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Pathogen profile

Developing tools to unravel the biological secrets of *Rosellinia necatrix*, an emergent threat to woody crops

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SUMMARY

White root rot caused by *Rosellinia necatrix* is one of the most destructive diseases of many woody plants in the temperate regions of the world, particularly in Europe and Asia. Recent outbreaks of *R. necatrix* around the globe have increased the interest in this pathogen. Although the ecology of the disease has been poorly studied, recent genetic and molecular advances have opened the way for future detailed studies of this fungus.

Taxonomy: *Rosellinia necatrix* Prilleux. Kingdom Fungi; subdivision Ascomycotina; class Euascomycetes; subclass Pyrenomycetes; order Sphaeriales, syn. Xylariales; family Xylariaceae; genus *Rosellinia*.

Identification: Fungal mycelium is present on root surfaces and under the bark, forming mycelium fans, strands or cords. A typical presence of pear-shaped or pyriform swellings can be found above the hyphal septum (with diameters of up to 13 μm). Sclerotia are black, hard and spherical nodules, several millimetres in diameter. Black sclerotia crusts may also form on roots. On synthetic media, it forms microsclerotia: irregular rough bodies composed of a compact mass of melanized, interwoven hyphae with no differentiated cells. Chlamydospores are almost spherical (15 μm in diameter). Synnemata, also named coremia (0.5–1.5 mm in length), can be formed from sclerotia or from mycelial masses. Conidia (3–5 μm in length and 2.5–3 μm in width) are very difficult to germinate *in vitro*. Ascospores are monostichous, situated inside a cylindrical, long-stalked ascus. They are ellipsoidal and cymbiform (36–46 μm in length and 5.5–6.3 μm in width).

Host range: This fungus can attack above 170 different plant hosts from 63 genera and 30 different families, including vascular plants and algae. Some are of significant economic importance, such as *Coffea* spp., *Malus* spp., *Olea europaea* L., *Persea americana* Mill., *Prunus* spp. and *Vitis vinifera* L.

Disease symptoms: *Rosellinia necatrix* causes white (or *Dematophora*) root rot, which, by aerial symptoms, shows a progressive weakening of the plant, accompanied by a decline in vigour. The leaves wilt and dry, and the tree can eventually die. White cottony mycelium and mycelial strands can be observed in the crown and on the root surface. On woody plant roots, the fungus can be located between the bark and the wood, developing typical mycelium fans, invading the whole root and causing general rotting.

Disease control: Some approaches have been attempted involving the use of tolerant plants and physical control (solarization). Chemical control in the field and biological control methods are still under development.

INTRODUCTION

Woody crops extend to all temperate areas. Among their phytopathological threats, the soil-borne fungi are gaining relevance. A few fungi cause root rot in woody plants in temperate areas; one of these is *Rosellinia necatrix* (Freeman and Stezjnberg, 1992), a cosmopolitan fungus from which quantitative data on losses are mostly lacking. However, some studies have estimated increasing losses caused by this fungus in apple trees (*Malus* spp.) (Agarwala and Sharma, 1966), glasshouse grapevines (*Vitis vinifera*) and Japanese pear trees (*Pyrus pyrifolia*) (Ten Hoopen and Krauss, 2006). Recently, *R. necatrix* was included in the fungi listed as regulated plant pests by the US Department of Agriculture Animal and Plant Health Inspection Service (Cline and Farr, 2006). Simultaneously, this fungus was included in the list of diseases already present in Australia (*New South Wales Government Gazette*, 1 September 2006, available at www.nsw.gov.au); since then, it has come to be considered as an emergent threat to many crops, such as cotton, nuts, apples and pears (Department of Primary Industries, Vic., Australia, 2008, available at http://new.dpi.vic.gov.au/agriculture/pests-diseases-and-weeds/plant-diseases/fruit-diseases/white-root-rot).

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In the Mediterranean region, white root rot can be especially damaging because of the co-occurrence of favourable environmental conditions for the development of the fungus and susceptible hosts. In this region, traditional crops, such as olives, almonds and vineyards, are being replaced by subtropical crops, such as avocado and mango. The inherent differences in watering, soil nutrition, crop management and other practices have been shown to play a role in the emergence of this pathogen (Pérez-Jiménez, 2006).

The ecology and biology of *R. necatrix* are unknown. A lack of information about the life cycle, including how it initiates root infection, among other concepts, has limited the study of this pathogen. The recent advent of molecular tools has allowed the development of new studies, which have elucidated aspects of the biology and ecology of the fungal infection. This review updates the current knowledge on *R. necatrix* biology, focusing on the molecular aspects that can also be used as research tools, the description of the host–pathogen interaction and different control approaches.

TAXONOMY

The genus *Rosellinia* (subdivision Ascomycotina; class Euascomycetes; subclass Pyrenomycetes; order Sphaeriales, syn. Xylariales; family: Xylariaceae) was identified by De Notaris in 1844, although its systematic site within the Pyrenomycetes was not always clear, as the stromatic character of its fructifications was unrecognized by many authors. *Dematophora necatrix*, asexual facies, was previously known as *Rhizomorpha necatrix*, when it was considered as an important threat to vineyards in Germany and France (Hartig, 1883). On the basis of the analysis of conidial morphology, Hartig (1883) speculated that the teleomorph of *D. necatrix* might belong in *Rosellinia* or a closely related genus. Afterwards, in 1892, when studying the ascocarps of *Rosellinia aquila*, Berlese included *D. necatrix* within the genus *Rosellinia* (Behdad, 1975) and, several years later, Prillieux (1902) confirmed this taxonomy after obtaining perithecia from fruit tree roots infected with *D. necatrix*. He named the teleomorph *R. necatrix*. Hansen *et al*. (1937) and Pérez-Jiménez *et al*. (2003) also confirmed the relationship between *D. necatrix* and *R. necatrix*. Prillieux (1904) was the first to give a detailed description of the teleomorph, together with its disease symptoms (Cline, 2010). A description of the development of the teleomorph was given by Nakamura *et al*. (2000) and Pérez-Jiménez *et al*. (2003). The taxonomic characteristics of this species have been reviewed by Francis (1985) and Petrini (1993).

HOSTS AND GEOGRAPHICAL DISTRIBUTION

Rosellinia necatrix has become a pathogen of increasing importance and, currently, has a wide range of host species. A list of 437 reports of fungus–host combinations with specific references is available at http://nt.ars-grin.gov/fungaldatabases/index.cfm, many of which are of economic interest. These include tropical (avocado, coffee, citrus and mango) and temperate (almond, apple, fig, kiwi, grape, olive, pear, peach, persimmon, sweet cherry and tea) fruit trees, nut tree crops (chestnut, pistachio and walnut), small fruits, such as the strawberry, narrow leaf (cedar, fir, pine, sequoia and yew) and wide leaf (holly, oak, poplar and elm) forest trees, herbaceous (daffodil and paeony) and woody (azalea, camellia and rose) ornamental plants and field crops (alfalfa and potato). Other field crops (beans and cotton) and some weeds have also been killed following artificial inoculations with this fungus.

