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Pathogen profile

Tobacco leaf spot and root rot caused by *Rhizoctonia solani* Kühn

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SUMMARY

Rhizoctonia solani Kühn is a soil-borne fungal pathogen that causes disease in a wide range of plants worldwide. Strains of the fungus are traditionally grouped into genetically isolated anastomosis groups (AGs) based on hyphal anastomosis reactions. This article summarizes aspects related to the infection process, colonization of the host and molecular mechanisms employed by tobacco plants in resistance against *R. solani* diseases.

Taxonomy: Teleomorph: *Thanatephorus cucumeris* (Frank) Donk; anamorph: *Rhizoctonia solani* Kühn; Kingdom Fungi; Phylum Basidiomycota; Class Agaricomycetes; Order Cantharellales; Family Ceratobasidiaceae; genus *Thanatephorus*.

Identification: Somatic hyphae in culture and hyphae colonizing a substrate or host are first hyaline, then buff to dark brown in colour when aging. Hyphae tend to form at right angles at branching points that are usually constricted. Cells lack clamp connections, but possess a complex dolipore septum with continuous parenthesomes and are multinucleate. Hyphae are variable in size, ranging from 3 to 17 μm in diameter. Although the fungus does not produce any conidial structure, ellipsoid to globose, barrel-shaped cells, named monilioid cells, 10–20 μm wide, can be produced in chains and can give rise to sclerotia. Sclerotia are irregularly shaped, up to 8–10 mm in diameter and light to dark brown in colour.

Disease symptoms: Symptoms in tobacco depend on AG as well as on the tissue being colonized. *Rhizoctonia solani* AG-2-2 and AG-3 infect tobacco seedlings and cause damping off and stem rot. *Rhizoctonia solani* AG-3 causes 'sore shin' and 'target spot' in mature tobacco plants. In general, water-soaked lesions start on leaves and extend up the stem. Stem lesions vary in colour from brown to black. During late stages, diseased leaves are easily separated from the plant because of severe wilting. In seed beds, disease areas are typically in the form of circular to irregular patches of poorly growing, yellowish and/or stunted seedlings.

Resistance: Knowledge is scarce regarding the mechanisms associated with resistance to *R. solani* in tobacco. However, recent evidence suggests a complex response that involves several constitutive factors, as well as induced barriers controlled by multiple defence pathways.

Management: This fungus can survive for many years in soil as mycelium, and also by producing sclerotia, which makes the management of the disease using conventional means very difficult. Integrated pest management has been most successful; it includes timely fungicide applications, crop rotation and attention to soil moisture levels. Recent developments in biocontrol may provide other tools to control *R. solani* in tobacco.

INTRODUCTION

The soil-borne basidiomycete fungus *Rhizoctonia solani* Kühn (teleomorph: *Thanatephorus cucumeris*) causes disease on many economically important crop plants worldwide. Isolates of this species-complex are traditionally classified into genetically isolated anastomosis groups (AGs) based primarily on hyphal anastomosis reactions (Sneh *et al*., 1991). Over the last 30 years, a comprehensive literature has been produced dealing with the taxonomy, molecular systematics, genetics, pathology and ecology of *Thanatephorus cucumeris* that reflects its broad host range and prevalence in different agroecosystems. The establishment of *R. solani* hyphal AGs has greatly assisted in

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identification and epidemiological studies (Carling, 1996; Ogoshi, 1987). Further support for these genetic groupings has come from more recent internal transcribed spacer (ITS) rRNA gene sequence polymorphism analyses (Carling *et al*., 2002; González *et al*., 2001; Kuninaga *et al*., 2000; Salazar *et al*., 2000). *Rhizoctonia solani* AG-2-2 and AG-3 are the main causal agents of leaf spot and root rot in tobacco (*Nicotiana tabacum* L.), where they cause damping off and stem rot in young transplants, sore shin in older field plants and a foliar disease named 'target spot' (Lucas, 1975; Sneh *et al*., 1996).

