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Selection of effective antagonists against *Rhizoctonia solani* (AG-3), the causal agent of Rhizoctonia disease of potato

by

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A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science.

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Short title

The biological control of Rhizoctonia disease of potato

Nasreen Zahan Kabir©

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FOREWORD

This thesis consists of eight parts. The first part is a general overview of the problem, the review on the previous work on the thesis topic and the goals of this research. Part two, three, four, five, six, and seven are the body of this thesis presented as complete manuscripts covering the entire research project. Part eight is a general discussion and conclusion of the whole manuscripts.

The general thesis format has been approved by The Faculty of Graduate Studies and Research of McGill University and following the condition outlined in the "Guideline Concerning Thesis Preparation", section B, **"Manuscripts and Authorship"**, which are as follows:

The candidate has the option, subject to the approval of their department, of including as part of the thesis the text, or duplicated published text, of an original paper, or papers.

- Manuscript-style thesis must still conform to all other requirements explained in the Guidelines Concerning Thesis Preparation.
- Additional material (procedural and design data as well as descriptions of equipment) must be provided in sufficient detail (e.g. in appendices) to allow clear and precise judgement to be made of the important and originality of the research reported.
- The thesis should be more than a mere collection of manuscripts published or to be published. It must include a general abstract, a full introduction and literature review and a final overall conclusion. Connecting texts which provide logical bridge between different manuscripts are usually desirable in the interest of cohesion.

All the work reported here was the responsibility of the candidate. The research was conducted under the supervision of Dr. Suha J.-Hare, Department of Plant Science, Macdonald Campus of McGill University. For consistency and convenience, all manuscripts follow the same format.

ABSTRACT

Selection of effective antagonists against *Rhizoctonia solani* (AG-3), the causal agent of Rhizoctonia disease of potato

M. Sc. Nasreen Z. Kabir Plant Science

Rhizoctonia solani (AG-3), the causal agent of Rhizoctonia disease of potato, overwinters as sclerotia on potato tubers. To develop a biocontrol strategy based on the prevention of the sclerotial germination, an isolation of microorganisms colonizing sclerotia of infected potato tubers (cultivars Norland, Atlantic and Souris), was conducted. A total of 259 soil fungi and 36 bacteria were isolated. The fungal and bacterial communities isolated from the different varieties of potato were fairly uniform. No differences were detected in the types of identified genera among different potato fields. *In vitro* screening was used to select effective antagonistic fungi against *Rhizoctonia solani.* Fifty fungal isolates were selected in order to cover all identified genera and potato variety and examined for their ability to inhibit germination of sclerotia which were incubated with the test fungus for 14 days. Twenty-four (24) fungal isolates were retained based on their ability to reduce sclerotial viability by more than 50% as compared with 100% viability of untreated sclerotia. These 24 isolates were further examined for their ability to protect Table beet seedlings against the pathogen in greenhouse soils. Based on their ability to protect Table beet seedlings from Rhizoctonia infections and to increase the number of secondary roots and root length isolates, F2, F11, F132, F158, and F258 were screened and test their efficacy to increase beet seed germination in field soils. Of the five isolates, 4 isolates had a seed germination rates between 42%-67%, whereas germination rates in soil infected by *R. solani* alone was 30%. Genera were identified as species of *Pestalotia* (F2), *Penicillium* (F11), *Gliocladium* (F132, and F158), *Trichoderma* (F258). Dual-interaction technique was used to examine

with light microscopy for their ability to parasitize hyphae of *R. solani* on agar media. F2, F11, F132 showed strong antagonistic activities; they grew toward *R. solani* hyphae, penetrate and colonized host hyphae. Substrate-agar plate experiments of extracellular enzymes (ECE) produced by selected mycoparasites were carried out to demonstrate mycoparasitic and saprophytic ability of antagonists. Chitinases, ß-1,3-glucanases, cellulases, and proteases were secreted by antagonists F2, F11, and F132 when grown on minimal synthetic medium containing *R. solani* cell wall fragments. Pronounced activity of cell wall lytic enzymes (chitinase, glucanase, cellulase, and protease) activity from liquid cultures of three selected antagonists were compared favourebly with those produced directly on agar plate. These mycoparasites were especially effective in reducing sclerotial germination and controlling Rhizoctonia disease of potato in field soils under growth chamber conditions.

RÉSUMÉ

Sélection d'antagonistes efficaces contre *Rhizoctonia solani*, le vecteur de la maladie de Rhizoctonia affectant la patate.

M. Sc. Nasreen Z. Kabir Plant Science

Rhizoctonia solani (AG-3), qui cause la maladie de Rhizoctonia affectant les pommes de terre, passe l'hiver sous la forme de sclerotes sur les tubercules. Pour développer une stratégie de biocontrôle contre la germination des sclerotes, nous avons isolé des microorganismes qui colonisent les sclerotes des tubercules de pommes de terre infectées des cultivars Norland, Atlantic et Souris. Un total de 259 champignons du sol et de 36 bactéries ont été isolés. Les communautées fongiques et bactériennes étaient relativement uniformes pour chaque variété de pommes de terre. L'échantillonage des genera identifiés parmis les différents champs de patates étaient identiques. Une sélection *in vitro* a été utilisée afin de choisir un antagoniste efficace contre *Rhizoctonia solani*. Cinquante (50) isolats fongiques ont été choisis et leur capacité à inhiber la germination des sclerotes ayant été incubées avec l'isolat pendant quatorze jours a été testée. Vingt-quatre (24) isolats fongiques ont été retenus selon l'habilité à réduire de plus de 50% la viabilité des sclerotes. Ces isolats ont ensuite été testés en serre pour vérifier leur capacité à protéger les semences de bettraves de tables contre l'agent pathogène. Les isolats F2, F11, F132, F158 et F258 ont présenté la meilleure protection contre les infections de *Rhizoctonia* et de plus ont accru le nombre de racines secondaires et la longueur des racines. Ces isolats ont été testés pour leur efficacité a augmenter la germination des semences de bettraves dans un sol provenant du champs. Les isolats ont été identifié au genre en tant qu'espèces de *Pestalotia* (F2), *Penicillium* (F11), *Gliocladium* (F132 et F158) et *Trichoderma* (F258). Sur les cinq isolats, quatre ont donné un taux de germination des semences de bettraves entre 42% et

67%, comparé au taux de germination de 30% dans les sols infectés par *Rhizoctonia solani* uniquement. En utilisant une méthode relativement facile et rapide de sélection sur plaque d'agar optimisée pour la détection individuelle d'enzymes extracellulaires, trois antagonistes (F2, F11 et F132) ont démontré une activité prononcée d'amylases, cellulases, chitinases, glucanases, lipases, mannases, pectinases et protéases. Des courbes temporelles de l'activité des cellulases, chitinases, glucanases et protéases ont été produites pour F2, F11 et F132 en utilisant une méthode colorimétrique. Une technique d'interaction réciproque a servi à observer par microscopie leurs habilités à réduire la croissance et à parasiter *R. solani* sur un médium d'agar. F2, F11 et F132 ont démontré une grande activité antagoniste: ils ont poussé vers les hyphes de *R. solani* pour infecter et coloniser les cellules de l'agent pathogène. Ces isolats ont été particulièrement efficaces pour réduire la germination des sclérotes sur les pommes de terre dans un sol provenant du champs et en conditions contrôlées.