Rosellinia necatrix is considered to be a cosmopolitan pathogen, as it is widely distributed on five continents in temperate, subtropical and tropical zones (Behdad, 1975; Farr *et al*., 2006; Petrini, 1993; Saccas, 1956; Sivanesan and Holliday, 1972). This pathogen is a limiting factor for avocado and apple crops in Israel and southern Spain, where its incidence has progressively increased to the point at which it is now considered to be the most important cause of endemic avocado root rot (López-Herrera, 1998). In Japan, it is considered to be one of the most serious pathogens affecting fruit trees, such as grapevines and Japanese pear and apple trees (Arakawa *et al*., 2002). *Rosellinia necatrix* is also considered to be a potential threat in Thailand (Thienhirun and Whalley, 2001).

LIFE CYCLE AND DISEASE SYMPTOMS

The asexual life cycle of *R. necatrix* (Fig. 1) occurs through two different spore types: chlamydospores and conidiospores. Chlamydospores can be found only under exceptional environmental conditions and are rarely found under natural and artificial conditions (Pérez-Jiménez *et al*., 2003). These spores are almost spherical and are 15 μm in diameter; they originate by condensation of the pyriform swellings in the protoplasm and subsequent formation of a cell wall (Makambila, 1976). Conidia originate at the ends of synnemata of conidiogenous cells, which are produced from either sclerotia (possibly related to pathogen survival in soil) or brown mycelial masses. The length of the synnemata varies between 0.5 and 1.5 mm. Conidia are solitary, one-celled, hyaline, elliptical, 3–5 μm in length and 2.5–3 μm in width, and are borne both apically and laterally to the conidiogenous cells (Petrini, 1993).

The sexual reproductive structures of *R. necatrix* (ascospores, Fig. 1) are formed inside the perithecium and, when they reach maturity, are expelled into a mucilaginous mass from the pore of the papilla located at the top of the perithecium. These structures have been found on infected apple, loquat and avocado tree roots (Lin and Duan, 1988; Makambila, 1976; Pérez-Jiménez *et al*., 2003; Teixeira de Sousa and Whalley, 1991). Their diameter ranges from 0.89 to 1.78 mm, and their height from 1.39 to 1.58 mm. Fresh perithecia are soft and spherical, with a gelatinous aspect and a honey colour. As they age, they contract and acquire a brown–black colour and a dry aspect as a result of hyphal and cell melanization. The asci are projected towards the interior of the perithecium (Pérez-Jiménez *et al*., 2003).

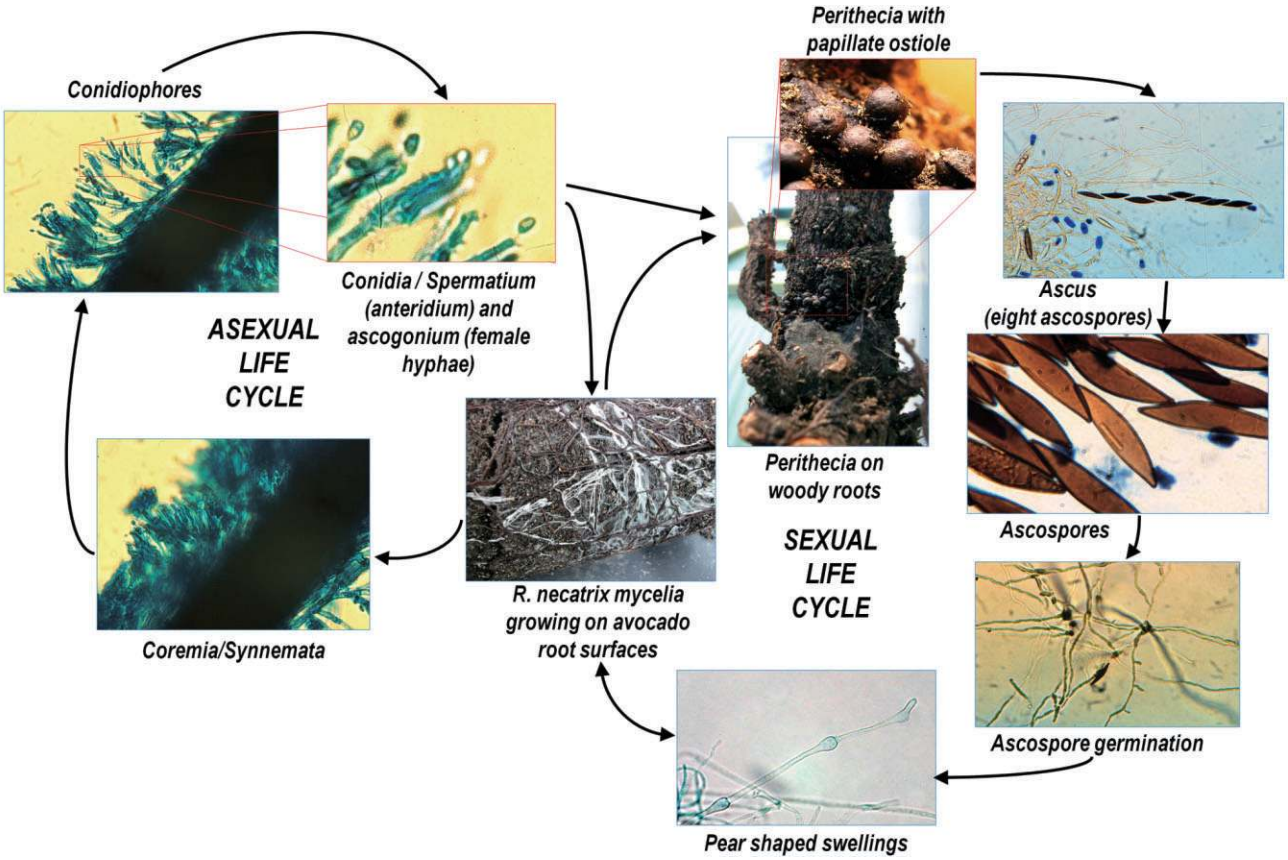

Fig. 1 Life cycle of *Rosellinia necatrix*, covering the sexual and asexual aspects of the cycle. The asexual life cycle occurs via two different spore types, chlamydospores (not considered because they are rarely found under natural conditions) and conidiospores. Conidia originate at the ends of synnemata of conidiogenous cells, which are produced from either sclerotia or brown mycelial masses. The sexual life cycle is mediated by ascospores, which can be easily found in infected tissues. The sexual reproductive structures of *R. necatrix* are formed inside the perithecium and, when they reach maturity, are expelled. As they age, they contract and acquire a brown–black colour and a dry aspect as a result of hyphal and cell melanization. The asci are projected towards the interior of the perithecium. Perithecia formation takes a long time under natural conditions and has never been achieved previously in a Petri dish.

Ascospores are monostichous and are located within a cylindrical long-stalked ascus (with a length ranging from 62.0 to 93.0 μm and width from 6.9 to 10.1 μm). They are ellipsoidal and cymbiform, and their sizes vary between 36 and 46 μm in length and 5.5 and 6.3 μm in width (Pérez-Jiménez *et al*., 2003; Petrini, 1993; Sivanesan and Holliday, 1972). Perithecia formation takes a long time under natural conditions and has never been achieved previously in a Petri dish.