The leaf spot and root rot in tobacco were described for the first time in US tobacco crops in 1904 (Lucas, 1975). Although this disease is not considered to be critically important for tobacco cultivation, it nevertheless occurs every year in many fields of this crop (Lucas, 1975). As a result of the broad host range reported for both *R. solani* AG-2-2 and AG-3, it is likely that this fungus may be dispersed in cultivated soils worldwide. However, little is known about the molecular components responsible for the susceptibility or resistance of *N. tabacum* to *R. solani*. This knowledge would be very desirable in order to obtain resistant genotypes that could be included in different integrated management strategies. This article summarizes aspects related to the infection process, colonization of the host and molecular mechanisms employed by tobacco plants in resistance against *Rhizoctonia* diseases.

TAXONOMIC HISTORY

In 1858, Julius Kühn described a fungus on diseased potatoes and placed it in the genus *Rhizoctonia*, which had been described earlier by the Swiss mycologist A. P. De Candolle (1815), naming it *Rhizoctonia solani*. As Kühn's original description of *R. solani* was brief and purportedly contained descriptions of a secondary organism (Parmerter and Whitney, 1970), and as the mycelia of some ascomycetes may closely resemble those of *R. solani* (Moreau and Moreau, 1956; Whitney and Parmeter, 1964), the description of *R. solani* was later revised (Duggar, 1915; Parmerter *et al*., 1967) to help minimize misidentification. The current species concept for this taxon suggests that the diagnostic features for the species are as follows: (i) hyphal pigmentation of different brownish tones; (ii) branching near the distal septum of cells in young vegetative hyphae; (iii) constriction of hyphae and the formation of septa near the point of origin of hyphal branches; (iv) dolipore septa; (v) the number of nuclei close to the tips of young vegetative hyphae is greater than two (Parmerter and Whitney, 1970; Sneh *et al*., 1996; Fig. 1C,D). Most, but not all, isolates have characteristics such as monilioid cells, sclerotia, rapid growth rate and pathogenicity (Sneh *et al*., 1996). In addition to the above morphological characteristics, the following features are never present and may be helpful to rule out other species with similar morphology: clamp connections, conidia, sclerotia differentiated into rind and medulla, and rhizomorphs (Parmerter and Whitney, 1970; Parmerter *et al*., 1967). Concerning its systematic relationships, *Thanatephorus* is actually included (together with the genera *Ceratobasidium* and *Waitea*) in the family Ceratobasidiaceae, considered by some authors (Roberts, 1999; Rogers, 1935; Weiss and Oberwinkler, 2001) as one of the most primitive group of Holobasidiomycetes, because of their characteristic basidial morphology, with large and sometimes septate sterigmata, close to some phragmobasidiomycetous groups. The evolutionary relationship between the genera *Thanatephorus* and *Ceratobasidium* remains controversial. Employing classical taxonomic approaches, some authors (Roberts, 1999; Stalpers and Andersen, 1996) have considered both genera as part of a generic complex, where delimitation between the two genera presents some difficulties, and differences in morphometric features and ecological behaviour are gradual along the several taxa within the two genera.

Isolates of *R. solani* can vary greatly in phenotypic and genotypic characteristics, but have been traditionally arranged in genetically related groups based on hyphal anastomosis criteria. To date, 14 different AGs have been recognized (González-García *et al*., 2006) for *R. solani*, although some authors (Roberts, 1999) have suggested the convenience of splitting the complex into several biological species. Thus, they have proposed four taxonomic epithets to cover all the 'classical' *R. solani* AGs. In this sense, *T. microsclerotium* (G.F. Weber) Boidin, Mugnier & Canales is proposed for AG-1B, *T. sasakii* (Shirai) C.C. Tu & Kimbr. is preferred to name AG-1A isolates, *T. praticola* (Kotila) Flentje for AG-4, and *T. cucumeris* is preferred to name most of the rest of the described AGs.

