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REVIEW

Use of *Coniothyrium minitans* as a biocontrol agent and some molecular aspects of sclerotial mycoparasitism

J. M. Whipps · S. Sreenivasaprasad · S. Muthumeenakshi · C. W. Rogers · M. P. Challen

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Abstract The use of the sclerotial mycoparasite *Coniothyrium minitans* as a biological control agent of diseases caused by sclerotium-forming pathogens especially *Sclerotinia sclerotiorum* is briefly reviewed. A number of studies have examined production and application methods, integrated control, ecology, and modes of action in order to understand the biology of the mycoparasite and enhance activity and reproducibility of use. Recently, development of a number of molecular-based techniques has begun to allow the examination of genes involved in mycoparasitism. Some of these procedures have been applied to identify pathogenicity genes involved in the infection of sclerotia of *S. sclerotiorum* by *C. minitans* and this work is discussed.

Keywords Biological control · *Coniothyrium minitans* · Mycoparasitism · Pathogenicity genes · Sclerotia · *Sclerotinia*

Introduction

Campbell (1947) first isolated the fungus *Coniothyrium minitans* from a sclerotium of *Sclerotinia sclerotium* in the USA and immediately recognised its potential as a biological control agent. It has subsequently been isolated from all continents except Antarctica, largely from sclerotia in soil (Sandys-Winsch et al. 1993; Monaco 1989). There are numerous phenotypes differing in colony morphology and a number of other biological characteristics (Sandys-Winsch et al. 1993; Jones and Stewart 2000; Grendene et al. 2002) but ability to parasitize sclerotia of *S. sclerotiorum* remains a key feature. Some isolates have begun to be characterised using molecular procedures such as random amplification of polymorphic DNA (RAPD) and simple sequence repeat (SSR) – PCR profiling, and rRNA gene sequencing (Goldstein et al. 2000; Ridgway and Stewart 2000; Muthumeenakshi et al. 2001; Grendene et al. 2002). Recently, based on anamorphic characteristics seen in vitro, and maximum parsimony analysis of ITS and SSU nrDNA sequences, *C. minitans* was reclassified as *Paraconiothyrium minitans* (Verkley et al. 2004). However, because of common usage, *Coniothyrium minitans* will continue to be applied throughout this paper.

J. M. Whipps (✉) · S. Sreenivasaprasad · S. Muthumeenakshi · C. W. Rogers · M. P. Challen
Warwick HRI, University of Warwick,
Wellesbourne,
Warwick CV35 9EF, UK
e-mail: John.Whipps@warwick.ac.uk

Coniothyrium minitans as a biological control agent

Coniothyrium minitans has the ability to infect sclerotia of many ascomycetous fungi including *Sclerotinia minor*, *Sclerotinia sclerotiorum*, *Sclerotinia trifoliorum*, *Sclerotium cepivorum* but not apparently, those of

Ciborinia camellia (Van Toor et al. 2005), nor of any basidiomycetous sclerotia (Whipps and Gerlagh 1992). It has been used successfully when applied to soil in the glasshouse and field to control *S. sclerotiorum* in numerous crops including lettuce, celery, sunflower, bean and oilseed rape (Budge and Whipps 1991; Huang and Hoes 1980; Gerlagh et al. 1999; Luth 2001), *S. minor* on lettuce and peanut (Partridge et al. 2006b; Rabeendran et al. 2006) and *S. cepivorum* on onion (Ahmed and Tribe 1977; McLean and Stewart 2000). Conidia have also been sprayed onto foliage to prevent ascospore infection and disease development in alfalfa, beans and oilseed rape (Huang et al. 2000; Gerlagh et al. 2004; Li et al. 2003a, 2005a, 2006), to foliage to diminish sclerotial production and survival in rotations of several crops (Gerlagh et al. 1999) as well as to crop debris to decrease sclerotial carryover (Budge et al. 1995). It also survives well in soil for several years and can be responsible for suppressiveness to Sclerotinia disease in some areas (Huang and Kozub 1991; Huang and Erickson 2002; Jones and Whipps 2002). However, as with many biological control agents, there is evidence that reproducibility of control is sometimes variable depending on environmental and biological factors, especially disease pressure (Budge and Whipps 1991; McQuilken et al. 1995). Consequently, numerous studies have been carried out to characterise different properties of *C. minitans* to optimise its use in biological control.