Recently, Ikeda *et al*. (2011) confirmed, by DNA polymorphism analysis of ascospore progenies, that *R. necatrix* has a heterothallic life cycle (Ikeda *et al*., 2011; Kanda *et al*., 2003). In this regard, diseased roots develop a synnemata, which produces conidia as spermatia. These spermatia spread to adjacent diseased roots to produce perithecia (Ikeda *et al*., 2011; Nakamura *et al*., 2002) (Fig. 1).

The roles of these three spore types in the epidemiology of the fungus are still not clear and appear to vary in different countries. In Spain, it is believed that the pathogen spreads through direct root contacts between host plants (Delatour and Guillaumin, 1985). In this regard, the mycelium and aggregate organs can complete a whole infection cycle (Figs 2 and 3).

A detailed description of the infection process of *R. necatrix* on avocado roots has been reported recently by microscopic visualization using green fluorescent protein (GFP) derivative strains of *R. necatrix* (Pliego *et al*., 2009). The fungus is dispersed throughout the soil by the mycelium and mycelial strands or along the infected roots. On touching a healthy root, the mycelia network proliferates, covering the root surface either as a diffuse mycelium or in the form of hyphal strands. Then, in root areas covered by a diffuse mycelium, mycelial aggregates are formed over the root surface. The penetration of avocado roots by *R. necatrix* CH53-GFP takes place at these sites (Fig. 3) through natural openings (lenticels), wounds or directly by forming a penetration sclerotium. Primary infection of these organs through the junctions between epidermal root cells occurs simultaneously at several random positions along the root

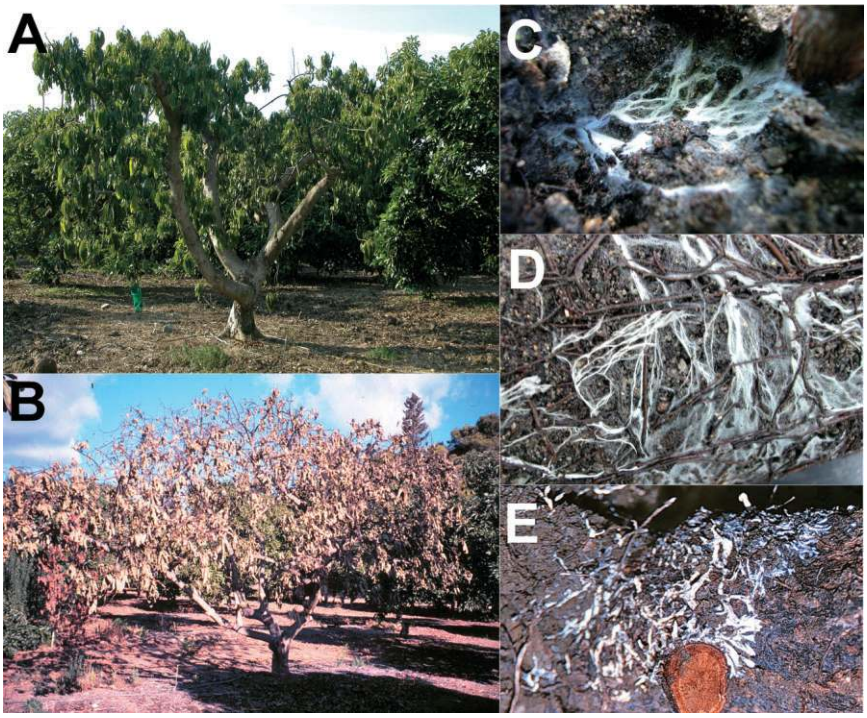

Fig. 2 Characteristic macroscopic symptoms caused by *Rosellinia necatrix* on avocado. (A) Healthy 15-year-old avocado tree. (B) Advanced aerial symptoms of *R. necatrix* root rot on a 15-year-old avocado tree: dry leaves attached to the wilted tree, sparse foliage and dry branches are observed. (C) Hyphal strands and cords of *R. necatrix* on the soil surface. (D) Hyphal strands and cords of *R. necatrix* spreading from colonized to healthy avocado roots in an avocado pot plant. (E) *Rosellinia necatrix* spreading on the wood in a symptomatic 15-year-old avocado tree.

longitudinal axis. *Rosellinia necatrix* hyphae invade and penetrate into the primary and secondary xylem (Pliego *et al*., 2009).

The development of the *R. necatrix* mycelium and mycelial strands depends on temperature, oxygen, moisture and organic matter contents, pH and soil microflora. *In vitro* studies have shown that the optimal growth temperature is 22–24 °C; it does not grow below 4 °C or above 32 °C (Abe and Kono, 1953; Anselmi and Giorcelli, 1990; Araki, 1967; Mantell and Wheeler, 1973; Pérez-Jiménez, 1997). Unlike most fungi, *R. necatrix* is not inhibited by high pH; *in vitro* growth occurs over a pH range of 4–9 (Makambila, 1978; Pérez-Jiménez, 1997; Ruano-Rosa, 2006), and growth in soil occurs over a pH range of 6–8 (Anselmi and Giorcelli, 1990; Gupta and Gupta, 1992). Soil moisture is the most important factor influencing the growth of the fungus and, in sandy–silt soils, the optimal growth of the mycelium is obtained at field capacity and is reduced when moisture approaches the wilting point (Anselmi and Giorcelli, 1990). Poor aeration is also unfavourable for disease occurrence, as the growth of *R. necatrix* mycelia is retarded when the oxygen content of the air is less than 10% (Araki, 1967). As a consequence, fungal distribution is often restricted to the topsoil (Abe and Kono, 1955; Makambila, 1976). Mycelial growth is strongly inhibited by light (Anselmi and Giorcelli, 1990; Makambila, 1976), and its development requires high levels of organic matter (Agarwala and Sharma, 1976; López and Fernández, 1966).

The virulence factors of *R. necatrix* are complex and not well understood, although it is well known that this pathogen is able to produce secondary metabolites that are toxic to plants and that can be found in filtrates obtained from liquid cultures of *R. necatrix*. Their toxicities have been tested on host seedlings: they caused black spots, discolouration, wilting, curling and blackening, and were also able to inhibit the germination of rice seeds (Abe and Kono, 1955, 1957). One metabolite is cytochalasin E (Chen, 1960; Cole and Cox, 1981; Kimura *et al*., 1989; Kshirsagar *et al*., 2001), which has physiological activity on mammalian cells, microorganisms and plants. However, because mutants that did not produce cytochalasin E did not affect the progress of the disease, this is not considered to be a main factor for *R. necatrix* pathogenicity (Kanematsu *et al*., 1997). Other secondary metabolites produced by *R. necatrix* filtrates include rosellichalasin (Kimura *et al*., 1989), rosellinic acid, rosnecatrone and diketopiperazines (Edwards *et al*., 2001). The production ratios of these secondary metabolites on fungal filtrates have been shown to vary among strains. In addition, important cellulolytic activities have been reported for *R. necatrix*, suggesting its capacity to metabolize existing sugars in vegetative cells, contributing to the infection process (Tourvieille de Labrouhe, 1986).