In general terms, teleomorphic stages are usually difficult to obtain *in vitro* for this group of fungi (Adams and Butler, 1983), and sexual fruitbodies are also scarce in natural substrates. Thus, the identification of *R. solani* isolates is typically based on the comparison of the anamorphic features mentioned previously. However, the species *T. cucumeris* has been designated as the teleomorphic counterpart for *R. solani* (Donk, 1956). Sexual fruitbodies of this taxon are typically characterized by the presence of a hypochnoid, thin basidiomata possessing a hymenium made up of successive layers of basidia rising from vertically branching, cymose hyphae just above the basal hyphae (subiculum), and typically with four sterigmata, sometimes septate, and about the same length as the metabasidia or shorter (González-García *et al*., 2006; Roberts, 1999; Sneh *et al*., 1996). Hymenia produce basidia bearing four ellipsoid to oblong, hyaline basidiospores [4–5.5(6.5) μm × 7–10 μm] (Roberts, 1999). Moore (1987) proposed the grouping of anamorphs with perfect stages into the genera *Thanatephorus* and *Waitea* in the genus *Moliniopsis* Ruhland, a higher priority generic name erected to accommodate *M. aderholdii*, an ancient synonym of *R. solani*. Subsequently,

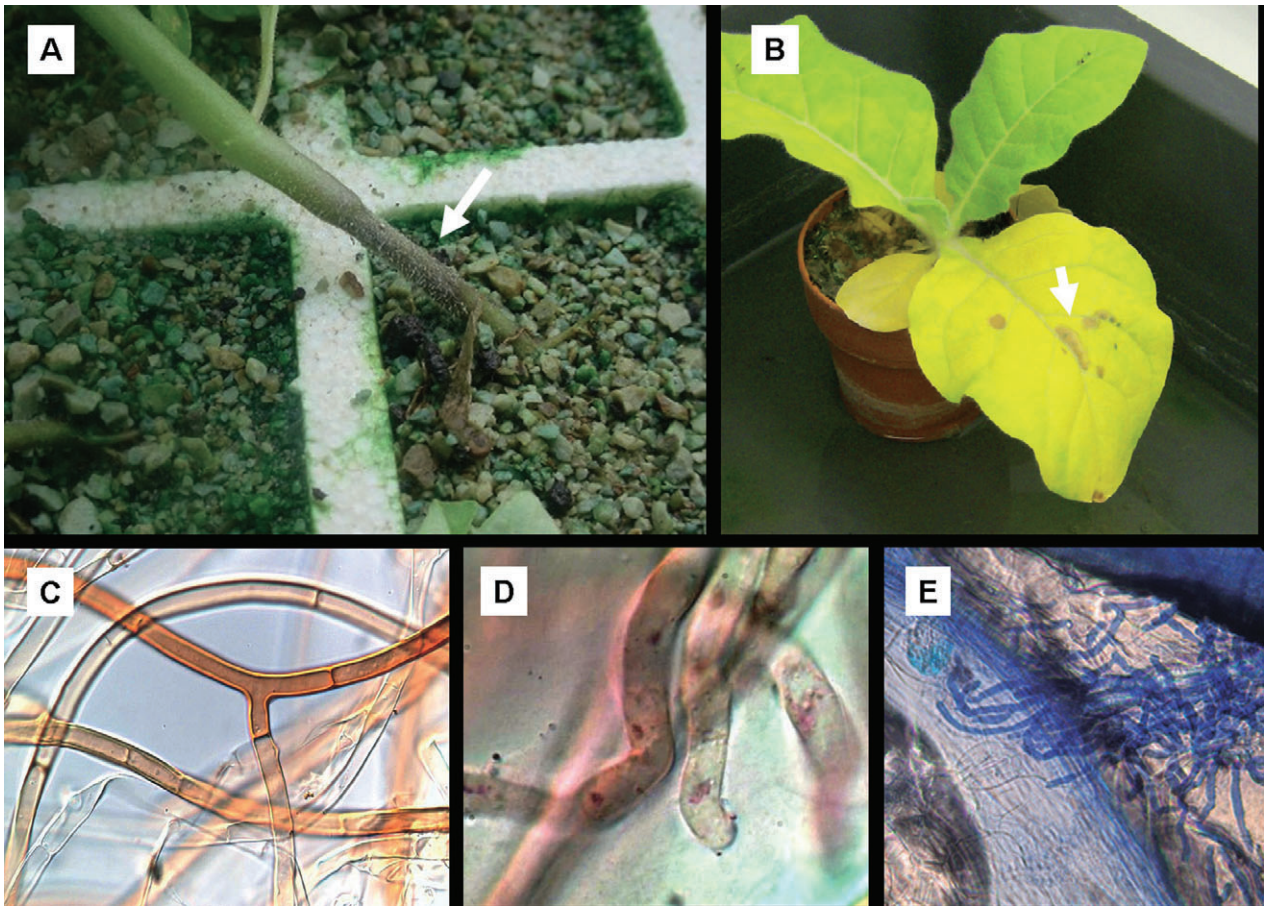

Fig. 1 Symptoms and morphological features of plant pathogenic *Rhizoctonia solani* (*Thanatephorus cucumeris*). (A) Damping off of a tobacco seedling in a floating tray. A characteristic necrosis symptom is visible on the infected stem (arrow). (B) Target spot in tobacco leaf (arrow). (C) Typical morphology of *R. solani* hyphae. (D) Hyphae of *R. solani* showing the number of nuclei stained with Giemsa. (E) Hyphal branching of *R. solani* colonizing a leaf of *Nicotiana tabacum*. The plant and fungal tissues were stained with trypan blue.