ACKNOWLEDGEMENTS AND DEDICATION

I wish to express my most sincere gratitude to Dr. Suha J.- Hare, professor of Plant Science and thesis supervisor, for her constant support, guidance, encouragement, enthusiasm, arranging the financial support during the course of this work, and ultimately inspiration.

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CHAPTER 1.

GENERAL INTRODUCTION AND REVIEW OF LITERATURE

1.1 General introduction

Potato *Solanum tuberosum* L. is considered one of the world's most important staple food crop producing more dry matter and protein per hectare than major cereal crops (Harris, 1992). Compared with cereal crops, its production exceeds that of barley, sorghum, millet, rye, and oat. In Canada, the total cultivated area under potato production is 144,700 hectares, producing 3,78,400 tons/ha. In the province of Québec alone, 25,000 hectares of agricultural land (soil type loamy sand and peat) are cultivated annually with early (Superior, Atlantic, Norland) and late (Kennebec) potato varieties, producing 25 tons/ha with a total commercial value of $200 million (Canadian Potato Production, 1995; 1996).

Potato is confronted with a wide range of pathogens wherever it is grown. Worldwide losses of potato due to diseases such as bacterial soft rot (*Erwinia carotovora* Dye), verticillium wilt (*Verticillium albo-atrum* Reinke & Bertheir and *V. dahliae* Kleb.), common scab (*Streptomyces scabies* Thaxter) and late blight (*Phytopthora infestans* (Mont.) de Bary) are important. In potato growing regions of Canada including the province of Québec, potato is a host of several fungal and bacterial diseases (Hooker, 1981), one of which severely affects the production and yield causing annual damage of $4 million (Banville, 1989). This disease is collectively known as Rhizoctonia disease of potato (stem canker and blackscurf). The causal agent of this disease is the soilborne fungus *Rhizoctonia solani* Kühn belonging to the anastomosis group AG-3 (Otrysko and Banville, 1992; Demirci and Döken, 1993).

The government of Québec, Ministère de l'Agriculture, des Pécheries, et de l'Alimentation, currently recommends treatment with fungicides throughout the growing season for control of Rhizoctonia disease (CPVQ, 1987). Because application of fungicides

can have deleterious effects to the environment and agro-ecosystems, it is desirable to find a non-chemical method to help control *R. solani* so that the number of fungicide applications can be reduced.

1.2 Review of literature

1.2.1 Biology and pathology of *Rhizoctonia solani*

Rhizoctonia solani Khün (Teleomorph: *Thanatephorus cucumeris* (Frank) Donk) has long been recognized as a destructive pathogen on a wide variety of economically important agricultural crops (Dodman and Flentje, 1970; Weinhold *et al.*, 1982). Several researchers now recognize *R. solani* as a complex of species which can be best delimited on the basis of their hyphal fusion i.e., anastomosis (Anderson, 1982; Ogoshi, 1987; Balali *et al.*, 1995). In general, these groups of species share the following characteristics: (i) multinucleate cells, pale to dark brown pigment, rapidly growing mycelium of relatively large diameter with branching near the distal septum of hyphae, (ii) formation of monilioid cells which are barrel-shaped cells, chains of these cells aggregate to produce vegetative resting structure, the sclerotia, (iii) sclerotia are usually of uniform texture and of varying size and shape, and (iv) lack of conidia or sexual spores.

1.2.2 Sclerotia

Principally, sclerotia are composed of compact masses of monilioid cells. Truly black sclerotia are not characteristic of *R. solani* (AG-3). The basic color of mature sclerotia is brown. Sclerotia are generally superficial but they may also form inside host tissue. Sclerotial formation in nature is rather common in many hosts, potato tubers being the best known, and infested potato tubers with sclerotia are considered the major source of inoculum of Rhizoctonia disease (Frank and Leach, 1980). The impact of the disease on tuber production is a very important consideration in reaching disease management.

1.2.3 Anastomosis group (AG-3)

Pathologists and mycologists rely on the anastomosis grouping (AG) system for identifying and grouping similar isolates of *R. solani* into a specific AG. This identification method is based on anastomosis between hyphae of isolates grown on solid culture media. To date, 11 anastomosis groups designated as 1 to 11 are recognized (Carling and Leiner, 1986; Ogoshi, 1987; Ogoshi *et al.*, 1990). AG-3 is a homogeneous group with no known sub-groups or interspecific groups (ISG). Isolates of AG-3 grow more slowly and generally are more tolerant to cool temperature than other AGs of *R. solani*. There is conclusive evidence that isolates of *Rhizoctonia solani* belonging to anastomosis group AG-3 are the major pathogens causing both lesions on the potato plant (stem canker) and sclerotial infestation of tubers (blackscurf). Although AG-3 is the most common group isolated from potato (Bandy *et al.*, 1988; Demirci and Döken, 1993; Bains and Bisht, 1995); isolates belonging to either AG-1, AG-2-1, AG-2-2, AG-4, AG-5 or AG-9 can be occasionally isolated from potato or soil where potatoes were grown. These groups exist as saprophytes and cause no damage to potatoes.

1.2.4 Rhizoctonia disease of potato

Stem canker and blackscurf are two phases of Rhizoctonia disease; affecting potato wherever it is grown (Banville, 1989; Read *et al.*, 1989; Hide *et al.*, 1992;).

Stem canker phase: The potato plants are infected with *R. solani* from mycelium either in the soil or from sclerotia on the tubers. However, significant source of stem canker inoculum is derived from sclerotia which are borne on seed tubers (Baker and Martinson, 1970). Penetration of plants by *R. solani* may be accomplished in a number of ways: through the cuticle and epidermis, through natural openings such as lenticels and through wounds (Dodman and Felntje, 1970). The stem or sprout rot of potato provides an excellent example of the occurrence of the disease. Severe infection occurs mostly on underground parts often causing girdling and collapse of stems and stolons. The disease

delays shoot emergence, decreases the number of stems and increases variation in stem height, and leads to stolon and sprout pruning (Hide *et al.*, 1985; Read *et al.*, 1989). In addition to the above symptoms, initiation of tubers and development of foliage are delayed and number of aerial tubers is increased.