Inoculum production and application

Once a screening exercise has been carried out to identify active isolates (Whipps and Budge 1990; Jones and Stewart 2000) it is important to optimise the quality and quantity of inoculum to be applied. Many experimental systems have applied solid substrate fermentation products directly to soil with concomitant arguments about cost effectiveness (Whipps and Gerlagh 1992). This process has also been used regularly to produce conidia which are then used in spray formulations (Gerlagh et al. 1999). Indeed, conidia produced by solid state fermentation are incorporated into the wettable granule formulation of the commercial *C. minitans* product, Contans® WG (de Vrije et al. 2001). Considerable efforts have been made to optimise the yield, viability, infectivity and surface characteristics of conidia in these systems with particular emphasis on physiological studies of substrate utilisation and influence of environmental conditions (Whipps and Gerlagh 1992; McQuilken et al. 1997b; Oostra et al. 2000; Ooijkaas et al. 1998, 1999; Smith et al. 1999; Jones et al. 2004b; Chen et al. 2005). Nevertheless, other workers have explored the potential to produce inoculum of *C. minitans* in liquid fermentation (McQuilken et al. 1997a; Cheng et al. 2003) and to improve infectivity by pre-germinating conidia prior to application procedures (Shi et al. 2004), and could be worth exploring further. There have also been numerous studies to compare application rates, timings and forms of inocula to obtain cost-effective disease control or infection of sclerotia (eg. Jones 2003a, 2004a; Gerlagh et al. 2003, 2004) and it is clear that these may be variable for each crop.

Integrated control

Another approach to improve efficacy has been to integrate *C. minitans* with other control treatments. For example, successful integration of *C. minitans* with the fungicide iprodione was obtained in glasshouse trials against Sclerotinia disease in lettuce (Budge and Whipps 2001). However, *C. minitans* is susceptible to numerous fungicides (Budge and Whipps 2001; Li et al. 2002; Partridge et al. 2006a) and so care must be taken with any strategy integrating fungicides with *C. minitans* unless a fungicide-tolerant isolate is available. In the study of Budge and Whipps (2001), use of a fungicide-tolerant isolate was not required as the mycoparasite in the soil was protected from the direct effects of foliar applied fungicide. *Coniothyrium minitans* has also been successfully integrated with combinations of *Trichoderma* spp. (Budge et al. 1995) for control of Sclerotinia disease in lettuce but under these glasshouse experiments, control reflected that of *C. minitans* rather than the *Trichoderma* spp. In this case, despite the fact that the *T. virens* used originated from a sclerotium of *S. minor*, the control reflected the temperature in the glasshouse and the temperature optima of the fungi involved. Thus, when temperatures in laboratory experiments were below 20°C only *C. minitans* was active in degrading sclerotia whereas when temperatures were increased above 25°C, *C. minitans* became inactive and *T. virens* became more active, dominating infection of sclerotia. Some small-scale studies have also explored the potential to integrate partial soil sterilisation (pasteurization) with

subsequent *C. minitans* application for longer-term control (Bennett et al. 2005).

Ecology

The ecology of *C. minitans* is gradually being revealed. It has long been known to have the ability to survive for several years in soil following application (Budge and Whipps 1991; Jones and Whipps 2002; Huang and Erickson 2007) but cannot grow through raw soil and utilise organic substrates in soil as a saprotroph (Williams 1996). Consequently, it must be viewed as an ecologically obligate mycoparasite. Tribe (1957) first proposed that the mycoparasite could survive for long periods of time in soil protected within sclerotia but only recently have a combination of experiments shown this hypothesis to be true. Firstly, studies using a strain genetically marked with GUS (β-glucuronidase (*uid A*)) and hygromycin resistance (hygromycin phosphotransferase (*hph*)) (Jones et al. 1999) showed that the mycoparasite infects a large proportion of sclerotia in soil rapidly from low population levels and that fungi colonising sclerotia already infected by *C. minitans* mask the detection of *C. minitans* rather than displacing it (Jones et al. 2003b). Indeed, only two conidia of *C. minitans* are needed to infect a sclerotium of *S. sclerotiorum* under ideal conditions (Gerlagh et al. 2003). Secondly, following colonisation of sclerotia of *S. sclerotiorum* by *C. minitans* in soil, the sclerotial medulla was largely converted to pycnidia of the mycoparasite with conidial droplets produced on the surface of a largely intact rind (Bennett et al. 2006). The pycnidia and dried conidial droplets were still present 6 months after infection and by 10 months approximately 13% of the conidia in dried droplets were still viable. Together these studies clearly show that *C. minitans* is a highly efficient sclerotial mycoparasite which uses sclerotia of *S. sclerotiorum* as reservoirs for survival.

