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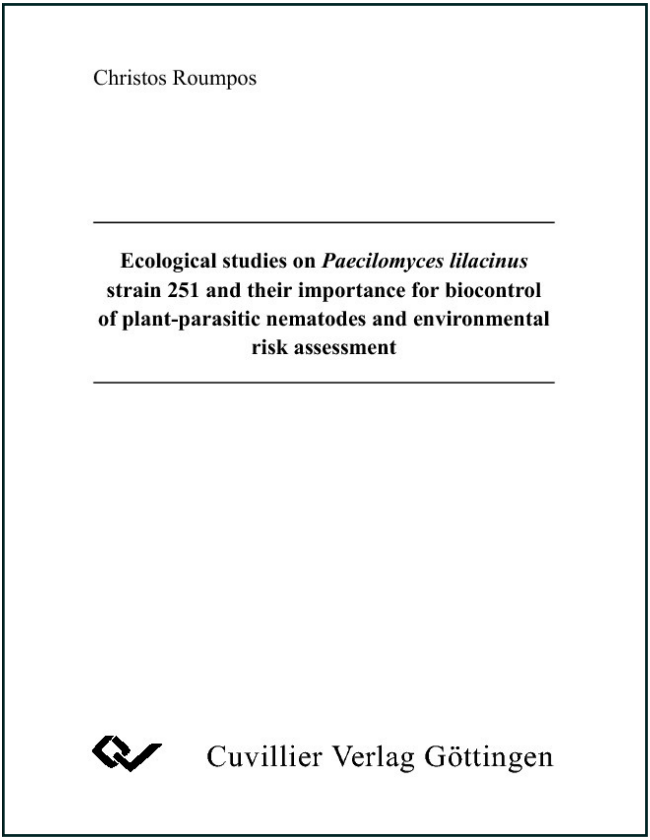

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1 Introduction

Agriculture has always been a struggle between the forces of natural biodiversity and the need to produce the essential supplies for human survival and well-being. With no doubt chemical pesticides have played a key role in the increase of agricultural productivity in the last century, by providing efficient control of plant pests and diseases. However, their heavy use has a negative impact on the ecosystem and is associated with environmental deterioration. Due to their side-effects, a number of synthetic pesticides are banned (e.g. organochlorines) or being phased out (e.g. methyl bromide). Public perception worldwide is that chemical pest control and inputs of synthetic pesticides in agricultural production need to be significantly reduced. Therefore, the development and implementation of alternative control strategies is needed.

Biological control, the exploitation of naturally occurring organisms, such as insects, bacteria, viruses and fungi for the control of crop pests, weeds and diseases is a realistic alternative to chemical control methods and should be considered as an essential element of modern agriculture. Unfortunately, there is still relatively little investment in research and development of biological control agents (BCAs) compared with that spent on the discovery of chemical pesticides (Whipps and Lumsden, 1989). Cook et al. (1996) identified two reasons for the underdevelopment of biocontrol agents: i) the technical difficulties of using microorganisms for biocontrol, owing to a lack of fundamental information on their ecology, which often results in inconsistent and poor control and ii) the costs of product development and regulatory approvals required for each strain, formulation and use. These factors often must be overcome by small-medium-size enterprises, having limited investment potential (Lüth, 2000). However, the demand for environmentally friendly and sustainable approaches in agriculture sets the stage for further development of BCAs and greater employment of biocontrol in agricultural production.

1.1 Fungal biocontrol agents

In recent years, there has been considerable interest in the use of fungi as biological control agents of pest insects, weeds, plant pathogens and nematodes (Butt et al., 2001). Some fungal antagonists have been developed as commercial biocontrol products and are considered promising alternatives to chemical pesticides for disease control in agricultural production systems (Whipps and Lumsden, 2001).