Blackscurf: The best known symptom of Rhizoctonia disease is the appearance of sclerotia on the surface of potato tubers. Dean (1994) described the symptom as "the dirt that won't wash-off". This description comes from the fungal masses (sclerotia), which serve as overwintering vegetative structures (Hooker, 1978). Infections originating from sclerotia cause increases in the number of malformed cracked tubers, the production of aerial tubers, and alteration in size and distribution of tubers. All these symptoms lead to lower marketable yields (Weinhold *et al.*, 1982; Otrysko and Banville, 1992).

1.3 Control

1.3.1 Chemical control

In many potato production areas including Canada and U.S.A., potato growers are advised to either (i) plant seed that is blackscurf free, (ii) plant in pathogen-free soils, (iii) prevent the introduction of disease, (iv) practice crop rotation, or (v) use fungicide seed treatment. The efficiency of soil treatments with various fungicides under laboratory, greenhouse and field conditions is a limited protection against the pathogen (Leach and Murdoch, 1985; Stevenson *et al.*, 1986; Platt, 1989). Severity of Rhizoctonia disease is substantially decreased by treating the seed tubers with organo-mercury compounds (Graham, 1960), formaldehyde (Weinhold *et al.*, 1978), meneb (Blaszczak *et al.*, 1978), thiabendazole, benomyl, iprodione, oxathin (Biehn, 1969; Humphreys-Jones, 1977) and with tolclofos-methyl or pencycuron (Oxley and Lang, 1987). Seed piece treatment with 2.5% benomyl was found to be as effective as a 11 kg/ha PCNB (pentachloronitrobenzene) soil treatment for Rhizoctonia disease control (Davis, 1973). On the other hand, soil treatment with thiabendazole, iprodion or benodanil (as wettable powders at 12 kg a.i./ha),

decreased both stem canker and blackscurf (Hide and Cayley, 1982). But wherever blackscurf was severe, none of the treatments controlled the disease (Biehn, 1969). Although several fungicides are effective in controlling disease, growers' concern about toxicity and the increasing costs of pesticides on the market has limited their use in disease control (Whipps, 1992). In addition, the need for an alternative to chemical disease control arises because of environmental problems associated with the use of chemical pesticides. Thus, there is an increased interest in the application of biological control agents (BCA) to control plant pathogens with beneficial advantages to the consumer and the environment. Pathologists are beginning to purposefully involve biological control programs for plant diseases.

1.4 Biological control

There are mainly two different approaches to biocontrol that are widely used. The first approach is based on the implementation of cultural practices to control disease, such as crop rotation, burial residues, flooding, fertilization to elevate or lower pH, and suppressive soil (Palti, 1981; Davis *et al.*, 1993) and the second is based on using naturally occurring antagonistic microorganisms (Chet *et al.*, 1981; Cook, 1988). Cook and Baker (1983) defined the second approach of biocontrol as "the reduction of the amount of inoculum or disease producing activity of a pathogen accomplished by or through one or more organisms other than man". These microorganisms include fungi, bacteria, virus, nematodes, protozoans which can control fungal pathogens (Burge, 1988).

1.4.1 Enhancement and introduction of antagonists

Due to the complex nature of *Rhizoctonia solani* infections and type of invasion, Rhizoctonia disease of potato is hard to control with any one means of control (Frank and Wilson, 1972). An economical and non-polluting method of reducing this disease incidence and the pathogen's saprophytic activity in the soil is crop rotation (Honeycutt *et al.*, 1996).

Several reports indicated that rotation with corn, wheat, oat or other grains reduced Rhizoctonia disease incidence on various crops including potato (Frank and Murphy, 1977; Specht and Leach, 1987). Soils with a two-year rotation of oat-potato had the lowest incidence of disease on the stems, roots, and tubers of potato, while plots or soils without rotation practices (control) had the highest pathogen activity and the greatest disease incidence (Honeycutt *et al.*, 1996). Another effective integrated approach is crop rotation combined with green amendments. This resulted in significant suppression of *R. solani* inoculum due to increased populations of antagonistic bacteria, actinomycetes and fungi (Linderman, 1970; Specht and Leach, 1987). In one study, it was found that high populations of actinomycetes suppressed *R. solani* because of their ability to produce antibiotics (Reddi and Rao, 1971). In another study, high populations of *Trichoderma* spp. was associated with suppression of *R. solani* (Liu and Baker, 1980). When *Trichoderma* species were combined with various management practices, the efficiency of *Trichoderma* was improved to control this pathogen (Lewis and Papavizas, 1980). However, when the two methods were combined, there was generally less disease than when they were used alone. In Québec, *R. solani* inoculum can be minimized, but not completely eradicated by using fungus-free seed tubers combined with a 3-year crop rotation with buckwheat and winter rye (Banville, 1989).

1.4.2 Suppressive soil

The term "suppressive soils" describes certain soils that are inhospitable to some pathogens (Schippers, 1992). Suppressive soils have been shown to subsequently decrease disease incidence. This type of soil has been effective in controlling damping-off of radish caused by *R. solani* belonging to AG-4 (Liu and Baker, 1980). After successive cultures of radish and cucumber, high numbers of *Trichoderma harzianum* Rifai propagules were found in infested soil, rendering it suppressive to *R. solani.* To date, suppression to *Rhizoctonia solani* (AG-3) on potato has not yet been demonstrated. A naturally

suppressive soil against *R. solani* was shown to have a high population of *Trichoderma* species including *T. hamatum* (Bonord.) Bainier.*T. hamatum* is a necrotrophic mycoparasite, causing lysis of hyphae of *R. solani* (Chet and Baker, 1981).