Moore (1996) restricted *Moliniopsis* to species with *Thanatephorus* teleomorphs. However, the name *Rhizoctonia* has been proposed to be conserved against *Moliniopsis* (Stalpers *et al*., 1998), making *Rhizoctonia solani* the current name for the most well-known and studied species in the complex.

SYMPTOMS IN TOBACCO

Rhizoctonia solani can survive for many years in soils by way of sclerotia or as a saprophyte, colonizing soil organic matter. Sclerotia and/or mycelium present in soil and/or plant tissue can eventually activate to produce vegetative hyphae that can attack a wide range of crops (Keijer, 1996). In certain situations, *R. solani* can produce basidiospores that will cause disease and also serve as a source for rapid and long-distance dispersal of the fungus. The basidiospores germinate to produce hyphae that infect leaves during periods of high relative humidity (Fig. 2). Although most *Rhizoctonia* diseases are initiated by mycelium and/or sclerotia, several important diseases of tobacco and other crops, such as beans and sugar beet, are a result of basidiospore infection (González-García *et al*., 2006; Harveson *et al*., 2009).

Rhizoctonia solani causes damping off and stem rot in young transplants and a disease of the lower stem and root, called 'sore shin', in older field plants (Shew, 1991; Sneh *et al*., 1996). Damping off (Fig. 1A) is the most widespread symptom caused by *R. solani* observed in tobacco. In general, seedlings are susceptible to parasitization during the first few weeks of their development, and become progressively less susceptible as they mature through the development of biochemical and physical defence mechanisms and/or barriers. In damping-off events, severely infected seeds usually do not germinate, and infected seedlings can be killed either before or after emergence. Seedlings killed after emergence often appear to have fallen over the soil surface as a result of excessive rainfall (Sneh *et al*., 1991). The transplantation of infected seedlings is a significant factor in

