2006) developed a method for separating the proteome of *T. harzianum* and *T. atroviride* from that of bean or tomato while being cultivated together under controlled conditions. They identified a large number of proteins involved in disease resistance processes during colonization of these plants by *Trichoderma* spp. The authors concluded that several resistance genes used for the recognition of microbial metabolites are up-regulated or activated by the presence of the beneficial fungus and probably allow the plant to recognize them (Woo *et al*. 2006).

Four different ABC-transporter encoding genes of *Trichoderma* spp., which are specifically expressed to a greater or less degree when the fungi are exposed to culture filtrates and antibiotics produced by *B. cinerea* and *P. ultimum*, have

been described by Woo *et al.* (2006). These *Trichoderma* spp. fungi are known to be able to compete strongly with other microbes and to possess a high innate degree of resistance to an astonishing assortment of chemicals and natural toxins produced by other microbes (Harman 2000, 2004). The results obtained by Woo *et al*. on the ABC-transporter genes may demonstrate the presence in these fungi of a large number and variety of effective membrane pumps; this would therefore help explain their remarkable resistance to such environmental and biochemical stresses.

It is now accepted that *Trichoderma* spp. fungi establish a strong molecular-based communication/interaction with infected plants (the "cross-talk" referred to previously). In these fungi there is a direct interaction between the living cells of plants and the fungal structures; this has been observed and demonstrated *in vivo*. The type of induced resistance mechanism so far demonstrated for *Trichoderma* spp. resembles that of the induced systemic resistance (ISR) caused by rhizobacteria but, in contrast, researchers have not yet established the mechanism by which *Trichoderma* spp. fungi provide a stimulus for the plant defence mechanism and remains an avirulent symbiont that grows together with the plant root system. It is suggested that *Trichoderma* spp. transfer to or even exchange with the crop plant various receptive molecules that are recognized and able to activate a variety of processes such as defence against phytopathogenic fungi and accelerated development or resistance to such pathogens. It has been demonstrated that *Trichoderma* spp. produce at least three different types of molecules, called elicitors.

The first type of elicitor produced may include various peptides and proteins, such as serine protease, xylanases, chitinases and glucanases. *Trichoderma* spp. fungi secrete them when the presence of the pathogen is sensed and the plant recognizes these molecules as foreign fungal molecules, thus activating the expression of resistance genes. The second type of elicitor may be avr-like proteins similar to those found in avirulent pathogens and the avr proteins of *Trichoderma* spp. itself. The third type of elicitor comprises molecules released from the pathogen and the plant cell wall by the *Trichoderma* cell wall degradation enzymes (CWDEs). These molecules would stimulate the biocontrol fungi and their antagonistic activity by activating the mycoparasitic gene expression cascade, but also act as elicitors when plant cells are exposed to them or when injected into the root and leaf under-surfaces (Woo *et al*. 2006).

With respect to recent research into the biology of phytopathogenic fungi, our group has accumulated a considerable amount of new information about the biology of *Botrytis cinerea* from various different proteomic approaches, which has resulted in the publication of five papers and more than ten congress communications during the last four years (Fernández-Acero *et al.* 2006b, 2007a, 2007b, 2009, 2010). In 2006, Fernández-Acero *et al*. (2006b) reported the first approach to the proteome analysis of *Botrytis cinerea*. Up to four hundred protein spots were resolved in 2-DE after optimizing a protocol for protein extraction using phosphate buffer followed by TCA-acetone precipitation. Due to the absence of genomic data on *B. cinerea*, the proteins had to be sequenced *de novo*, and 21 protein spots were positively identified; these correspond to MDH and GADPH, and some of them are associated with virulence, including these latter two and cyclophilin. This study constituted the basis of subsequent research and was the first approach to analysing the proteome of this important fungus.