1.4.3 Introduction of antagonists for biological control of *R. solani*

The second approach of biological control can be achieved with the introduction of antagonists. This approach is mainly a means to retard the production of the pathogen's inoculum. Several microorganisms including bacteria and fungi have been shown to be effective antagonists of *Rhizoctonia solani* AG-3 (Roy, 1989). Recently, Turhan (1990), screened several soil fungi belonging to the genera *Botryotrichum, Coniothyrium, Dicyma* for their ability to parasitize hyphae of *R. solani.*

Trichoderma and Gliocladium spp. are two fungal genera which have been exhaustively studied for their biocontrol potential (Beagle and Papavizas, 1984; Papavizas, 1985). Within the *Trichoderma* genus, species such as *T. harzianum* Rifai, *T. hamatum* (Bonord.) Bainier, and *T. viride* Pers. Fr. have demonstrated excellent antagonistic activity against *R. solani.* This activity has been related to mycoparasitism and /or antibiosis as two mechanisms of biocontrol (Chet, 1987). *T. harzianum*, the most commonly studied species was tested with varying degrees of success against Rhizoctonia damping-off of beans, tomatoes, eggplants, and beets (Hader *et al.*, 1979). It was found that *T. harzianum* in combination with *T. hamatum* directly attacked and was capable of lysing mycelia of *R. solani.* Also under field conditions, *T. harzianum* significantly increased the yield of beans and decreased disease incidence. These two isolates were shown to produce cell wall degrading enzymes namely, ß-1,3 glucanases and chitinases.

The genus *Gliocladium* contains many mycoparasitic species which are considered good biocontrol agents against soil-borne pathogens. *G. virens* J.H. Miller, J.E. Giddens, & A.A. Foster successfully controlled Rhizoctonia damping-off of cotton seedlings and root rot of white bean (Tu and Vaartaja, 1981; Howell, 1982; Howell and Stipanovic,

1995). Production of the antibiotics gliotoxin and glovirin by the biocontrol fungus *G. virens* has been associated with its efficacy as a biocontrol agent of seedling diseases incited by *R. solani* (AG-4).

Another antagonist that shows promise as an effective biocontrol agent against Rhizoctonia disease of potato, is the recently isolated obligate mycoparasite, *Verticillium biguttatum* (Jager and Velvis, 1985; Boogert, 1989; Morris *et al.*, 1992). First isolated from tuber-borne sclerotia in the Netherlands by Jager *et al.* (1979), this mycoparasite was shown to quickly establish itself on the host fungus by colonizing the sclerotia, and killing the moniliod cells (Velvis and Jager, 1983). The efficiency of *V. biguttatum* in reducing the incidence of stem and stolon canker, was demonstrated *in vitro* and in soils amended with *V. biguttatum* singly or in combination with the free living nitrogen-fixing bacteria, *Azotobacter chrococeum* Beijerinck (Meshram and Jager, 1983). Another potentially good mycoparasite of *R. solani* is the newly discovered fungus, *Stachybotrys elegans* (Pidopl.) W. Gams, (Benyagoub, 1993). *S. elegans* was able to attack sclerotia and hyphae of *R. solani* isolates belonging to all AGs. Viability of treated sclerotia with conidial suspension *S. elegans* significantly decreased compared with untreated controls. *S. elegans* was also shown to parasitize inter- and intracellularly sclerotial and hyphal cells thus rendering the host cells non viable (Benyagoub *et al.*, 1993). Recent results proved that the cell-wall degrading enzymes chitinases and glucanases are involved in the process of mycoparasitism (Tweddell *et al.*, 1994, 1995).

1.5 Hypothesis

Until now, no indigenous and potential biocontrol agent against *Rhizoctonia solani* (AG-3) on potato have been documented in Québec. Therefore, we made a collection of microorganisms isolated from field-grown sclerotia on potato grown in different regions of Québec. We hypothesized that the reduction in sclerotial viability of *R. solani* (AG-3) can be successfully achieved by the introduction of antagonists cohabiting the same niche as the pathogen. We focused our efforts exclusively on the destruction of sclerotia since they are considered the major source of inoculum in the soil.

1.6. Objectives

To accomplish this, our specific objectives were to:

(1) isolate and identify soil microorganisms from tuber-borne sclerotia of *Rhizoctonia solani*, (2) evaluate their effectiveness in rendering the hyphae and sclerotia non-viable under *in vitro* conditions, (3) study the mechanisms involve in the sclerotial germination inhibition of *R. solani* by selected antagonists, (4) study the ability of selected antagonists to produce lytic enzymes under induced conditions and evaluate their competitive saprophytic ability and (5) evaluate the efficiency of selected antagonists in controlling the development of Rhizoctonia disease under growth chamber conditions.

CHAPTER 2.

ISOLATION OF MICROORGANISMS FROM TUBER-BORNE SCLEROTIA AND THEIR EFFICACY TO INHIBIT SCLEROTIAL GERMINATION OR REDUCE VIABILITY OF *RHIZOCTONIA SOLANI*

2.1 Introduction

Sclerotia are the means by which a viable state is maintained by many fungi in the absence of a suitable host or of conditions favouring active growth (Coley-Smith and Cooke, 1971). The fungus *R. solani* (AG-3) survives in soil in the form of thick-walled mycelium associated with organic debris and in the form of sclerotia on potato tubers (Boosalis and Scharen, 1959). The dense cellular contents, pigmentation and rather impermeable walls of the sclerotial cells makes them resistant to adverse environmental conditions. They are considered as a main source of inoculum for Rhizoctonia disease of potato incited by fungus *R. solani* (AG-3) (Frank and Leach, 1980).

In addition to their importance as the primary source of inoculum, sclerotia accumulate relatively high concentrations of carbohydrates, fats, and proteins during growth which provide good nutritional niche for microorganisms in the soil. Consequently, soil organisms with potential value in biological control can be easily selected by their ability to colonize and degenerate living sclerotia (Naiki and Ui, 1972). These microorganisms can be isolated by direct hyphal isolation technique (Warcup, 1960), or using the baiting technique (Dos and Dhingra, 1982). Fungi frequently isolated from sclerotia of *Sclerotinia* spp., and *Rhizoctonia* spp., are *Trichoderma* spp., *Gliocladium* spp., *Coniohrium* spp., *and Penicillium* spp. (Zazzerini and Tosi, 1985; Schmiedeknecht, 1993). Of these, *Gliocladium* and *Trichoderma* killed a very high percentage of sclerotia and significantly reduced the intensity of the disease. To date, no work has been carried out to study the antagonistic activity of microorganisms isolated from sclerotia of *R. solani* (AG-3) in Québec.