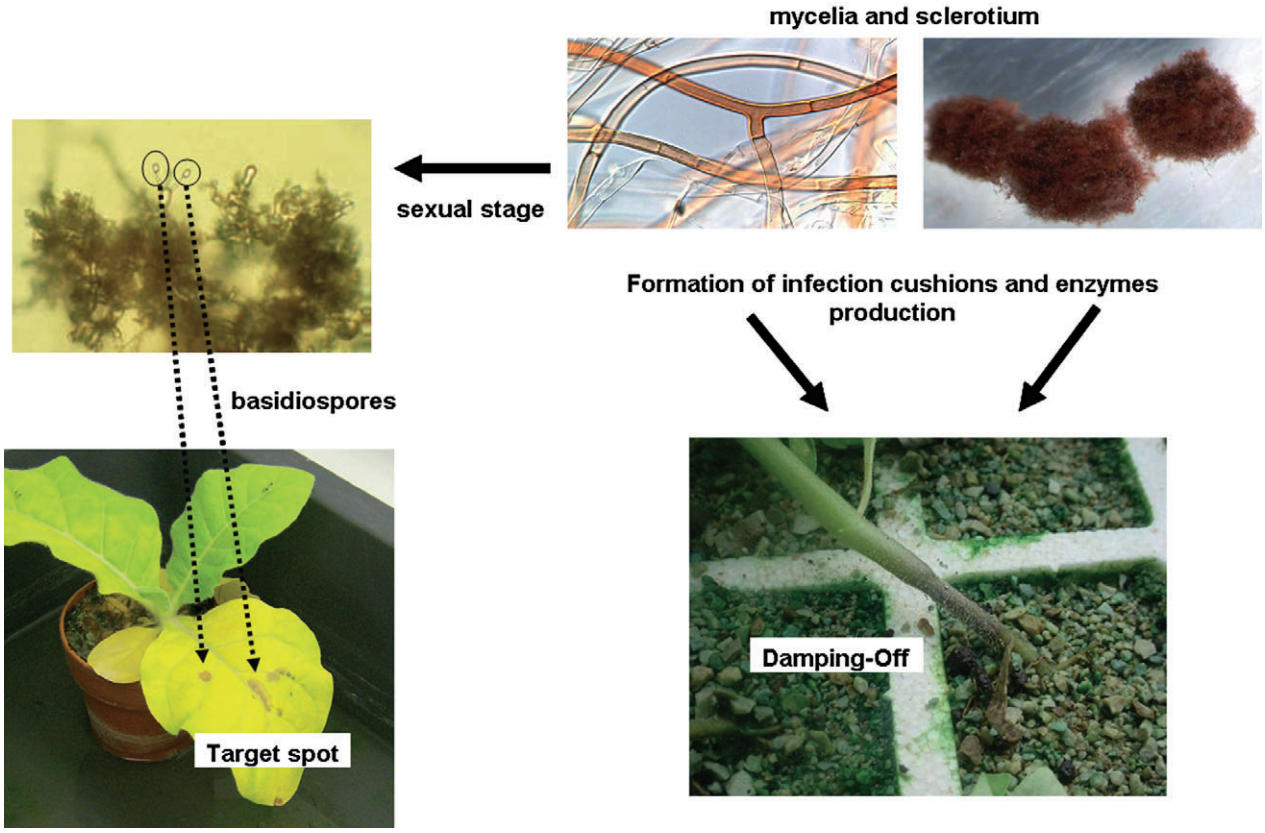

Fig. 2 Disease cycle of *Rhizoctonia solani* and (*Thanatephorus cucumeris*) on tobacco.

the spread of sore shin in field plants; however, the latter infections can also be caused by *R. solani* that is already present in the field (Elliott *et al*., 2008). Isolates of *R. solani* causing stem and root rot symptoms have typically been associated with AG-1, AG-2-2 and AG-4 (Stevens *et al*., 1993).

Target spot is a foliar disease that first appeared in the USA in the 1980s and causes economically important losses in tobacco production (Elliott *et al*., 2008; Shew, 1991). This disease is caused by infection with basidiospores of *T. cucumeris* that are produced from hymenia on the soil surface or infected plant tissue (Elliott *et al*., 2008; Shew and Main, 1990). Symptoms begin as small water-soaked lesions on leaves which can expand to large circular spots with concentric rings (Fig. 1B). Target spot can occur on tobacco seedlings in high-humidity environments, particularly when leaves have grown close together to form a canopy in glasshouse environments. In some severe cases, the pathogen may grow from leaf tissue into the stem, resulting in plant death. Isolates of *R. solani* causing stem and root rot symptoms have been characterized by anastomosis as groups AG-1, AG-2-2 and AG-4, whereas target spot is typically associated in the glasshouse and the field as group AG-3 (Stevens *et al*., 1993).