Later, Fernández-Acero *et al*. (2007b) carried out a comparative analysis between two *B. cinerea* isolates differing in both virulence and toxin production, in an attempt to find differences between mycelia extracts from the two isolates that could be related to the differences in virulence. The results reported in that study were in agreement with previous identifications of proteins. MDH proteins were identified and found to be over- expressed in the more virulent strains used in that work (*B. cinerea* CECT-2100). The role of MDH as a pathogenicity factor had been suggested previously, because this enzyme catalyzes the reversible conversion of oxalacetate and malate; oxalacetate has been shown to be an oxalic acid precursor which has been described as a pathogenicity factor in *B. cinerea* (Lyon *et al.* 2004). In fact, the influence of pH on the expression and secretion of virulence factors, such as cell wall degrading enzymes, and the biosynthesis of phytotoxins (botrydial and dihydrobotrydial, secreted exclusively in acidic media), support the hypothesis that MDH is likely to play a key role in the cascade of events leading to plant cell death: MDH therefore is probably essential for the whole infection process (Fernández-Acero *et al*. 2007b).

A transcriptional regulator protein of the metE/meth family was found to co-migrate with MDH. This was also found in the proteome of *B. cinerea* in 2006, but now it was under-expressed in the less pathogenic strain of *B. cinerea* (*B. cinerea* CECT-1.11) (Fernández-Acero *et al.* 2006b, 2007b). The variability of these fungicide targets may be involved in the basic molecular structure of the different fungicide resistance phenotypes described for *B. cinerea*, as suggested by Fernández-Acero *et al.* (2007b). Several GAPDHs and a cytosolic cyclophilin were also identified in that work, and these were only present in the more virulent *B. cinerea* strain. GAPDH is involved in oxidative metabolism and the results support the hypothesis that this metabolism may be more active in the pathogenic than in the non-pathogenic strain. In the case of cyclophilin, the role of this protein as a virulence factor in *B. cinerea* has previously been described by Viaud *et al*. (2003), and it is associated with the later stages of infection, such as penetration or plant colonization. Thus, the proteomic analyses of two strains of *B. cinerea*, which differ in virulence as well as in toxin production, was very useful for relating various different cellular components involved in the infection process (Fernández-Acero *et al*. 2007b).

In a subsequent work, Fernández-Acero *et al*. (2009) describe an analysis of the proteins produced by *B. cinerea* during cellulose degradation. It is known that *B. cinerea* is capable of secreting many different cell wall degrading enzymes; therefore since cellulose is one of the major components of the plant cell wall, the authors adopted this experimental approach in their attempt to identify proteins with crucial roles in this degradation. A total of 267 spots were identified; a list with the identifications is available in the World-2PAGE public database (www.expasy.org/world-2dpage/; accession number: 0005-"*B. cinerea* mycelium cultured in carboxymethyl cellulose"). Proteins that could play a significant role in plant infection were identified, specifically *B. cinerea* peptidyl-prolyl *cis-trans* isomerase and glyceraldehydes 3-phosphate dehydrogenase (Fernández-Acero *et al*. 2009) (**Fig. 4**).

In 2009, Shah *et al.* (2009) published an approach to the secretome of *Botrytis cinerea* using a high-throughput LC-MS/MS approach. The authors used a cellophane membrane and a media supplemented with extract of tomato, strawberry and/or *Arabidopsis* leaf extract. *Botrytis cinerea* secreted proteins were identified such as transport proteins, carbohydrate metabolism proteins, peptidases, and oxidation/reduction proteins used by *B. cinerea* secreted proteins for plant infection and colonization (Shah *et al*. 2009). Our group has developed a proteomics approach for studying the secretome of *B. cinerea*; recently, an article has been published showing interesting results that open the door to understanding the protein that *B. cinerea* secretes to the external environment and its induction pathways (Fernández-Acero *et al*. 2010). This work constitutes the first proteomic approach to the secretome of this phytopathogenic fungus based on 2-DE combined with MS/MS analysis. The secretome has been studied using carbon sources and plant-based elicitors under controlled culture conditions. The different elicitors, as explained in preceding paragraphs, are molecules that activate the plant defences and accelerate the development of resistance against phytopathogenic fungi; these have been used, in this proteomic approach, to stimulate the fungal infection mechanisms in order to identify the