Based on the above studies, we hypothesized that potential antagonists are present on sclerotia and should provide a large diversity of genera thereby increasing the chance of finding potential biocontrol isolates. Therefore, our objective was to isolate and identify the microorganisms present on sclerotia isolated from potato varieties grown in Québec soil. They were identified by means of spore morphological characteristics and tested for their potential as biocontrol agents. Species of *Alternaria, Cladosporium, Fusarium, Gliocladium, Penicillium, Pestalotia, Pythium, Trichoderma,* and *Verticillium* were most frequently isolated in this study. They were evaluated for their antagonism *in vitro* against *R. solani.*

2.2 Materials and Methods

2.2.1 Varieties of potato

Blackscurf infected potatoes (*Solanum tuberosum* L.) were collected from two different potato growing regions of Québec and from one region of Prince Edward Island (PEI) . Four potato varieties were screened: Atlantic and Norland (Baie comeau, Québec), Souri (L'Assomption, Québec) and Mouraska (PEI). Ten (10) potatoes heavily infested with sclerotia were selected from each variety per region except for the variety Norland, in which 13 infested potatoes were selected.

2.2.2 Isolation technique

A total of 100 sclerotia per potato variety per region were removed with a sterile scalpel. Sclerotia were placed in petri plates containing Potato Dextrose Agar (PDA), and Nutrient Agar (NA) to isolate fungi and bacteria, respectively (see Appendices AI a,b). To isolate *Trichoderma* and *Gliocladium*, the selective media of Elad *et al.* (1981c) was used (Appendices AII). To isolate inter- and intracellular colonizers, sclerotia from Mouraska and Atlantic were surface sterilized using 1% sodium hypochlorite solution, then crushed and placed on appropriate selective media (Appendices AI & AII). Irrespective of the type of culture media used, a total of six sclerotia per medium-plate were cultured. All plates were incubated at room temperature in complete darkness except for those containing selective media. Sclerotia cultured on *Trichoderma* and *Gliocladium* selective medium were incubated in a controlled incubator at 24^{0}C.

Daily observation and isolation of various microorganisms growing from the cultured ungerminated sclerotia was carried out. To ensure a pure culture of the microorganisms, three to four consecutive sub- culturing had to be carried out. The final sub-culturing of fungi was carried out on full-strength PDA plate without antibiotics and plates were incubated at 24^{0}C for 2 weeks. To obtain pure bacterial cultures, the final sub-culturing

was done on full strength Nutrient Agar without antibiotics at 37^{0}C for 24-48 hours. Isolated fungi were identified to the genus level (Table 2) according to their phialide and spore morphology (Raper and Thoms, 1948; Barron, 1972; Domsch, 1980; Nelson, 1983; Barnett and Hunter, 1987). All fungal isolates were stored on either oat-soil or PDA slants at 4^{0}C (Appendices AIII a,b), while the bacterial isolates were stored on Nutrient broth-medium in vials at -80^{0}C (Appendices AV).

2.2.3 Selected fungal isolates and culture conditions

Since it is practically impossible to test all isolated microorganisms for their pathogenicity on the sclerotia of *R. solani,* we limited the number of isolates to 50. These isolates were carefully chosen to ensure non-biased selection and to cover all identified genera isolated from all potato varieties (Table 3). They were tested for their ability to inhibit sclerotial germination.

The pathogen *R. solani* (AG-3) and all selected fungal isolates (Table 3) were maintained at 24^{0}C on PDA. For the pathogenicity test, laboratory-grown sclerotia of *R. solani* were produced on PDA in petri dishes at 24^{0}C and were harvested from the agar surface after 5-8 weeks using a sterile scalpel and placed in sterile petri dishes.

2.2.4 The viability test of *R. solani* sclerotia

Ten uniform-sized sclerotia of *R. solani* (3-5 mm diam.) were placed on the surface of PDA which was overgrown with mycelium of a one-week-old colony of one of the selected fungal isolates. The cultures were incubated at room temperature in complete darkness for 14 days. Untreated sclerotia served as the control. The viability of *R. solani* sclerotia was estimated after 14 days by placing them on water agar for 24 hours at room temperature, and hyphal germination was detected using a stereo microscope (x 40). Three replicate-plates and total of 30 sclerotia were used for each isolate. The sclerotia were classified according to the method of Velvis *et al.* (1988). The data were first subjected to log

transformation and treatment means were statistically analyzed using the student numan kuels (SNK) test (Steel and Torrie, 1980).

2.2.5 Assay procedure

The sclerotia were classified according to the number of outgrowing hyphae: 0, 1-5, 6-10, 11-25, and > 25 hyphae. The percent sclerotial viability (%SV) was calculated according to the formula of Velvis *et al.* (1988): %SV = [(1-5)x1 + (6-10)x2 + (11-25)x3 + (>25)x4]/4 x 100/TS (TS = total sclerotia). The maximum value of V is 100.

2.3 Results

2.3.1 Collection of microorganisms from sclerotia

A total of 36 bacterial isolates were obtained from three varieties of potatoes: Nine from Atlantic, 11 from Norland, and 16 from Souri. Forty-four (44%) of them were identified as Gram-positive or Gram-negative (Table 1). A total of 259 fungi were isolated from tuber-borne sclerotia, among which 7 fungi were isolated from sclerotia that were surface sterilized and crushed. Ninety-three (93) fungal isolates originated from Atlantic, 95 from Souri, 61 from Norland, 7 from Mouraska, and 3 from an unknown variety. Out of these, 147 isolates grew on the *Trichoderma* and *Gliocladium* selective medium. Eighty-six (86%) of the total isolates were identified to the genus level (Table 2). The most common genera were: *Penicillium* spp. (44%), *Gliocladium* spp. (22%), *Trichoderma* spp. (7%), *Fusarium* spp. (5.8%), *Pythium* spp. (4%), and some others namely *Zygorrhyncus* spp. (1.15%), *Cladosporium* spp. (0.78%), *Verticillium* spp. (0.78%), *Alternaria* spp. (0.39%), and *Pestalotia* spp. (0.39%). The total percentages were calculated from the total number of isolates of a given genus divided by the total number of isolates found; multiplied by one hundred.

2.3.2 Effect of fungal isolates on *R. solani*

Of the 50 fungal isolates tested in dual cultures, most isolates proved to be active antagonists and some strongly inhibited the germination of sclerotia (Table 3). Unless stated otherwise, the selected fungal isolates which proved to be active against *R. solani* sclerotia will be referred to hereafter as antagonists.