INFECTION PROCESS

Regardless of the type of host plant, in most of the disease events caused by *R. solani* isolates, the generalized infection process includes some consecutive steps identified as adhesion, penetration and colonization (Keijer, 1996). However, experimental data available on infection processes have shown that there are few differences among AGs with regard to the infection process in *R. solani* (González-García *et al*., 2006). The initial step in the infection process is characterized by hyphal growth over the surface of the host plant. Such growth is easily washed off the host. In contrast, the attachment of hyphae in subsequent infections steps is characterized by flattened hyphae that are closely associated with the host. After attachment, hyphae grow following contiguous epidermal cells (Armentrout and Downer, 1987; Marshall and Rush, 1980), a probable response of *R. solani* to the topography of the host surface, leakage of host nutrients or other stimulatory components, or a combination of the above (Armentrout *et al*., 1987). T-shaped hyphal branching is typically one of the morphological precursors prior to the formation of infection structures, such as short swollen hyphae, appressoria, infection cushions or repetitive T-shaped branches, in part as a

specific response to the host (Fig. 1E). Finally, infection pegs are produced that allow the fungus to penetrate and enter intact plant tissue through the cuticle and epidermal cell walls or, more rarely, through the stomata or wounds (Matsuura, 1986; Weinhold and Sinclair, 1996).

During the course of infection, the fungus produces extracellular hydrolytic enzymes capable of degrading the cell wall in advance of the invading hyphae. It has been shown that, during the earlier stages of the infection process, *R. solani* AG-4 secretes pectinolytic and cellulolytic enzymes, such as endopectin lyase, which have been reported to be associated with tissue degradation in later stages of infection (Marcus *et al*., 1986). Altogether, at least 10 different extracellular enzymes have been identified to be produced by *R. solani* (Bertagnolli *et al*., 1996; Lister *et al*., 1975). Together with cell wall damage, changes in the cytoplasm of cortical cells can be detected before colonization events occur. For example, cytological changes in infected plant cells include the formation of reaction zones, plasmolysis and the collapse of the cytoplasm (González-García *et al*., 2006). As the pathogenic process of *R. solani* is characterized by the death of plant cells, both before and after penetration and colonization events, it is interesting to speculate that the fungus relies on both a necrotrophic and hemibiotrophic lifestyle for pathogenicity, as has been shown previously with other fungal pathogen models (Bolton *et al*., 2006).

Infection by basidiospores occurs in target spot of tobacco. Although the infection process has not been studied in detail in tobacco, Naito and Sugimoto (1978) followed the course of basidiospore infection of *R. solani* AG-2-2 IV in sugar beet, which may be similar to infection in tobacco. In sugar beet, basidiospores originate from basidia on the soil surface or on host plants, and land on the surface of the host plant. After germination, penetration occurs via appressoria. From such primary infection sites, invading hyphae grow on the leaf surface and enter stomata to create secondary lesion sites. Spores are formed, thereby completing the life cycle and generating inoculum to infect other susceptible hosts (Naito and Sugimoto, 1978).

NICOTIANA SPP. DEFENCE AGAINST *R. SOLANI* INFECTION

Several constitutive factors (formed prior to infection), including cuticle and epicuticular wax thickness (Reddy, 1980; Yang *et al*., 1992), cell wall calcium content (Bateman and Lumsden, 1965) and tolerance of cuticle and epicuticular wax to pathogen enzymes and toxins, have been reported to function in resistance against *R. solani* (Kenning and Hanchey, 1980). In addition, induced mechanisms of resistance (formed after infection), such as hypersensitive responses (HRs) (Marshall and Rush, 1980) and an increased production of pathogenesis-related proteins (PRPs), may also be involved (Anuratha *et al*., 1996).

So far, the interaction of *R. solani* with rice is the best studied pathosystem (Chang-Jiang *et al*., 2008; Lee *et al*., 2006). Unfortunately, there is little information regarding the mechanisms associated with resistance to *R. solani* in plants of the Solanaceae family to which *Nicotiana* spp. belong. Genetic resistance to *R. solani* in tobacco is scarce. Recently, 97 genotypes of tobacco and related *Nicotiana* spp. were evaluated for seedling resistance to stem rot and target spot caused by *R. solani* (Elliott *et al*., 2008). Significant differences in disease incidence were initially found among the genotypes for both stem rot and target spot. However, resistance to target spot was not observed when disease pressure was high. Partial resistance to stem rot was observed in several genotypes in repeated tests (Elliott *et al*., 2008). A cDNA library, using suppression subtractive hybridization, was generated from transcripts that were differentially expressed during a compatible and incompatible interaction between *N. tabacum* cv. 'Sumatra' and *R. solani* (Chacón *et al*., 2010). This allowed the isolation of a protein kinase cDNA that was downregulated during a compatible and upregulated during an incompatible interaction. A functional study showed that this cDNA was directly related to resistance to *R. solani* (Chacón *et al*., 2010).