Plants infected by *R. necatrix* normally show both aerial and root symptoms that arise as a consequence of damaged roots and the delivery of toxic compounds inside the plant vascular system (Fig. 2). The first symptom that can be observed on larger infected root surfaces is the existence of white cottony mycelium and white or black mycelial strands. Invasion extends through the cambium and wood to the trunk. On woody plants, the fungus is located between the bark and the wood, developing very typical white mycelial fans (Fig. 2C–E). Later, the white mycelium turns

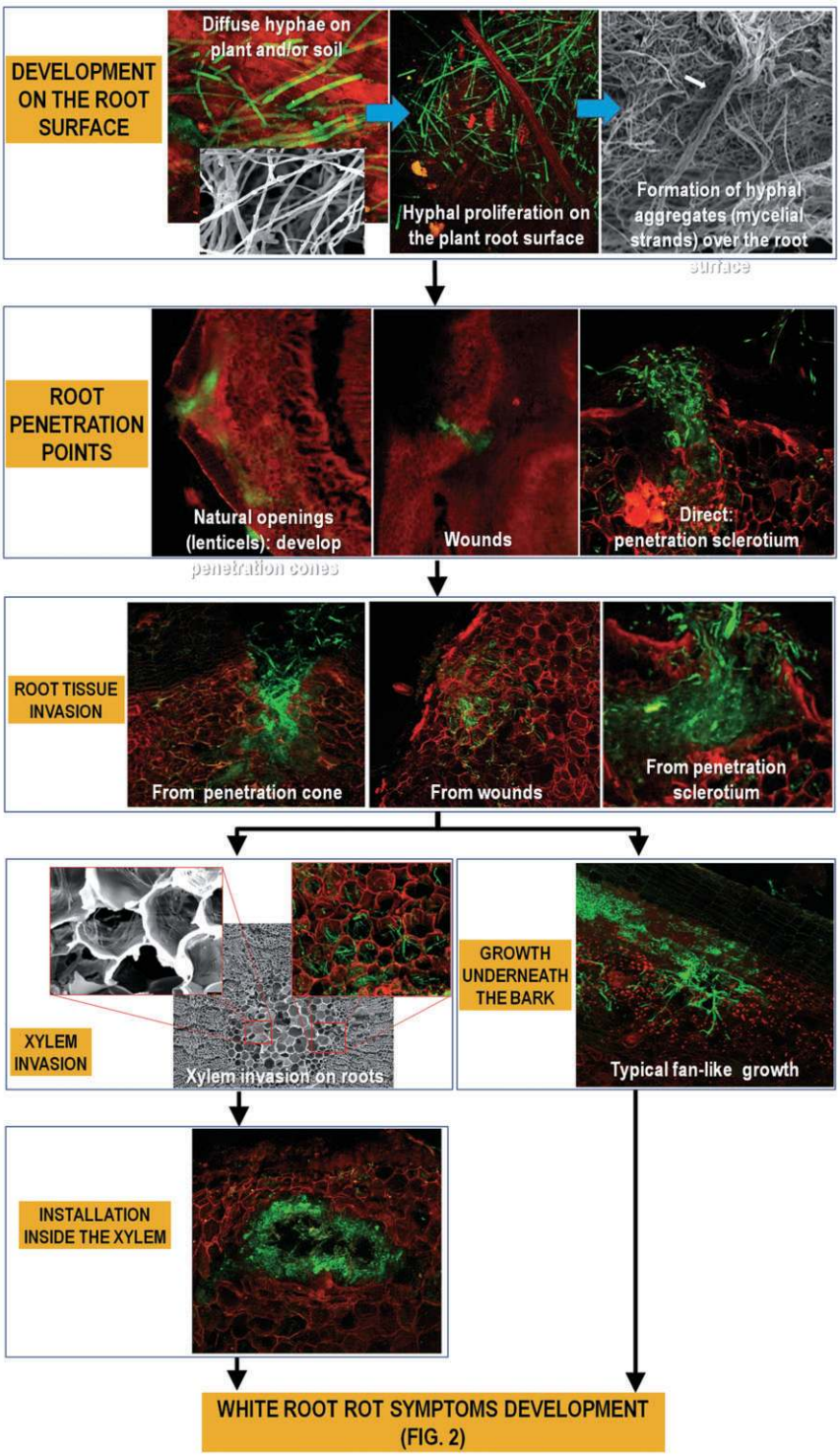

Fig. 3 Root infection course of *Rosellinia necatrix* in avocado. This study was conducted used the green fluorescent protein (GFP)-tagged *R. necatrix* CH53 strain and visualized by confocal laser spectroscopy and scanning electron microscopy. The whole process takes around 24 days in 1-year-old avocado plantlets. The root infection by *R. necatrix* initiates with fungal development on the root surface without penetration. Then, root penetration can occur in different ways, including natural openings, surface wounds and by direct penetration. Once inside the root, *R. necatrix* spreads to the inner parts, resulting in xylem invasion, as well as underneath the bark, leading to full symptom expression, as shown in Fig. 2.

greenish-grey or black, and the pathogen forms indefinite plaques within the bark and loosely aggregated strands of associated hyphae, which invade the whole root system, causing a general rotting. Subsequently, the roots acquire a dark brown colour.

Infected trees do not always show aerial symptoms, making the diagnosis of this fungus extremely difficult. The evolution of the symptoms expressed by the aerial system, especially on fruit trees, can occur either quickly or slowly. In the first case, and in a very short period of time, infected trees may suddenly decline in vigour, leaves wilt and dry and, finally, trees eventually die. In the second case, symptoms develop more slowly and, consequently, retarded growth can be observed with infected trees. Sparse foliage may be observed in these trees, together with wilting of the leaves, chlorosis and the death of twigs, branches and leaves. These symptoms worsen every year, when moisture and temperature are favourable, and trees eventually die (Guillaumin *et al*., 1982).

RESEARCH TOOLS

The recent development of molecular tools that can be applied to *R. necatrix* have opened up the way to new approaches to unravel unknown aspects of its biology. Using different molecular techniques, improvements have been made in the early detection, visualization of plant–pathogen interactions and control by virus infections.

Genetic tools

In the case of *R. necatrix*, DNA extractions require a 7-day pregrowth period of mycelia on potato dextrose agar (PDA) in the dark at 25 °C. Small blocks containing mycelia are excised from the colony and transferred onto new PDA plates containing a sterile cellulose membrane. After incubation at 25 °C for 5 days, mycelia are frozen and ground with a pestle. Mycelia cells can be lysed with lysis buffer containing: 1 M tris(hydroxymethyl)aminomethane (Tris)-HCl, pH 8, 0.5 M ethylenediaminetetraacetic acid (EDTA), 5 M NaCl, sodium dodecylsulphate (SDS) (0.5%) and β-mercaptoethanol (Choi and Nuss, 1992), and DNA can be extracted with phenol–chloroform–iso-amyl alcohol. DNA extractions of *R. necatrix* have also been successfully performed using the E.Z.N.A. Plant Miniprep Kit (Omega Bio-Tek Inc., Norcross, GA, USA), following the manufacturer's instructions (Armengol *et al*., 2010). No RNA extractions of *R. necatrix* have been performed to date.