Fungi possess different modes of action as natural enemies. One of the control strategies is parasitism, where the fungus directly attacks and feeds on other organisms. The entomopathogens *Beauveria bassiana*, *Metarhizium anisopliae* and *Paecilomyces fumosoroseus* are known to be pathogens of a wide range of crop pests (Johnson and Goettel, 1993; Tounou et al., 2003), while *Trichoderma* spp. are intensively studied mycoparasites, used against a broad spectrum of soil-borne or foliar diseases (Harman, 2000). Furthermore, many fungi produce secondary metabolites, which contain antibiotics that inhibit the growth or reduce the competitive ability of other organisms. Gliotoxin, an antibiotic produced by *Trichoderma virens* is known to play an important role in the biological control of *Pythium* and *Rhizoctonia* damping-off diseases (Lumsden et al., 1992). Another mode of action of fungal antagonists involves competition for resources, whereby the fungal BCA colonises plant surfaces and subsequently prevents the invasion of the pathogen. Competition for space, nutrients and oxygen is implicated in the biocontrol of tomato *Fusarium* wilt by *Penicillium oxalicum* (De Cal and Melgarejo, 2001). Likewise, the application of *Phlebiopsis gigantea* on the freshly cut surfaces of pine stumps preempts the establishment of *Heterobasidion annosum*, the cause of annosus root rot of pine (Pratt et al., 1999). Finally, BCAs may also induce changes in the plant that enhance plant resistance, resulting in induced resistance. Non-pathogenic strains of *Fusarium oxysporum* have been shown to induce systemic resistance against the burrowing nematode *Radopholus similis* in banana plants (Vu Thi Thanh, 2005).

1.1.1 Nematophagous fungi as biocontrol agents of plant-parasitic nematodes

Plant-parasitic nematodes (PPN) cause high yield losses annually worldwide in many important crops like rice, wheat, barley, maize, banana and vegetables (Ferraz and Brown, 2002). The loss of important non-fumigant nematicides and the phase out of the fumigant methyl bromide, which until recently have been considered panaceae for nematode control, has led to more holistic integrated nematode management approaches, that depend on the combination of chemical, cultural and biological methods to regulate nematode populations (Sikora et al., 2005). Nematicides, fumigants or non fumigants, are combined with physical and cultural methods, like solarization and soil heating, flooding, fallow, crop rotation with non-hosts, tolerant or resistant cultivars, incorporation of organic amendments to suppress nematode densities (Sikora and Fernández, 2005). Public demand for more environmentally friendly approaches in agriculture has provided a strong impetus for the exploitation of microbial agents and the management of the antagonistic potential of agricultural ecosystems for the biological control of plant-parasitic nematodes (Sikora, 1992; Kerry, 2000). Soil microorganisms, like bacteria, fungi, actinomycetes, viruses and rickettsia are a valuable resource of microbial control agents of PPNs that has only been partially exploited and needs to be further investigated. Nematophagous fungi are natural enemies of nematodes, an essential component of the nematode antagonistic microflora and comprise many potential biological control agents of PPNs. They consist of three main groups of fungi: i) the nematode-trapping or predatory fungi, ii) the endoparasitic fungi and iii) the facultative parasites or opportunistic fungi (Siddiqui and Mahmood, 1996).

The predatory fungi form different types of traps, such as adhesive branches, knobs or rings, with which they capture vermiform living nematodes in soil, and subsequently kill and feed on them. Among the predatory fungi, *Arthrobotrys* and *Monacrosporium* species have received the most attention. However, the non-specific nature of the nematode-trapping fungi in predation, their slow growth and their requirement for high amounts of nutrients were proven to be drawbacks in their success as candidates for commercial production (Jatala, 1986). Additionally, these fungi are difficult to manipulate so as to ensure that they produce traps when the infective nematode juveniles are migrating towards roots (Kerry, 2001).

The endoparasitic fungi are obligate parasites that complete their life cycle within the body of their hosts and use their spores to infect nematodes. Spores either adhere to the cuticle and subsequently infect, like in the case of *Hirsutella rhossiliensis* and *Drechmeria coniospora* or are swallowed by nematodes e.g. *Harposporium* spp. Many studies have shown the potential of *H. rhossiliensis*, the most promising fungal agent of this group, to suppress nematode populations (Tedford et al., 1993) and currently research focuses on the enhancement of the biocontrol properties of the fungus by improved formulations (Slaats et al., 2005).