Nicotiana plumbaginifolia Viv. plants silenced for the ATP-binding cassette transporter gene *NpPDR1* showed an increased sensitivity to *R. solani* infection compared with wild-type plants. The infiltration of pathogen suspension through the leaf stomata of mature wild-type plants of *N. plumbaginifolia* induced *NpPDR1* expression after 4 days, and no symptoms were observed in infiltrated zones. Similarly, the expression of an *NpPDR1* orthologue in leaves of mature wild-type plants of *N. tabacum* was activated by *R. solani* (Bultreys *et al*., 2009).

Resistance to *R. solani* in genetically modified plants under glasshouse conditions has also been reported. The first success in the production of a *Rhizoctonia*-resistant plant was obtained with tobacco expressing an endochitinase from bean (Broglie *et al*., 1991). This was soon followed by a study in which a cauliflower mosaic virus (CaMV) 35S-driven tobacco chitinase A was overexpressed in *N. sylvestris* Speg. & Comes. Transgenic plants showed less colonization and a smaller loss of fresh weight than controls (Vierheilig *et al*., 1993). Recently, a chitinase 1 gene from the entomopathogenic fungus *Metarhizium anisopliae* (Metschn.) Sorokïn was expressed in tobacco under the control of the CaMV 35S promoter. Transgenic plants showed enhanced resistance to *R. solani*, providing the first example of transgenic plants inducing resistance through the expression of a chitinase from *Metarhizium anisopliae*, an entomopathogenic and acaricide fungus (Kern *et al*., 2010). Overexpression of individual or combined cDNAs encoding the barley (*Hordeum vulgare* L.) class II chitinase, class II β-1,3-glucanse and type I ribosome-inactivating protein,

Table 1 Genes from *Nicotiana* spp. involved in *Rhizoctonia solani* defence.

Gene	Effects on disease development	Reference
Class II chitinase, class II β-1,3-glucanase and type I ribosome-inactivating protein	Overexpression in transgenic plants increases protection against diseases	Jach *et al*. (1995)
Type III calmodulin	Knockdown of this gene compromises disease resistance	Takabatake *et al*. (2007)
NpPDR1	Silencing of this gene increases sensitivity to the fungus in *Nicotiana plumbaginifolia* and *N. tabacum*	Bultreys *et al*. (2009)
GhZFP1 (CCCH-type zinc finger protein)	Overexpression in transgenic plants enhances resistance	Guo *et al*. (2009)
NtPK protein kinase	Overexpression enhances resistance to damping off produced by an aggressive *R. solani* strain, and silencing compromises the resistance to a nonaggressive *Rhizoctonia solani* strain	Chacón *et al*. (2010)