To date, few studies have focused on the genetic analysis of *R. necatrix* because of the difficulty in generating mutations in this pathogen. Most mutagenesis studies (UV, chemical and transposon insertional mutagenesis) are based on the isolation of mononucleated cells, such as conidia. Nakamura *et al*. (2002) described the production of conidioma from *R. necatrix* in axenic culture under near-UV light irradiation. To accomplish this, pieces of sterilized Japanese pear twig were placed on 7-day-old oatmeal agar culture in plates, which were further incubated for 5 days and then illuminated with near-UV light. Synnemata and conidia were produced (Pérez-Jiménez *et al*., 2003); however, the *in vitro* germination of conidia or chlamydospores has not yet been reported. The germination of ascospores has been achieved and described by Hansen *et al*. (1937) and Teixeira de Sousa and Whalley (1991); however, the sexual stage of the fungus cannot be reached *in vitro*.

Kanematsu *et al*. (1997) used UV irradiation mutagenesis to obtain *R. necatrix* cytochalasin E (CE) mutants. After stirring in a Petri dish, mycelial fragments were exposed to short-wavelength (254-nm) UV light at a distance of 30 cm from the lamp (15 W) for 60 s. All mycelial fragments were tested for the production of CE by thin layer chromatography (TLC), and two mutants producing smaller amounts of CE were isolated.

DNA-mediated transformation

The capacity to introduce homologous or heterologous genes into *R. necatrix* can also increase our understanding of how this pathogen causes disease. In recent years, successful transformations of *R. necatrix* have been achieved either via *Agrobacterium*- or $CaCl_2$–polyethylene glycol (PEG)-mediated protoplast transformation. In both cases, the *hph* (hygromycin B phosphotransferase) gene, under the control of the glyceraldehyde 3-phosphate dehydrogenase promoter (P*gpd*) or the tryptophan promoter (P*trp*C) and the *trp*C terminator (T*trp*C) from *Aspergillus nidulans*, was used as a marker gene because of its functionality in *R. necatrix* cells.

Protoplast transformation of *R. necatrix* strains was first described by Kanematsu *et al*. (2004). They reported an optimized protocol that yielded a total of $(0.5–1) \times 10^7$ protoplasts per gram of wet mycelia with 0.01%–0.03% regeneration frequencies among the four tested strains. In this protocol, the utilization of disaggregated young mycelia, 0.6 M mannitol osmotic solution, an enzyme osmoticum mixture containing Zymolyase 100T and 1% lysing enzymes played a key role in the production of round and clearly visible protoplasts. Transformation of *R. necatrix* protoplasts using $CaCl_2$–PEG occurred in 20 min at 20 °C in the presence of 10 mM $CaCl_2$ and 60% PEG 4000. Highly purified and concentrated (10 μg/μL) DNA plasmids pAN7.1 and pSH75 (containing the *hph* gene) were required for the transformation experiments. The optimized protocol proved to be useful for all *R. necatrix* isolates tested, although the transformation efficiency was relatively low (two to five transformants per experiment), probably as a result of the difficulty in preparing protoplasts from mycelia of this pathogen because of the low rates of protoplast formation and the slow regeneration process.

More recently, eight Spanish and Japanese isolates of *R. necatrix* strains exhibiting different degrees of virulence have been assessed for their capacities to generate high protoplast yields and

to be transformed with plasmid pCPXHY1eGFP. This plasmid contains the *egfp* gene, encoding the enhanced green fluorescent protein, under the control of the P*gpd* promoter and the T*gpd* terminator of the *A. nidulans gpd* gene, with the *hph* gene as a selectable marker. Protoplast generation and transformation were performed by increasing the amount of lysing enzymes to 1.5% with respect to previously reported methods (Kanematsu *et al*., 2004). However, similar protoplast yields were obtained. A critical role for Zymolyase 100T was confirmed during these experiments, as low protoplast yields were obtained in its absence. HygB-resistant transformants from five of the eight tested strains were obtained. The transformation efficiency varied significantly among the different *R. necatrix* isolates. The number of transformants (per 10 μg of plasmid DNA) was in the range 1–20. As a consequence, the appropriate transformation system must be optimized for each individual strain, as for many filamentous fungi. The transformation of *R. necatrix* protoplasts, generated from the same strains as used in this study, was also attempted using a binary vector system composed of plasmids Pgpd-gfp and Pan7-1, carrying the *egfp* and *hph* genes, respectively; however, no transformants were obtained for any of these strains, suggesting that the utilization of one vector containing both genes is more efficient than co-transformation experiments with two plasmids.

Agrobacterium-mediated transformation of *R. necatrix* mycelia has recently been optimized with regard to the *Agrobacterium* strain and co-cultivation temperature (Kano *et al*., 2011). Two derivative plasmids of pAN26 (pAN26_BI121 and pAN26_CB1300) were used for the transformation of three different *A. tumefaciens* strains (LBA4404, EHA105 and AGL-1) at three different co-cultivation temperatures (17, 20 and 25 °C). The optimal temperature for transformation of *R. necatrix* was 25 °C, a temperature also found to be near optimal for the T-DNA transfer machinery in *A. tumefaciens*. Among the three *Agrobacterium* strains assayed, AGL-1 showed high efficiency with all *R. necatrix* strains when using the vector pAN26_CB1300. *Agrobacterium*-mediated transformation resulted in single-copy and random T-DNA integrations in each transformant, suggesting that this method is a useful insertional mutagenesis and gene disruption tool in this pathogen.

Identification of the pathogen

Because of the difficulties in the diagnosis of white root rot, especially on woody crops, large efforts have been made to facilitate the early detection of the pathogen. As a result of the increasing importance of *R. necatrix*, early detection methods and treatment of the pathogen have been developed as tools for disease control. This has motivated researchers to develop methods to differentiate healthy uninfected plants from those at an early stage of infection, but with no visible symptoms. Microbiological techniques, such as isolation of the pathogen from infected roots, are difficult because of the lack of selective media and the occurrence of large numbers of saprophytic microorganisms. Sztejnberg *et al*. (1987) developed the avocado leaf disc colonization method to isolate and assess inocula levels of *R. necatrix* in naturally and artificially infected soils; however, this is a laborious procedure, and expertise is needed to identify the fungus (Petrini, 1993).

Baiting methods have also been used traditionally to detect *R. necatrix* in the soil around mulberry trees. Tanaka (1965) reported that the insertion of twigs into the soil at a depth of 15 cm for 2 months was effective for the diagnosis of white root rot. Recently, this technique has been optimized by Eguchi *et al*. (2009) who, following the insertion of twigs near trunk bases at soil depths of 6–20 cm, were able to trap *R. necatrix* from 80% and 75% of infected Japanese pear and apple trees, respectively. These results suggest that the bait method can aid in the detection of this pathogen in trees at early stages of infection; in addition, its use is recommended to detect the recurrence of the fungus after fungicide treatment.

However, many recent studies have focused on the development of rapid and sensitive detection methods in roots and soils by high-throughput genetic techniques, such as those based on real-time Scorpion-polymerase chain reaction (PCR) (Schena and Ippolito, 2003). This technology combines the sensitivity of conventional PCR with the generation of a specific fluorescent signal, providing real-time analysis of the reaction kinetics and allowing the quantification of specific DNA targets. A nested Scorpion-PCR with conventional primers (R3–R8) and Scorpion probe (R15 Scorpion R18, Table 1) detected *R. necatrix* in infected soils in approximately 6 h. These primers, specific for *R. necatrix*, were designed from the internal transcribed spacer (ITS) regions of the ribosomal RNA genes and were assessed using a large number of fungal contaminants in *Rosellinia*-infected tissues. The ITS regions ITS1 and ITS2 from *R. necatrix* 18S rRNA are available from the National Center for Biotechnology Information (NCBI), with accession numbers AB017657 and AB017658, respectively. The molecular detection technique proved to be more sensitive and reliable than the traditional detection methods described above and, because it is not affected by external factors, it is less time consuming and does not require expertise for fungal identification (Ruano-Rosa *et al*., 2007; Schena *et al*., 2002; Schena and Ippolito, 2003).