Finally, an important group of nematophagous fungi with many biocontrol candidates are the facultative parasites or opportunistic fungi. They are usually soil saprophytes that have also the potential to colonize sedentary stages mainly of the root-knot and cyst nematodes. *Pochonia chlamydosporia* (Syn. *Verticillium chlamydosporium*) has been widely tested for the control of several *Meloidogyne* species (Kerry, 2001; Atkins et al., 2003) and at present an indigenous isolate (RES 392) is under commercial development in Cuba.

Paecilomyces lilacinus

Paecilomyces lilacinus (Thom.) Samson 1974 (Deuteromycotina: Hyphomycetes) is a nematode egg pathogenic fungus, which has been shown to be effective against a wide spectrum of plant-parasitic nematodes and is considered one of the most promising biological control agents for the practical management of PPNs (Jatala, 1986; Kerry and Evans 1996; Siddiqui and Mahmood, 1996; Cannayane and Sivakumar, 2001). The fungus is a common soil hyphomycete, reported from numerous parts of the world, but more frequently from warm regions (Samson, 1974; Domsch et al., 1980). It has been isolated from a wide range of habitats including cultivated and uncultivated soils, forests, grassland, desert soil, estuarine sediments and sewage sludge. The species has a wide temperature range from 8 to 38°C with optimal growth in the range of 26-30°C. It also has a wide pH tolerance, which makes it competitive in a broad spectrum of agricultural soils, and allows it to grow on a variety of substrates. *Paecilomyces lilacinus* forms a dense, septate and profusely-branched hyaline mycelium which gives rise to conidiophores. These bear phialides, from the end of which spores are formed in long chains.

Paecilomyces lilacinus attacks mainly sedentary stages and to a lesser extent juveniles of root-knot and cyst nematodes. There are also reports of control of other nematode species by the fungus (Walters and Barker; 1994). During infection, the fungal hyphae grow and form a mycelial network around the nematode egg. Appressoria are formed, which enable the penetration of the fungal hyphae into the egg (Dunn et al., 1982; Holland et al., 1999; Morton et al., 2004). Penetration of the eggshell is a result of mechanical as well as enzymatic activities. *Paecilomyces lilacinus* produces degradative enzymes like proteases and chitinases (Gupta et al., 1993; Bonants et al., 1995; Khan et al., 2003), which are believed to be involved in the infection process and particularly in the degradation of the nematode eggshell (Khan et al., 2004).

The first report of *P. lilacinus* as an effective parasite of *Meloidogyne incognita* and *Globodera pallida* was by Jatala et al. (1979). Since then, many greenhouse and field studies have been conducted in a number of countries with different isolates of *P. lilacinus* that have demonstrated the potential of the fungus to suppress populations of root-knot and cyst nematodes (Dube and Smart, 1987; Cabanillas and Barker, 1989; Gomes Carneiro and Cayrol, 1991; Mittal et al., 1995). The fungus is applied as spores in large quantities to the soil, where it parasitizes the nematode eggs reducing the nematode multiplication and providing population control.

Paecilomyces lilacinus strain 251 (PL251) was originally isolated from a *Meloidogyne* eggmass in the Philippines (Davide and Zorilla, 1983). At present it is commercially formulated and already on the market in the USA under the product name MeloCon^{TM} WG (Anonymous, 2005b; Lüth, 2005). In Europe, PL251 is currently undergoing registration procedure and will be registered under the product name BioAct$^{®}$ WG (Anonymous, 2003; Kiewnick et al., 2005). Solid-state fermentation technology, where fungal growth occurs on solid substrate in the absence of free liquid, is used to produce a water-dispersible granule formulation of PL251 conidia with glucose as a carrier (Kiewnick, 2001). Another formulation of PL251 is commercially available in South Africa under the product name *Pl* plus$^{®}$ and consists of fungal spore powder together with a specially formulated liquid growth medium to promote the growth of the fungus when added to the soil. The efficacy of PL251, mainly against root-knot and cyst nematodes, has been shown in many greenhouse and field experiments (Holland et al., 2003; Kiewnick and Sikora, 2003; 2004; Kiewnick, 2004; Kiewnick et al., 2004b). Research now focuses on the potential of *P. lilacinus* to control other plant parasitic-nematodes, such as the burrowing nematode *Radopholus similis* in banana (Kiewnick et al., 2004a).