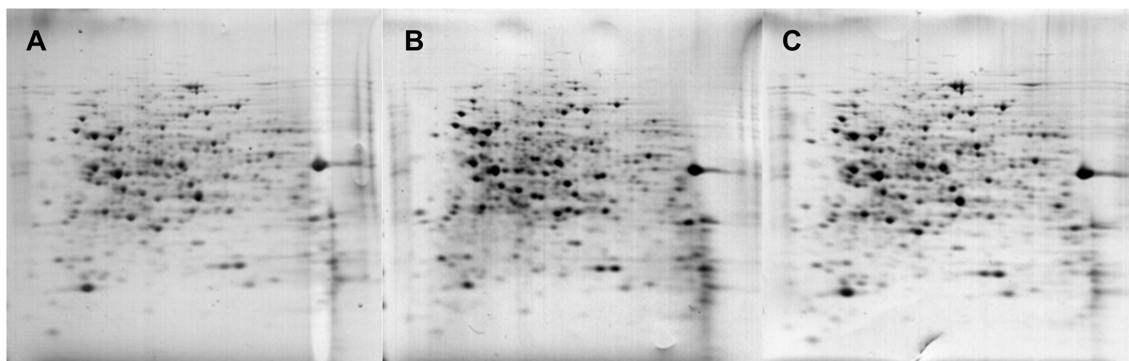

Fig 4 2-DE CBB-stained gels from mycelial extracts of *B. cinerea* 2100 growing in different carbon sources. (**A**) Glucose, (**B**) pectin, (**C**) starch. Proteins were separated on 17 cm, pH 3–10 non-linear gradient IPG gels (IEF) and 14% polyacrylamide gels (SDS-PAGE).

protein secreted by the fungus (Fernández-Acero *et al.* 2010). More than seventy protein spots have been identified in this work. The proteins identified from the *B. cinerea* secretome were assigned to categories according to their involvement in different biological processes, including carbohydrate metabolic processes, metabolic processes, and cell wall organization and biogenesis. Classifying the proteins by function, hydrolase activity, catalytic activity, carbohydrate binding, and binding activity, among others, were found. A detailed study of the spots found in our approach show a clear relationship between the proteins identified and the virulence data obtained from *B. cinerea*. Many proteins identified in this work have not yet been functionally assigned, but their regulation under the conditions assayed demonstrates their functional significance in the mechanisms of infection used by *B. cinerea*, and the study will advance our functional understanding of *Botrytis* pathogenesis.

All of these new proteomics approaches and the results achieved by the several research groups working with biocontrol agents or directly with the phytopathogenic fungi, improve our understanding of the complex biology of these microorganisms. The proteome-mining approaches are particularly promising from a biotechnological perspective because the kinds of biomolecule synthesized and secreted by these filamentous fungi can be studied in depth, and may be future alternatives as biocontrol molecules, instead of using biological organisms that depend to a greater or less extent on the particular environmental conditions for their growth. In addition, the proteins identified from these studies can be used as therapeutic targets to enable the design of more specific and ecological fungicides, based on targeted molecular research; some of the proteins identified may even be useful for developing a procedure for field diagnosis of plant infections (Fernández-Acero *et al.* 2010).

CONCLUSIONS

Strawberry is a commercially important crop which production and marketing generate hundreds of millions of Euros. The commercial viability of the crop is continually subject to various risks, one of the most serious of which is the diseases caused by phytopathogenic organisms. Until the last decade, the cultivation of strawberry has followed lines of action established in the 1950s and 60s. During the last years the advances in control measures applied in agriculture, and the new laws and regulations imposed by the international community, the traditional approaches are changing towards a more rational kind of agriculture.