Coniothyrium minitans exhibits no ability to infect healthy plants even on cut tissues (Gerlagh et al. 1996) although it can grow on foliage, petals and into stems precolonised by *S. sclerotiorum* (Gerlagh et al. 1994; Li et al. 2003a) and survive on petals of oilseed rape for several days (Yang et al. 2007). However, it is dispersed by water splash directly and as aerosols (Williams et al. 1998) and by animals including slugs, collembolans, mites and sciarid larvae (Whipps 2001).

It could be argued that despite its longevity in soil, *C. minitans* has few effects on the soil microbial population as it is an ecologically obligate mycoparasite. However, recent findings that *C. minitans* produces antimicrobial metabolites in culture (McQuilken et al. 2003; Li et al. 2005b) suggest this may not be the case and some studies to examine the impact of *C. minitans* on the soil microbial population have begun. Introduction of *C. minitans* at 10^3 or 10^6 colony forming units (cfu) g^{-1} soil resulted in no changes in culturable populations of bacteria over a 30-day period and only a small decrease (0.1 $\log_{10}$ cfu g^{-1}) in culturable fungal populations with the higher application rate (Bennett et al. 2003). Populations of *C. minitans* did not change over this period. Similarly, preliminary data using a genetically marked strain to enhance detection, indicate once again that *C. minitans* survives in soil for 6 months with little loss in cfus and that there is little impact on the microbial populations determined by microbial fingerprinting using PCR-denaturing gradient gel electrophoresis (Rogers and Whipps, unpublished). These data seem to confirm the concept that any effect of *C. minitans* on the natural microflora is minimal.

Modes of action

Although hyphal–hyphal interactions between *C. minitans* and numerous other fungi have been observed in vitro, the two major interactions in vivo reflect the competition or mycoparasitism that occurs between mycelium of *C. minitans* and *Sclerotinia* on petals, pollen or infected plant tissues (Li et al. 2002, 2003b), and the mycoparasitic attack of mycelium of *C. minitans* on host sclerotia (Whipps and Gerlagh 1992), the latter being most common. There have been no detailed microscopical or biochemical studies of the interactions on plant tissues and few studies of sclerotial mycoparasitism since the subject was last reviewed (Whipps and Gerlagh 1992). Cell wall degradation and host tissue collapse has long been associated with production of cell-wall degrading enzymes such as chitinases and glucanases by *C. minitans* as part of the mycoparasitic process and recently molecular studies have shown the increased expression of a β-1, 3 glucanase gene *cmg1* by *C. minitans* during infection of sclerotia of *S. sclerotiorum* (Giczey et al. 2001). *Coniothyrium minitans* has the ability to produce a wide spectrum of cell-wall degrading enzymes in culture when grown on complex

substrates such as purified fungal and Oomycete cell walls (Dahiya et al. 1998; Inglis and Kawchuk 2002; Kaur et al. 2005) and some mutants with enhanced β-glucanase activity have been obtained (Zantinge et al. 2003). Recently, the finding that *C. minitans* produces antimicrobial metabolites in culture (McQuilken et al. 2003; Li et al. 2005b) opens up the possibility that these compounds could be involved in the mycoparasitic or competitive processes occurring during these interactions in vivo. However, other than the study by Giczey et al. (2001), no good evidence for expression of specific enzymes, genes or metabolites during the sclerotial infection process by *C. minitans* has been obtained. The challenge to address this lack of knowledge using modern molecular techniques is considered in the next section.

Examining molecular aspects of sclerotial mycoparasitism

To date, there are less than 10 gene sequences of *C. minitans* available in public databases and the molecular tool-kits for gene discovery and gene function analysis in this challenging dual-ascomycete interaction system are only beginning to be developed. Recently, protocols for rapid and reliable transformation of *C. minitans* based on restriction enzyme mediated integration and *Agrobacterium tumefaciens*-mediated transformation were developed. These techniques were applied for insertional mutagenesis of *C. minitans* leading to the identification of 11 sclerotial mycoparasitism mutants (Rogers et al. 2004; Li et al. 2005b). Using inverse PCR, sequences flanking the site of insertion in some of the REMI mutants have been identified (Rogers et al. unpublished). For example, the sequence recovered in a non-mycoparasitic mutant R2427 showed high homology to the *PIF1* DNA helicase gene in yeasts and filamentous fungi. Functional complementation analysis by reintroduction of the wild-type *PIF1*gene into the mutant led to the restoration of sclerotial mycoparasitism. *PIF1* gene is likely to play a major role in maintaining mitochondrial integrity in response to oxidative stress, based on the recent work published in yeasts (Doudican et al. 2005).