Some isolates of *P. lilacinus* were recognised as rare, opportunistic human pathogens and were reported to cause infections to humans (Blackwell et al., 2000; Nayak et al., 2000; Safdar, 2002). Most cases occurred in patients with impaired host defences or following surgical procedures (Gutiérrez-Rodero et al., 1999). Although fungal infections caused by this species occur very rarely (Gutiérrez-Rodero et al., 1999), concerns were raised about human health risk that could derive from a biopesticide on the basis of this fungus. However, it could be proven that the commercial strain 251 differs from the human clinical isolates and does not present a significant human health risk (Anonymous, 2005a). Mammalian safety tests, including acute oral, dermal and pulmonary toxicity tests in rats and irritation studies in rabbits, have been conducted and demonstrated the safety of this strain (Goettel et al., 2001; Anonymous, 2004). Furthermore, *P. lilacinus* strain 251 is not able to survive at normal human body temperature (Anonymous, 2005a), alleviating scientific concerns for human health risks posed by the fungal biocontrol agent.

1.2 Factors affecting biocontrol

One of the major limitations of biocontrol is inconsistency in efficacy and low levels of control that are often observed, in comparison to the relative consistent record of chemical pesticides (Whipps and Lumsden, 2001). To make BCAs more attractive as alternatives to chemical pesticides it is necessary to reduce the variability in their performance. One of the reasons for the inconsistent results of biocontrol is often our inadequate understanding of the ecology of the biological control agents in the field. Better knowledge of the ecology of a BCA can contribute to an improved understanding of the effect of environmental factors on its survival and efficacy. This in turn can enable scientists to design appropriate biocontrol strategies supportive of the applied agents and conducive to existing environmental conditions at treatment.

In general, the success of biocontrol of a pathogen or a pest is related to the persistence of the antagonist once it has been applied. In a field trial, Thomas et al. (1997) investigated the residual infectivity of an oil formulation of *Metarhizium anisopliae*. They concluded that apart from the initial infectivity of the applied inoculum, infection caused by the persisting fungal propagules could contribute substantially to the total mortality of grasshopper populations. Paulitz (2000) suggested that the most critical aspect affecting efficacy of biological control is maintainance of the necessary population densities of the biocontrol agent over time to protect the plant. The longer the antagonist persists, the higher is the probability of an encounter between the BCA and its target host. Understanding the critical factors that impact the persistence of a BCA is important in order to fully exploit its biological control potential and to improve applications strategies (Bidocka, 2001). The performance of biocontrol is also affected by the movement of the inoculum of a BCA once it has been applied (Parke et al., 1986). Optimum dispersal and distribution of the agent around the plant can result in enhanced biocontrol. Therefore, it is important to monitor the movement of the applied conidia and determine the factors that affect their dispersal.

The interactions of an antagonist with the microflora and microfauna at the site of application can also affect its performance. Inglis et al. (1998) reported a higher prevalence of mortality of grasshoppers caused by *Beauveria bassiana*, when the females were ovipositing into sterilized than non-sterilized soils and attributed this effect to the fungistasis in non-sterile soils. Likewise, *Trichoderma harzianum* was found to decrease more rapidly in the “mycorrhizosphere”, suppressed by the arbuscular mycorrhizal fungus *Glomus intraradices* and competition for nutrients was proposed as the mechanism of interaction (Green et al., 1999). Vegetation is also a biotic factor that can have a critical impact on the efficacy of biocontrol. Bourne and Kerry (1999) showed that the efficacy of *Pochonia chlamydosporia* is influenced by the susceptibility of the host plant to the nematode and its ability to support growth of the fungus in its rhizosphere.

Fluctuations in the performance of a biocontrol agent can arise from a variety of causes reflecting the biological nature of the biocontrol microorganism. Improving our understanding of the BCA ecology is critical to understand the success or failure of biocontrol and design appropriate management strategies. Application strategies can have a profound impact on the efficacy of fungal BCAs but this has often been a neglected area that should attract more interest from the scientific community.