Determination of genetic diversity

A useful tool has been developed in studies undertaken to identify intraspecific diversity within field populations of fungal plant pathogens. These studies have provided valuable information about the reproductive strategy of the pathogen and its dispersal process (Cilliers *et al*., 2000; Couch and Kohn, 2000; Kohn *et al*., 1991; Kull *et al*., 2004; López *et al*., 2008; Wu and Subbarao,

Table 1 Primers utilized for the identification of *Rosellinia necatrix* and the generation of polymorphisms.

Primer	Sequence	Used for	Reference
R2F	CAAAACCCATGTGAACATACCA	^{I}PCR	Schena *et al*. (2002)
R3F	CGAAGTGCCCTACCCTGTTA	^{I}PCR	Schena *et al*. (2002)
R8R	CCGAGGTCAACCTTTGGTATAG	^{I}PCR	Schena *et al*. (2002)
R15F	CCATAGGCGAGATGAGAAATC	$^{I\text{-}Scorpion}$PCR	Schena and Ippolito (2003)
S18R	CAGCCCCTCGAAGTCAGT	$^{I\text{-}Scorpion}$PCR	Schena and Ippolito (2003)
ISSR1	5′HBH$(AG)_7$A	^{P}ISSR-PCR	Armengol *et al*. (2010)
ISSR2	5′DBDA$(CA)_7$	^{P}ISSR-PCR	Armengol *et al*. (2010)
ISSR4	5′YHY$(GT)_7$G	^{P}ISSR-PCR	Armengol *et al*. (2010)
ISSR5	5′BDB$(ACA)_5$	^{P}ISSR-PCR	Armengol *et al*. (2010)
CA8CT	GCGCGCGCGCGCGCGCCT	^{P}ISSR-PCR	Armengol *et al*. (2010)
CA8G	CACACACACACACACAG	^{P}ISSR-PCR	Ikeda *et al*. (2005)
GTG	GTGGTGGTGGTGGTG	^{P}ISSR-PCR	Ikeda *et al*. (2005)
AS4	TGTGGGCGCTCGACAC	^{P}UP-PCR	Ikeda *et al*. (2005)
OPC10^{P}	5′-TGTCTGGGTG-3′	^{P}RAPD	López *et al*. (2008)
OPC13	AAGCCTCGTC	^{P}RAPD	López *et al*. (2008)
OPF3	CCTGATCACC	^{P}RAPD	López *et al*. (2008)
OPF12	ACGGTACCAG	^{P}RAPD	López *et al*. (2008)
BaLccF	GGNCANTTYTGGTAYCAYWSNCA	^{P}RFLP	Kanda *et al*. (2003)
BalccR	TGNCCRTGNARRTGRAANGGRTG	^{P}RFLP	Kanda *et al*. (2003)

I, used for identification; ISSR, inter-simple sequence repeat; P, used to generate polymorphisms; PCR, polymerase chain reaction; RAPD, random amplification of polymorphic DNA; RFLP, restriction fragment length polymorphism; UP, universally primed.

2006). The determination of genetic diversity can be achieved by the analysis of mycelial interactions and/or at the DNA level using inter-simple sequence repeat (ISSR) or random amplified microsatellites (RAMS) techniques.

Mycelial interactions can be determined by the direct assessment of heterokaryon formation, whereby genetically different nuclei coexist in a common cytoplasm, and by the direct assessment of the inability to form a heterokaryon, referred to as 'heterokaryon incompatibility', usually through barrage formation (Anagnostakis, 1977). Studies on mycelial incompatibility in *R. necatrix* have been addressed traditionally through barrage formation in pairings among both multiple and single ascosporic fungal isolates on culture media (Aimi *et al*., 2002; Arakawa *et al*., 2002; Ikeda *et al*., 2005; Inoue *et al*., 2011; Pérez-Jiménez *et al*., 2002) or by using fluorescence microscopy (Aimi *et al*., 2002). Mycelial barrage lines varied between different strains, suggesting that mycelial incompatibility triggers a number of cell reactions that depend on combinations of sibling pairing (Ikeda *et al*., 2011). Hyphal interactions between compatible and incompatible *R. necatrix* pairs have been analysed recently using light and electron microscopy (Inoue *et al*., 2011). It was observed that the hyphae of *R. necatrix* died without anastomosis soon after contact in incompatible combinations, whereas perfect hyphal anastomosis occurred in compatible pairs. These results suggest the presence of at least one extracellular incompatibility factor that is recognized by the cell surface receptors of opposite individuals (Inoue *et al*., 2011).

To detect differences among *R. necatrix* strains at the DNA level, the telomere-associated DNA sequence pTel46, isolated from *Coprinus cinereus*, was used as a marker for restriction fragment length polymorphism (RFLP) analysis. Hybridization of digested *R. necatrix* genomic DNA with the telomeric probe (plasmid pTel46 containing 34 repeats of TTAGGG) identified polymorphisms among *R. necatrix* strains isolated from various fields and single ascospores. RFLP analysis was in agreement with mycelial compatibility group (MCG) studies performed on culture media, as mentioned above (Aimi *et al*., 2002). The phenol oxidase gene, *rpo*1, has also been used as a marker for RFLP analysis to detect genetic diversity among *R. necatrix* single ascospore cultures isolated from the same perithecium. This gene is expected to be present as a single-copy gene in the haploid genome and to be diverse among *R. necatrix* strains. Primers BaLccF and BaLccR amplified an 1180-bp fragment of the *R. necatrix rpo*1 gene (Table 1). The use of this marker by Kanda *et al*. (2003) led to the suggestion of the existence of a heterothallic life cycle in white root rot fungus, recently confirmed by Ikeda *et al*. (2011) through comparisons of DNA polymorphisms between ascospores and their respective parental isolates using primers AS4 and GA8G (see Table 1).

ISSR PCR has been proven to be a powerful tool that can provide valuable information on the genetic diversity of *R. necatrix* (Armengol *et al*., 2010; Ikeda *et al*., 2005, 2011). Four ISSR primers (Table 1) yielded clear, easy-to-score polymorphic bands from 17 *R. necatrix* isolates representative of different hosts in the Valencia province in Spain (Armengol *et al*., 2010). ISSR analysis was in agreement with MCG determinations. Random amplification of polymorphic DNA (RAPD) amplifications have also been performed to identify intraspecific diversity among ascospores of the same perithecium (Kanda *et al*., 2003) and to study diversity among Spanish isolates of *R. necatrix* from different avocado-growing areas. The amplifications used four primers (Table 1) that yielded polymorphic fragments among the *R. necatrix* strains

studied; however, RAPD clustering showed low stability and no correlation with MCG determinations and virulence groups (López *et al*., 2008; Ruano-Rosa *et al*., 2007).