Further, using degenerate PCR and macroarrays of a genomic cosmid library of *C. minitans*, *PKAC* and *PMK1* genes implicated in fungal pathogenicity and signalling, have been isolated and fully sequenced with a view to characterising their role in signalling. In a separate study to identify the genes regulating key processes in sclerotial mycoparasitism, PCR-based suppression subtractive hybridisation (SSH) has been used (Muthumeenakshi et al. 2007). Following SSH between cDNA samples from *C. minitans* grown in culture and *C. minitans* colonising autoclaved sclerotia (simulated mycoparasitism), a cDNA library enriched for genes upregulated during this process was established. Sequencing of 672 cDNA clones and bioinformatic analysis led to the identification of 251 unisequences (putative genes) and their functional categorisation. The genes identified belonged to diverse functions such as signalling and cellular communication, cell wall degradation and hydrolysis of energy reserves, production of anti-microbial metabolites, detoxification, stress response and nutrient utilisation in *C. minitans* (Fig. 1). Nearly 35 putative genes encoding a whole range of hydrolytic enzymes were present among the *C. minitans* unisequences identified. Cell walls of the *S. sclerotiorum* sclerotia are a major barrier that the mycoparasite *C. minitans* has to overcome both to kill the host and to access the nutrients and a number of these hydrolytic enzymes are likely to play an active role in this process. It is also likely that some of these enzymes will play a role in remodelling the cells of *C. minitans*, as the mycoparasite grows through the sclerotial host. Further, Lu et al. (2004) expressed the *C. minitans* xylanase gene in *Arabidopsis* plants, providing a basis for investigat-

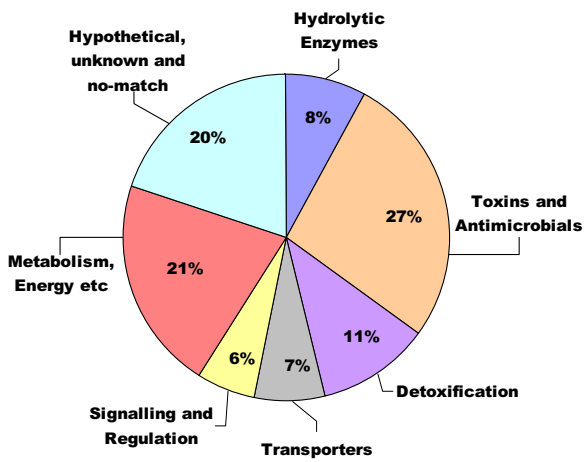

Fig. 1 Functional categorisation of gene transcripts expressed by *Coniothyrium minitans* during sclerotial mycoparasitism

ing transgenic resistance to *S. sclerotiorum* and other plant pathogens.

Various strategies were adopted to successfully perform expression analysis of *C. minitans* genes during sclerotial mycoparasitism (Muthumeenakshi et al. 2007). For example, specific probe sequences to avoid cross hybridisation were selected, by comparing the sequence of the *C. minitans* cDNAs with the genome sequence of *S. sclerotiorum*. Moreover, as the cDNA samples of *C. minitans* colonising live sclerotia yielded large quantities of RNA from the host fungus, slot blot analysis of known concentrations of the cDNA samples was carried out with different *C. minitans* probes and the cDNA ratio between the mycoparasite and the host was determined to be 1:6. Gene expression analysis carried out utilising dot blots and virtual Northerns of the unisequences, revealed different levels of upregulation of various *C. minitans* genes during sclerotial mycoparasitism. Experiments carried out with candidate genes suggest that RNAi and knock-out technologies are feasible for gene function analysis in *C. minitans*.

Overall, some of the genes identified in *C. minitans* such as those linked to signalling and detoxification belong to conserved gene families and pathways implicated in fungal-plant interactions. On the other-hand, the wide range of hydrolytic enzyme genes identified are likely to be important in fungal–fungal interactions and different to those expressed by fungal plant pathogens. Interestingly, less than 30% of the *C. minitans* genes identified showed homology to ESTs generated in *Trichoderma* spp. (Vizcaino et al. 2006). Moreover, nearly 20% of the genes are of unknown function suggesting that these could play novel roles in *C. minitans*, which shows a different eco-nutritional mode compared to other mycoparasites such as *Trichoderma* spp. These advances in gene discovery and gene function analysis in *C. minitans*, have laid a firm platform for comparative genomic investigations into the molecular mechanisms involved in sclerotial mycoparasitism. This knowledge is likely to provide strategies for a better exploitation of fungal biocontrol agents in future plant disease management.

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