In this review we have discussed recent advances in molecular biology, which are being applied in molecular technologies, to provide new strategies and weapons for combating the diseases that affect the strawberry – specifically those caused by fungal pathogens. The development of new methods for studying and controlling phytopathogenic fungi has occurred in parallel with the development and improvement of molecular technologies. New and more accurate methods for the detection, identification and monitoring of fungal pathogens, even directly from plant materials, have been demonstrated. Real-time PCR assays offer increased sensitivity compared with current gel-based PCR for the diagnosis of these pathogens. Diagnosis based on real-time PCR gives a rapid, sensitive and accurate result in 1–2 days, allowing high-throughput and inexpensive screening of samples. These methods also permit the detection of pathogens from plant material before any symptoms are apparent, and have proved to be a useful tool for studying the epidemiological routes of these strawberry pathogens in fields and nurseries.

Many research groups are devoting considerable efforts to identifying new species of antagonism organisms with capacity to act as biocontrol agents. The soil microflora is a complex microenvironment. Research is needed to clarify which of the pathogens adversely affect growth and cause yield reductions in field planting, how they are distributed in the production area, the time of year that they have the most significant impact on the strawberry plant, the effect of environmental parameters on disease, and the influence of cropping practices on disease severity (Martin *et al.* 2002). The success of biological approaches to controlling plant diseases must be judged by their performance under field conditions. Although we have shown in this review that several research projects have achieved promising results in terms of the antagonist activity of several species of bacteria and fungi, the main problem is reliability. Unfortunately, discrepancies exist between the antagonistic effect under *in vitro* conditions and the corresponding *in situ* efficacy, and although a good efficacy is shown in greenhouse assays under controlled conditions, this is not well correlated with the efficacy under field conditions, because many factors influence the growth and activity of the prospective biocontrol agents. Further studies are necessary to understand the mechanisms of action of antagonism in microorganisms and thereby to improve the biocontrol practices based on those mechanisms.

Advances in proteomics studies and proteome-mining approaches are providing a compendium of tools for a better understanding of the complex biological metabolisms presented by plants, biocontrol agents, and phytopathogenic fungi. Given the achievements reported for these techniques in several fields, including plant pathology, some authors have described the current period as the "post-genomic era" (Fernández-Acero *et al.* 2007a). We have presented the results obtained by several groups in this field, and their contributions in identifying proteins that may be involved in pathogenesis. Proteomics is a promising field for discovering new factors of pathogenicity and for dissecting infection mechanisms. In the near future, the proteins identified will be used as therapeutic targets, allowing the design of more specific and ecological fungicides based on targeted molecular research. Some of the proteins identified may even be used to develop a procedure for field diagnosis

of plant infections (Fernández-Acero *et al.* 2010).

After reading this review, one might believe that solutions to most of the problems associated with strawberry fungal pathogens have now been found, but nothing could be further from the truth. A broad selection of alternative improved methods has been considered in the current review. Moreover, more accurate and reliable protocols will be published in the coming years. However, the phytopathogenic organisms will continue evolving and new species and new pathotypes of the same pathogens will appear in the field, better adapted to changing environmental conditions and more resistant to fungicides and biomolecules/ toxins produced by biocontrol agents. It is necessary to continue undertaking research projects aimed at developing new methods with practical application, but it is also necessary to continue studying the complex biology of these microorganisms, in order to gain a better understanding that will enable strategies to be devised for their future control, and for the preservation of the highest possible quality of the strawberry crop in the future.

ACKNOWLEDGEMENTS

We would like to thank the Spanish Government, the Ministerio de Ciencia y Tecnología, and the Junta de Andalucía (Regional Government) for the financing of most of the work presented in this Review article, which was carried out by Garrido *et al*. and Fernández-Acero *et al.* (Spanish DGICYT AGL2006-13401-C02-02/AGR and AGL2009-13359-C02-02; Junta de Andalucía, PO7-FQM-02689).

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