driven by the CaMV 35S promoter, in tobacco resulted in enhanced protection against *R. solani* (Jach *et al*., 1995; O'Brien *et al*., 2001). Overexpression of sarcotoxin IA, a bactericidal peptide from *Sarcophaga peregrina*, enhanced the resistance of transgenic tobacco plants to bacterial (*Erwinia carotovora* ssp. *carotovora* and *Pseudomonas syringae* pv. *tabaci*) and fungal pathogens (Mitsuhara *et al*., 2000). Interestingly, the heterologous overexpression of the *Gastrodia* antifungal protein (GAFP; gastrodianin) provides a broad-spectrum resistance to various pathogens, such as *R. solani*, *Phytophthora nicotianae* Breda de Haan and *Meloidogyne incognita* Kofoid & White, but no resistance was observed against *Ralstonia solanacearum* (Cox *et al*., 2006). A cDNA clone from a novel CCCH-type zinc finger protein (GhZFP1) of *Gossypium hirsutum* L. was isolated from salt-induced cotton using differential hybridization screening. Overexpression of *GhZFP1* in transgenic *N. tabacum* cv. NC89 enhanced tolerance to salt stress and resistance to *R. solani* (Guo *et al*., 2009). Transgenic plants expressing the thaumatin gene from *Thaumatoccus daniellii* (Benn.) Benth, under the control of the CaMV 35S promoter, displayed enhanced resistance and delayed disease symptoms against fungal diseases caused by *Pythium aphanidermatum* (Edson) Fitzp. and *R. solani* (Rajam *et al*., 2007). Finally, a decrease in resistance to a compatible strain of *R. solani* was observed in type III knockdown tobacco lines targeting the calmodulin (CaM) gene *NtCaM13*. The expression of jasmonic acid (JA)- and/or ethylene (ET)-inducible basic PR genes was not affected in this line, suggesting that type III CaM isoforms are probably involved in basal defence against necrotrophic pathogens, independent of JA and ET signalling (Takabatake *et al*., 2007). These results indicate a complex response to challenge by *R. solani* that involves the simultaneous induction of proteins from multiple defence pathways (Table 1).

MANAGEMENT MEASURES

The control of this soil-borne fungus has been difficult to achieve using traditional means, such as breeding plants for resistance, crop rotation and fungicides. Generally, the most successful practices include integrated pest management and, in this case, it is very important to know the main sources of inoculum in order to control them. The growth of healthy transplants is an essential step in tobacco production. The main system for the production of tobacco seedlings worldwide is through the use of floating trays. Such Styrofoam trays are filled with a soil-less medium, seeded with pelletized seed, and floated on a shallow water reservoir. These trays are perforated on the bottom to allow for water and nutrient uptake. This soil-less medium procedure has been identified as the principal source of inoculum because it can harbour *R. solani* sclerotia and hyphae (Gutierrez *et al*., 1997). This makes it important to quantify the pathogen potentially present in soil-less medium before its use and to implement proper disinfection before re-use (Gutierrez *et al*., 1997).

Integrated pest management also relies on timely chemical application. For example, fungicides must be applied prior to infection to control diseases caused by *R. solani* AG-2-2 IIIB and IV in sugar beet. Recently, Bolton *et al*. (2010) identified soil temperature and moisture thresholds as necessary for infection. Because *R. solani* is affected by these environmental parameters, growers may be able to evaluate soil conditions for the optimal application of fungicide. The fungicide mancozeb was used to control damping off and target spot in glasshouse production. However, the fungicide iprodione demonstrated, in a comparison of the activity of several fungicides against *R. solani*, excellent control of these diseases (Csinos and Stephenson, 1999).

Finally, biological control is another important aspect of *R. solani* management in tobacco. The efficacy of this method has been demonstrated in *R. solani* using *Trichoderma* sp. (Cole and Zvenyika, 1988; Elad *et al*., 1980; Hadar *et al*., 1979). Isolates from *T. harzianum* Rifai reduced the growth of *R. solani* and enhanced the disease control in tobacco plants (Cole and Zvenyika, 1988). Seed quality and levels of cultivar resistance are other aspects to be taken into account. All of the above management measures will not totally eliminate the pathogen, but will cause a reduction in inoculum levels. However, once the plant is infected during a cropping season, management options become limited.

CONCLUDING REMARKS

A deeper understanding of the molecular interaction between *Nicotiana* spp. and *R. solani* will further our knowledge of the defence mechanisms that occur during fungal infection. The genome sequence should open up new possibilities, and important improvement for the future would be to determine the main biochemical and molecular mechanisms involved in the pathogenicity and virulence of *R. solani*. A knowledge of the possible mechanisms and genes suppressed in the host by the virulent pathogen may further our understanding of potential defences of the host. The identification of crucial genes may lead to useful tools for molecular breeding or for the development of transgenic varieties. It may also aid in the elimination of susceptible individuals during initial stages in the management of diseases.

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