Microscopic examination of individual sclerotia showed that the antagonists grew profusely and completely colonized the sclerotia. In some instances, due to heavy colonization by the antagonists, the sclerotia lost their color and disintegrated. More than 50% of the isolates caused a significant reduction in sclerotial viability (Table 3). Among

the 50 isolates tested, fifteen completely inhibited sclerotial germination, rendering them non-viable (%V= 0). These belonged to *Trichoderma* (7 isolates), *Gliocladium* (4 isolates), *Pestalotia* (1 isolate), *Pythium* (1 isolate), *Fusarium* (1 isolate) and *Verticillium* (1 isolate) species. Nineteen other isolates did not completely kill the sclerotia but significantly reduced their viability by 36% - 97%. The majority of these isolates belonged to *Gliocladium* (7 isolates), and *Penicillium* (5 isolates) followed by *Fusarium* (3 isolates), and unidentified (4 isolates). The rest of the isolates including some that were not identified had no effect on sclerotial viability. In most cases, the pathogenicity of isolates within the genus varied significantly. For instance, seven isolates of *Trichoderma* (F138, F218, F137, F250, F258, F233, F231) killed 100% of the sclerotia, whereas the other isolates (F236, F230, F256, F148) did not affect sclerotium germination. Similarly, 4 isolates of *Gliocladium* (F251, F6, F76, F139) killed all the sclerotia whereas the rest varied in their pathogenicity (Table 3).

Table 1: Bacteria isolated from tuber-borne sclerotia of three different potato varieties*

Bacteria designation number					
Potato varieties					
Souri	Identification	Atlantic	Identification	Norland	Identification
B2SO(4)NA+ sc	NI	B6AT(3)NA+sc	NI	B17NO(1)NA+sc	NI
B3SO(2)NA+sc	G- cocci	B8AT(1)NA+sc	G-cocci	B18NO(2)NA+sc	NI
B5SO(2)NA+sc	G+ rod	B10AT(4)NA+sc	NI	B20NO(1)NA+sc	NI
B7SO(1)NA+sc	NI	B11AT(6)NA+sc	NI	B23NO(6)NA+sc	G+ rod
B36SO(10)NA+sc	G+ rod	B12AT(4)NA+sc	NI	B4NO(6)NA+sc	G+ rod
B38SO(5)NA+sc	G- cocci	B15AT(2)NA+sc	NI	B24NO(4)NA+sc	NI
B39SO(6)NA+sc	NI	B16AT(8)NA+sc	G+rod	B25NO(6)NA+sc	G+ rod
B1SO(6)NA+sc	NI	B19AT(7)NA+sc	NI	B26NO(8)NA+sc	NI
B40SO(6)NA+sc	G+ rod	B21AT(10)NA+sc	NI	B27NO(7)NA+sc	NI
B42SO(4)NA+sc	NI			B31NO(5)NA+sc	NI
B46SO(7)NA+sc	G+ rod			B32NO(7)NA+sc	G+ rod
B47SO(7)NA+sc	G+ rod				
B48SO(7)NA+sc	NI				
B49SO(4)NA+sc	G+ rod				
B50SO(5)NA+sc	G+ rod				
B51SO(10)NA+sc	G+ rod				

* Bacterial isolation were carried out at room temperature.

NA = Nutrient agar with appropriate antibiotic (cychlohexamide 100 mg /L of medium).

B = Designated number of bacteria ; Sc = Sclerotia ; SO = Souri, AT = Atlantic, NO = Norland.

NI = Not Identified; G- = Gram-negative, G+ = Gram-positive.

Table 2: Fungal isolates recovered from tuber-borne sclerotia and identified to the genus level*

Isolate No.	Genus	Isolate No.	Genus
F18 AT (6) PDA+ sc	?	F45 NO (6) PDA+ sc	*Fusarium*
F25 AT (4) PDA+ sc	?	F54 SO (3) T&G sc	*Fusarium*
F26 NO (3) PDA+ sc	?	F55 SO (3) T&G sc	*Fusarium*
F28 NO (10) PDA+sc	?	F96 MO (7) PDA+StscC	*Fusarium*
F29 NO (9) PDA+ sc	?	F146 SO (5) PDA+ sc	*Fusarium*
F31 NO (7) PDA+ sc	?	F149 SO (9) PDA+sc	*Fusarium*
F36 NO (9) PDA+ sc	?	F150 SO (6) PDA+ sc	*Fusarium*
F43 NO (9) PDA+ sc	?	F216 SO (5) PDA+ sc	*Fusarium*
F44 NO (7) PDA+ sc	?	F232 SO (8) PDA+ sc	*Fusarium*
F46 NO (7) PDA+ sc	?	F240 SO (6) PDA+ sc	*Fusarium*
F52 SO (2) T&G sc	?	F160 AT (5) PDA+ sc	*Gliocladium*
F62 AT (2) PDA+ sc	?	F162 AT (2) PDA+ sc	*Gliocladium*
F80 AT (1) PDA+ Stsc	?	F166 AT (10) PDA+ sc	*Gliocladium*
F86 AT (3) PDA+ sc	?	F158 AT (2) T&G sc	*Gliocladium*
F87 AT (6) PDA+ stsc	?	F168 AT (2) T&G sc	*Gliocladium*
F97 NO (6) PDA+ sc	?	F175 AT (2) T&G sc	*Gliocladium*
F102 NO (8) T&G sc	?	F177 AT (8) T&G sc	*Gliocladium*
F112 SO (3) T&G sc	?	F35 NO (3) PDA+ sc	*Gliocladium*
F141 SO (10) PDA+ sc	?	F7 SO (3) PDA+ sc	*Gliocladium*
F191 AT (7) T&G sc	?	F19 AT (5) PDA+ sc	*Gliocladium*
F201 SO (10) PDA+ sc	?	F33 AT (10) PDA+ sc	*Gliocladium*
F208 NO (6) PDA+ sc	?	F39 NO (3) T&G sc	*Gliocladium*
F235 AT (10) T&G sc	?	F56 SO (2) T&G sc	*Gliocladium*
F246 NO (6) PDA+ sc	?	F64 AT (8) PDA+ sc	*Gliocladium*
F257 NO (11) T&G sc	?	F68 AT (10) T&G sc	*Gliocladium*
F258 NO (10) T&G sc	?	F74 AT (9) PDA+ sc	*Gliocladium*
F67 NO (10) PDA+ sc	?	F76 SO (3) T&G sc	*Gliocladium*
F140 SO (8) PDA+ sc	?	F78 NO (4) T&G sc	*Gliocladium*
F65 PDA+ sc	?	F81 NO (7) PDA+ sc	*Gliocladium*
F103 NO (4) T&G sc	?	F83 NO (7) PDA+ sc	*Gliocladium*
F113 SO (1) T&G sc	?	F84 SO (3) PDA+ sc	*Gliocladium*
F171 AT (2) T&G sc	?	F88 NO (4) T&G sc	*Gliocladium*
F204 AT (8) T&G sc	?	F89 SO (1) T&G sc	*Gliocladium*
F220 AT (7) T&G sc	?	F90 NO (4) T&G sc	*Gliocladium*
F98 AT (2) T&G sc	?	F108 AT (7) T&G sc	*Gliocladium*
F153 SO (6) PDA+ sc	?	F118 AT (2) T&G sc	*Gliocladium*
F253 SO (6) PDA+ sc	*Alternaria*	F133 AT (3) PDA+ sc	*Gliocladium*
F136 SO (8) PDA+ sc	*Cladosporium*	F130 AT (8) T&G sc	*Gliocladium*
F140 SO (8) PDA+ sc	*Cladosporium*	F132 NO (8) T&G sc	*Gliocladium*
F239 SO (6) T&G sc	*Fusarium*	F134 AT (5) PDA+ sc	*Gliocladium*
F4 AT (5) PDA+ sc	*Fusarium*	F139 SO (8) PDA+ sc	*Gliocladium*
F8 SO (1) PDA+ sc	*Fusarium*	F11 AT (2) PDA+ sc	*Penicillium*
F17 SO (7) PDA+ sc	*Fusarium*	F12 AT (2) PDA+ sc	*Penicillium*
F32 SO (3) PDA+ sc	*Fusarium*	F13 AT (5) PDA+ sc	*Penicillium*