1.3 Risk assessment of biological control agents

There has been much debate about the potential risks that biological introductions may pose to humans and environment. In the more than 100 years history of biocontrol, many species of natural enemies of plant pests and pathogens have been used, with more or less success (Gurr and Wratten, 2000). Until recently, only a few cases showed negative effects associated with the release of a BCA (Lynch et al., 2001; Van Lenteren et al., 2003). Experience indicates that any adverse non-target effects are likely to be short-term and transitory, and are eliminated by terminating the use of the BCA (Cook et al., 1996). A number of reports have been written reviewing the environmental impact and the unintended effects of applied biocontrol agents (Gullino et al., 1995; Boisvert and Boisvert, 2000; Strasser et al., 2000; Migheli, 2001; Lynch et al., 2002; Winding et al., 2004) and different methods have been proposed to assess the risks of releasing a biocontrol organism (Ryder and Correll, 1995; De Jong et al., 1999; Harris et al., 1999). In some cases, reviews of non-target effects (Brimner and Boland, 2003) have been seriously criticised (Kiss, 2004). While some authors believe that biocontrol can pose risks, many are more skeptical and critical of the quality of evidence. This indicates that identifying and assessing the potential risks that BCAs may pose to humans and environment, as well as interpreting the results of risk assessment studies is still a matter of controversy.

Van Lenteren et al. (2003) proposed a risk assessment methodology and a risk index for evaluation of exotic natural enemies used in inundation biological control and applied it to a number of biocontrol agents. The risk assessment consists of three steps: i) the risk identification and evaluation procedure, ii) a risk management plan dealing with risk reduction and iii) a risk benefit analysis of the antagonist as well as of other alternative pest management methods. It was suggested that the following ecological factors determining the environmental impact of an introduced organism should be evaluated: establishment, dispersal, host specificity, as well as the direct and indirect effects of the BCA on other organisms in ecosystem.

The potential of a natural enemy to establish and disseminate in the environment will determine the probability of temporal and spatial encounter between the BCA and non-target species. Risk of adverse effects can be defined as the combination of the agent’s toxicity and the probability of exposure of non-target organisms (Cook et al., 1996). Thus, the risk of using an agent with some known hazard can be reduced by limiting exposure, and the use of agents with no known hazards and high exposure presents little or no risk. Given the above, it becomes critical to investigate how long a BCA persists and its degree of spread in the environment following application.

Host range should be determined before the release of the biocontrol agent in the environment. Lack of host specificity is not a desirable feature for a BCA, because it increases the potential for detrimental impacts on non-targets. Because of the wide host range of *Sclerotinia sclerotiorum*, used in biological control of the creeping thistle *Cirsium arvense*, it became crucial to model and predict the survival of the soil-borne sclerotia and the dispersal of the air-borne ascospores of the fungus, in order to assess the phytosanitary risks that the use of the mycoherbicide may pose to non-target arable plants (De Jong et al., 1999). In contrast, if a natural enemy is very specific, attacking only one or few related hosts, risk is limited. As it is not possible to test all target and non-target species to susceptibility to a biocontrol organism, susceptibility tests are conducted on a selection of potential hosts and the risks to other species are predicted from the results (Harris et al., 1999). Species closely related to the targeted species are most likely to be attacked and should be included in host spectrum tests (Louda et al., 2003).

The evaluation of the direct and indirect effects of a natural enemy on non-targets is an essential element in the risk assessment process. The North American Microbial Biocontrol Working Group identified four safety issues associated with biological control: i) competitive displacement of non-target organisms, ii) allergenicity, iii) toxigenicity and iv) pathogenicity to non-target organisms (Cook et al., 1996). Competitive displacement occurs when the introduced species expels or replaces native non-target species through competition for space or nutrients. Allerginicity may occur in vertebrates that develop sensitivities to spores or formulations of the BCAs. Workers in production facilities exposed repeatedly to high concentrations of spores of fungi such as *Beauveria* spp. or *Metarhizium* spp. may develop hypersensitive reactions (York, 1958). The release of antibiotics or alkaloids may have toxic effects on non-target species. The various toxins of *Bacillus thurigiensis* affect a number of arthropod species that are not pests, but