DISEASE CONTROL TOOLS

This fungus is capable of living in the soil in a dormancy stage for many years and produces disease only when environmental conditions are favourable (López-Herrera, 2000). In some cases, the disease arises following the establishment of new crops in soils in which susceptible crops have been cultivated previously and where infected debris has remained in the soil, maintaining the fungus in an inactive dormant stage. This situation has been observed in new plantings of subtropical crops (e.g. avocado, mango and loquat) established in soils containing infected debris from previous susceptible crops (e.g. olive, almond and grapevine) in the Mediterranean area (López-Herrera and Zea-Bonilla, 2007). Thus, any preplanting treatment must have a long-term effect, and postplanting treatments must not adversely affect the crop plant; therefore, control of the fungus must be considered whilst simultaneously using cultural, physical, chemical and biological approaches.

Cultural methods and genetic resistance

Cultural control methods include the removal and burning of diseased plants (Mendoza *et al*., 2003) and periodic testing for the presence of the pathogen in the soil, which is now possible using the PCR-based detection techniques described above (Ruano-Rosa *et al*., 2007; Schena and Ippolito, 2003; Schena *et al*., 2002). In addition, hot water treatment can be used to disinfect plant material used for propagation and to kill the pathogen within the rhizosphere, exploiting the fact that *R. necatrix* is very sensitive to heat. Using this method (53–55 °C hot water for 25–30 min), efficient control of *R. necatrix* on tiger nut (*Cyperus esculentus*) tubers (García *et al*., 2004) and on Japanese pear trees (Eguchi *et al*., 2008) has been achieved. Alternate irrigation protocols are currently ongoing and may greatly help to control *R. necatrix* in avocado trees at the initial stage of infection, when less than 50% of the shallow root system is dead (Farré *et al*., 2005).

In several cases, genetic resistance has been attempted via selection programmes searching for plants tolerant to *R. necatrix* (Lee *et al*., 2000). Mansoori and Dorostkar (2008) selected seedlings of *Vitis vinifera*, which seems to be tolerant to *R. necatrix*, although more studies must be performed to verify this. Commercial *P. cinnamomi*-tolerant avocado rootstocks (e.g. 'Duke7', 'Thomas' and 'Toro Canyon') have been shown to be highly susceptible to *R. necatrix* under artificial inoculation (Pérez-Jiménez, 2006) and, currently, a selection programme of commercial rootstocks tolerant to this pathogen is being carried out in Spain; however, as yet, there are no available selected plants to establish on avocado orchards (Barceló-Muñoz *et al*., 2007).

Physical control

Solarization is a hydrothermal process in which wet soil, located over infected roots, is covered with a plastic transparent sheet to absorb solar radiation. Its main effect is the exposure of the pathogen to high temperatures. Moreover, Freeman *et al*. (1990) have reported that additional biological and chemical control mechanisms can be induced during solarization. Soil solarization has been shown to be effective in the control of white root rot in Spanish avocado orchards. However, the incidence of re-infection and its expense make this approach only useful locally. The cumulative effects of temperature and exposure time appear to play a key role in reducing and eradicating *R. necatrix* inoculum viability in soil (López-Herrera *et al*., 1998, 1999). Moreover, Raj and Sharma (2009) have reported effective control of *R. necatrix* in apple tree saplings previously inoculated with beneficial microorganisms (*Azotobacter chrococcum* and vesicular–arbuscular mycorrhizal fungi) following planting in solarized soil.

Chemical control

Although the pathogen has been identified for many years, chemical control is still experimental because none of the compounds already tested seem to protect plants against the pathogen. Sharma and Agarwala (1967) studied the effects of several antibiotics on the *in vitro* growth of *R. necatrix*. Of the four antibiotics tested, only aureofungin inhibited pathogen growth completely when added at the highest concentration used (40 μg/mL). A few years later, Mantell and Wheeler (1973) described the effects of several fungicides on soils colonized by *R. necatrix* mycelia. Treatments with metham-sodium (Vapam 4S) and formaldehyde were the most effective in controlling fungal growth. Formaldehyde also favoured the development of *Trichoderma*, a genus with significant biocontrol potential against this fungus.

The wine industry has given special focus to the early control of *R. necatrix* with ferrous sulphate, mercury compounds, formaldehyde and carbon disulphide (Ten Hoopen and Krauss, 2006). In Japan, producers of glasshouse grapevines and Japanese pear trees have controlled *R. necatrix* successfully with thiophanate-methyl; however, this compound has largely been substituted with phenylpiridinamine fluazinam (Kanadani *et al*., 1998; Nitta *et al*., 1998), which is more costly but remains stable in the soil for much longer than thiophanate-methyl (Ten Hoopen and Krauss, 2006). This fungicide, together with Benomyl, carbendazim and thiophanate-methyl, has been evaluated *in vitro* against *R. necatrix*. The fungistatic effect of fluazinam compared with the other systemic fungicides tested was demonstrated. This is in agreement with *in vivo* assays on avocado plants, which demonstrated the

high efficacy of fluazinam for the control of white root rot compared with the other fungicides tested (López-Herrera and Zea-Bonilla, 2007).

However, Bonilla *et al*. (1995) and Aranzazu *et al*. (1999) have reported that, in certain cases, disease symptoms were more severe after the application of fungicides, perhaps as a result of the deleterious effects of these products on beneficial microflora or because the fungicide did not properly reach the target zone. It appears that drenching and deep soil injection are the most effective delivery techniques for fungicides (Sugimoto, 2002).

Biological control

Microbial control of soil-borne pathogens has received a great deal of attention recently, and numerous reviews have been published on this subject. Although relatively few studies have focused on the biological control of *Rosellinia*, a slowly growing body of available literature has arisen recently (Ten Hoopen and Krauss, 2006; Table 2).

Table 2 Biocontrol agents used to control *Rosellinia necatrix*.

Control agent	Reference
	Fungi
Beauveria bassiana	Reisenzein and Tiefenbrunner (1997)
Clonostachys spp.	Mendoza *et al*. (2003)
Glomus spp	Bhardwaj *et al*. (2000); Watanabe (1991)
Sordaria spp.	Freeman *et al*. (1986)
Trichoderma spp.	Hermosa *et al*. (2000); Sharma and Sharma (2001); Ruano-Rosa *et al*. (2010)
	Bacteria
Agrobacterium spp.	Yasuda and Katoh (1989)
Bacillus spp.	Cazorla *et al*. (2001, 2007); Sharma and Sharma (2002); González-Sánchez *et al*. (2010)
Pantoea agglomerans	Valdebenito (2001); Carmatti-Sartori *et al*. (2008)
Pseudomonas spp.	Yasuda and Katoh (1987); Valdebenito (2001); Cazorla *et al*. (2001); Cazorla *et al*. (2006); Pliego *et al*. (2007, 2011); González-Sánchez *et al*. (2010)
	Organic materials
Violacein from *Janthinobacterium lividum*	Shirata *et al*. (2000)
Culture filtrate of *Bacillus amyloliquefaciens*	Yoshida *et al*. (2001)
Carboxylic acid from *Enterococcus faecalis*	US Patent and Trademark Office (n° 20030170218, 2003)
	Mycoviruses
MyRV3	Kanematsu *et al*. (2010); Osaki *et al*. (2002); Wei *et al*. (2003, 2004)
Partitivirus RnPV1	Kanematsu *et al*. (2010); Sasaki *et al*. (2006); Sasaki *et al*. (2005)
Megabirnavirus RnMBV1	Chiba *et al*. (2009)
	Insects
Mycophagous collembolan	Shiraishi *et al*. (1993)

Adapted from Ten Hoopen and Krauss (2006).