F167 AT (5) T&G sc	*Gliocladium*	F10 AT (5) PDA+ sc	*Penicillium*
F170 AT (8) T&G sc	*Gliocladium*	F15 AT (6) PDA+ sc	*Penicillium*
F173 AT (5) T&G sc	*Gliocladium*	F16 SO (2) PDA+ sc	*Penicillium*
F179 SO (5) T&G sc	*Gliocladium*	F20 NO (1) PDA+ sc	*Penicillium*
F180 AT (7) T&G sc	*Gliocladium*	F21 AT (4) PDA+ sc	*Penicillium*
F183 AT (2) T&G sc	*Gliocladium*	F22 SO (2) PDA+ sc	*Penicillium*
F188 SO (1) T&G sc	*Gliocladium*	F23 NO (1) PDA+ sc	*Penicillium*
F189 NO (4) T&G sc	*Gliocladium*	F24 AT (7) PDA+ sc	*Penicillium*
F190 SO (1) T&G sc	*Gliocladium*	F30 NO (7) PDA+ sc	*Penicillium*
F221 SO (5) T&G sc	*Gliocladium*	F40 NO (3) T&G sc	*Penicillium*
F225 SO (1) T&G sc	*Gliocladium*	F41 NO (3) T&G sc	*Penicillium*
F227 AT (3) T&G sc	*Gliocladium*	F47 NO (7) PDA+ sc	*Penicillium*
F228 SO (7) T&G sc	*Gliocladium*	F49 SO (2) T&G sc	*Penicillium*
F237 AT (2) T&G sc	*Gliocladium*	F51 AT (5) PDA+StscC	*Penicillium*
F238 SO (5) T&G sc	*Gliocladium*	F53 AT (10) T&G sc	*Penicillium*
F243 SO (4) T&G sc	*Gliocladium*	F58 AT (10) PDA+ sc	*Penicillium*
F248 SO (6) T&G sc	*Gliocladium*	F60 AT (9) PDA+ sc	*Penicillium*
F249 SO (6) T&G sc	*Gliocladium*	F61 MO (4) PDA+StscC	*Penicillium*
F251 SO (9) T&G sc	*Gliocladium*	F63 AT (6) PDA+ sc	*Penicillium*
F252 AT (7) T&G sc	*Gliocladium*	F66 NO (4) PDA+ sc	*Penicillium*
F254 SO (10) PDA+ sc	*Gliocladium*	F69 AT (3) PDA+ sc	*Penicillium*
F259 SO (6) T&G sc	*Gliocladium*	F70 AT (10) T&G sc	*Penicillium*
F109 AT (7) T&G sc	*Gliocladium*	F71 AT (10) T&G sc	*Penicillium*
F110 NO (4) T&G sc	*Gliocladium*	F72 SO (3) T & G sc	*Penicillium*
F128 AT (7) T&G sc	*Gliocladium*	F73 AT (10) T&G sc	*Penicillium*
F6 AT (5) PDA+ sc	*Gliocladium*	F75 SO (2) T&G sc	*Penicillium*
F223 SO (7) T&G sc	*Gliolcadium*	F77 NO (3) T&G sc	*Penicillium*
F2 AT (1) PDA+ sc	*Pestalotia*	F85 SO (3) PDA+ sc	*Penicillium*
F1 AT (9) PDA+ sc	*Penicillium*	F91 SO (1) T&G sc	*Penicillium*
F3 AT (9) PDA+ sc	*Penicillium*	F92 SO (1) T&G sc	*Penicillium*
F5 AT (4) PDA+ sc	*Penicillium*	F93 NO (4) T&G sc	*Penicillium*
F9 AT (5) PDA+ sc	*Penicillium*	F94 SO (1) T&G sc	*Penicillium*
F101 NO (4) T&G sc	*Penicillium*	F99 AT (1) PDA+ sc	*Penicillium*
F104 AT (2) T&G sc	*Penicillium*	F100 NO (8) T&G sc	*Penicillium*
F116 AT (2) T&G sc	*Penicillium*	F106 AT (2) T&G sc	*Penicillium*
F117 AT (5) T&G sc	*Penicillium*	F107 AT (7) T&G sc	*Penicillium*
F119 AT (8) T&G sc	*Penicillium*	F114 NO (4) T&G sc	*Penicillium*
F120 AT (2) T&G sc	*Penicillium*	F115 MO (1) PDA+ sc	*Penicillium*
F121 AT (5) T&G sc	*Penicillium*	F195 AT (7) T&G sc	*Penicillium*
F122 SO (3) T&G sc	*Penicillium*	F196 AT (5) T&G sc	*Penicillium*
F123 NO (4) T&G sc	*Penicillium*	F197 AT (8) T&G sc	*Penicillium*
F124 NO (8) T&G sc	*Penicillium*	F198 NO (8) T&G sc	*Penicillium*
F127 AT (7) T&G sc	*Penicillium*	F199 NO (8) T&G sc	*Penicillium*
F129 SO (1) T&G sc	*Penicillium*	F200 AT (5) T&G sc	*Penicillium*
F131 NO (8) T&G sc	*Penicillium*	F226 NO (6) PDA+ sc	*penicillium*
F137 SO (8) PDA+ sc	*Penicillium*	F229 SO (7) T&G sc	*Penicillium*
F142 SO (7) T&G sc	*Penicillium*	F241 SO (4) PDA+ sc	*Penicillium*
F144 SO (7) T&G sc	*Penicillium*	F247 SO (6) PDA+ sc	*Penicillium*
F145 SO (7) T&G sc	*Penicillium*	F260 SO (1) T&G sc	*Penicillium*