Virus genome-like pieces of double-stranded RNA (dsRNA) found in certain fungal strains have been shown to reduce the aggressiveness of their host fungus. They have been the subject of research interest because of their potential as biocontrol agents (BCAs) against plant pathogenic fungi, known as virocontrol, and have also been proposed for the control of *Rosellinia* (Matsumoto *et al*., 2002). Approximately 20% of collected *R. necatrix* isolates were found to be dsRNA positive and were presumed to be infected by mycoviruses (Ikeda *et al*., 2004). These dsRNAs belong to the Totiviridae, Partitiviridae, Reoviridae (Osaki *et al*., 2002) and Chrysoviridae families, together with some unassigned viruses (S. Kanematsu and A. Sasaki, unpublished results, NARO, National Institute of Fruit Tree Science, Morioka, Japan).

Among several fungal genera investigated, *Trichoderma* is well known for its mycoparasitic capabilities. It has received the most attention for its biocontrol potential against *R. necatrix* (Harman, 2000; Lorito *et al*., 2004). Several *Trichoderma* isolates were obtained from avocado roots and were selected by their antagonism *in vitro* (Ruano-Rosa *et al*., 2003) and *in vivo* (Ruano-Rosa *et al*., 2003) against *R. necatrix*. Some of these *Trichoderma* isolates have been characterized, and were identified as *T. atroviride*, *T. virens*, *T. harzianum* and *T. cerinum* species (Ruano-Rosa, 2006; Ruano-Rosa and López-Herrera, 2009; Ruano-Rosa *et al*., 2010). Biological control tests have been performed on avocado plants in individual, binary, tertiary and quaternary applications; better protection from avocado white root rot disease was more frequently conferred in combined experiments (Ruano-Rosa and López-Herrera, 2009; Ruano-Rosa *et al*., 2010).

Several studies have focused on bacterial species with the capacity to control *R. necatrix*. Large numbers of bacteria have been isolated from the soil and roots of avocado, peach and apple trees that exhibit antagonistic activity *in vitro* against *R. necatrix*, particularly species of the genera *Pseudomonas* and *Agrobacterium*. Promising results were obtained *in vitro* and under glasshouse conditions after dipping apple tree roots in bacterial suspensions of *Pantoea agglomerans* (Carmatti-Sartori *et al*., 2008; Valdebenito, 2001). In another approach, different bacterial strains were isolated from the rhizosphere of healthy avocado trees and were selected by different strategies (Pliego *et al*., 2011). *Pseudomonas fluorescens* PCL1606 was one of the most interesting strains isolated: it produces the antifungal compound 2-hexyl-5-propyl resorcinol (HPR), which has been shown to be directly involved in the biocontrol capacity of this bacterium (Cazorla *et al*., 2001, 2006). Other antagonistic strains of *Bacillus subtilis* have also been selected for their antagonistic activity *in vitro* against *R. necatrix*. Some *B. subtilis* strains displayed successful biocontrol activity, which has been correlated with the production of antibiotics, such as iturins, fengycin and surfactins (Cazorla *et al*., 2007). Another strain, *Pseudomonas pseudoalcaligenes* AVO110, was selected by efficient competitive root colonization (Kamilova *et al*., 2005; Pliego *et al*., 2007). This strategy

yielded a low frequency of antibiotic-producing strains and contributed to the isolation of rhizobacterial strains with a wider range of potential biocontrol traits and capabilities for slowing down the infection process under glasshouse conditions (Pliego *et al*., 2007). Recent studies using a screening methodology, based on the isolation of bacterial strains directly through plant protection tests, in the avocado–*R. necatrix* system have suggested that antagonism is a prevalent trait in biocontrol bacteria selected through this strategy (González-Sánchez *et al*., 2010; Pliego *et al*., 2011). Moreover, a comparative analysis of the different methods used for the screening of biocontrol agents against *R. necatrix* has confirmed that screening for antagonism, one of the more straightforward methods used for the selection of bacterial biocontrol agents, is a valid strategy for this experimental system (Pliego *et al*., 2011).

CONCLUSIONS AND FUTURE PROSPECTS

Rosellinia necatrix is widely distributed throughout temperate and tropical climates, and is becoming an important threat to a wide range of different host plants. Disease control is difficult because of the features exhibited by this pathogen, such as resistance to drought, survival capacity in acidic soils, colonization potential for numerous hosts, deep penetration into the soil and its resistance to various common fungicides. All of these circumstances make it necessary to use molecular methods for the early detection of the pathogen and its level of inocula in the soil, in conjunction with traditional techniques for the isolation from plant roots and growth in adequate media.

Chemical control methods are still experimental; meanwhile, the utilization of biological control is becoming a realistic alternative to chemicals. A virocontrol mechanism developed by Dr Naoyuki Matsumoto's group (Matsumoto, 1998) seems to be a promising strategy for reducing the performance cost required for the application of biocontrol agents in orchards (Ghabrial and Suzuki, 2009). However, the high heterogenic incompatibility system found among different orchards prevents mycoviruses from spreading among different fungal strains, because viruses are apparently transmitted through mycelial fusion between the same MCGs.

However, success in the application of biocontrol agents is variable, presumably as a result of the strongly varying conditions in the field; namely, the expression of many biocontrol traits is strongly influenced by biotic (Lee and Cooksey, 2000) and abiotic (Duffy and Défago, 1999; Thomashow and Weller, 1996) conditions. Indeed, it is generally agreed that the application of biocontrol products is more successful under better controlled glasshouse conditions than in the open field (Paulitz and Bélanger, 2001). In this regard, the development of successful and reproducible biocontrol agents against *R. necatrix* must consider the interactions established on at least three different trophic levels (i.e. the pathogen, the biocontrol agent and the root) in the specific application environment. Currently, researchers can perform the aforementioned studies on *R. necatrix* by using epifluorescence microscopy techniques in combination with the *gfp* reporter gene.

Because control strategies are expensive and, as yet, do not always resolve the infection, currently, the control of white root rot depends largely on attempts to exclude the pathogen through the use of *R. necatrix*-free propagating materials and planting in non-infested soils. Thus, all methods available for the detection of the pathogen in infected soils and roots have been described. In addition, we encourage a continuation of the search for tolerant rootstocks for this pathogen.

Although the production of viable sexual progeny for the genetic analysis of *R. necatrix* remains far from routine, the tools developed for the genetic transformation of *R. necatrix* (Aimi *et al*., 2005; Kanematsu *et al*., 2004; Kano *et al*., 2011; Pliego *et al*., 2009) will be very useful in future research. In particular, improvements in these transformation protocols will facilitate the analysis of the physiological and molecular processes involved in *R. necatrix* pathogenicity and biology, and will contribute to the understanding of the molecular dialogue between host and pathogen.

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