F152 SO (5) T&G sc	*Penicillium*	F261 SO (6) PDA+ sc	*Penicillium*
F154 SO (6) PDA+ sc	*Penicillium*	F205 SO (5) PDA+ sc	*Penicillium*
F155 AT (10) T&G sc	*Penicillium*	F48 SO (2) T&G sc	*Prenicillium*
F156 AT (2) T&G sc	*Penicillium*	F143 SO (7) T&G sc	*Pythium*
F157 SO (3) T&G sc	*Penicillium*	F95 MO (4) PDA+StscC	*Pythium*
F161 AT (10) T&G sc	*Penicillium*	F147 SO (4) PDA+ sc	*Pythium*
F163 NO (4) T&G sc	*Penicillium*	F217 NO (11) T&G sc	*Pythium*
F164 NO (8) T&G sc	*Penicillium*	F218 NO (11) T&G sc	*Pythium*
F165 NO (8) T&G sc	*Penicillium*	F50 MO (7) PDA+StscC	*Pythium*
F169 AT (5) T&G sc	*Penicillium*	F57 SO (2) T&G sc	*Pythium*
F172 AT (5) T&G sc	*Penicillium*	F79 MO (1) PDA+StscC	*Pythium*
F174 AT (5) T&G sc	*Penicillium*	F125 SO (5) T&G sc	*Pythium*
F176 AT (5) T&G sc	*Penicillium*	F126 SO (5) T&G sc	*Pythium*
F178 NO (6) PDA+ sc	*Penicillium*	F135 NO (4) T&G sc	*Trichoderma*
F181 AT (7) T&G sc	*Penicillium*	F138 SO (8) PDA+ sc	*Trichoderma*
F182 AT (7) T&G sc	*Penicillium*	F148 SO (9) PDA+ sc	*Trichoderma*
F184 AT (7) T&G sc	*Penicillium*	F151 AT (8) T&G sc	*Trichoderma*
F185 AT (7) T&G sc	*Penicillium*	F159 NO (3) T&G sc	*Trichoderma*
F186 AT (7) T&G sc	*Penicillium*	F230 SO (8) T&G sc	*Trichoderma*
F187 NO (4) T&G sc	*Penicillium*	F231 NO (12) T&G sc	*Trichoderma*
F192 SO (10) PDA+ sc	*Penicillium*	F233 SO (8) T&G sc	*Trichoderma*
F193 SO (10) PDA+ sc	*Penicillium*	F234 SO (6) T&G sc	*Trichoderma*
F194 SO (8) PDA+ sc	*Penicillium*	F236 SO (8) T&G sc	*Trichoderma*
F202 SO (5) PDA+ sc	*Penicillium*	F242 NO (11) T&G sc	*Trichoderma*
F203 SO (6) PDA+ sc	*Penicillium*	F244 SO (8) PDA+ sc	*Trichoderma*
F206 SO (1) T&G sc	*Penicillium*	F245 NO (12) T&G sc	*Trichoderma*
F207 AT (8) T&G sc	*Penicillium*	F250 NO (12) T&G sc	*Trichoderma*
F209 SO (8) T&G sc	*Penicillium*	F255 SO (8) PDA+ sc	*Trichoderma*
F210 AT (8) T&G sc	*Penicillium*	F256 NO (12) T&G sc	*Trichoderma*
F211 SO (5) PDA+ sc	*Penicillium*	F42 NO (9) PDA+ sc	*Trichoderma*
F212 SO (10) PDA+ sc	*Penicillium*	F59 MO (2) PDA+StscC	*Verticillium*
F213 SO (5) PDA+ sc	*Penicillium*	F14 SO (3) PDA+ sc	*Verticillium*
F214 AT (8) T&G sc	*Penicillium*	F27 NO (8) PDA+ sc	*Zygorhynchus*
F215 SO (5) PDA+ sc	*Penicillium*	F105 SO (8) PDA+sc	*Zygorhynchus*
F219 AT (7) T&G sc	*Penicillium*	F37	*Zygorhynchus*
F222 SO (4) T&G sc	*Penicillium*		
F224 SO (4) T&G sc	*Penicillium*		

* Sclerotia were incubated at room temperature.

F = Fungal number proceeding; No. in bracket = Designated potato; MO = Mouraska; AT = Atlantic; NO = Norland; SO = Souri; Sc = Sclerotia; StscC = Sterile crushed sclerotia; Stsc = Sterile not crushed sclerotia. PDA+ = Potato Dextrose Agar with antibiotics (Chloramphenicol and Novobiocin 100 mg /L of medium); Non identified isolates indicated as (?); T&G = *Trichoderma* and *Gliocladium* medium.

Table 3: Antagonistic activity of fungal isolates against sclerotium viability of *Rhizoctonia solani**

Isolate No.	Genus	Viable sclerotia (%V)	Isolate No.	Genus	Viable sclerotia (%V)
F217NO	*Pythium*	100a**	F7	*Gliocladium*	23def
F236SO	*Trichoderma*	100a	F158AT	*Gliocladium*	21def
F230SO	*Trichoderma*	100a	F32SO	?	20def
F102NO	?	100a	F67NO	?	20def
F256NO	*Trichoderma*	100a	F8SO	*Fusarium*	12def
F202NO	*Penicillium*	100a	F11NO	*Penicillium*	12def
F48SO	*Pythum*	100a	F20NO	*Penicillium*	7ef
F56SO	*Gliocladium*	100a	F216SO	*Fusarium*	5f
F57SO	*Pythium*	100a	F130AT	*Gliocladium*	5f
F24AT	?	100a	F189NO	*Gliocladium*	3f
F150SO	*Fusarium*	100a	F231NO	*Trichoderma*	0f
F225SO	?	100a	F233SO	*Trichoderma*	0f
F198NO	*Penicillium*	100a	F251SO	*Gliocladium*	0f
F148SO	*Trichoderma*	100a	F4AT	*Fusarium*	0f
F39NO	*Gliocladium*	93a	F138SO	*Trichoderma*	0f
F180SO	*Gliocladium*	71ab	F218NO	*Trichoderma*	0f
F108AT	*Gliocladium*	64b	F6AT	*Gliocladium*	0f
F88NO	*Gliocladium*	53bc	F147SO	*Pythium*	0f
F78NO	?	41cd	F250NO	*Trichoderma*	0f
F146SO	*Fusarium*	36dce	F76SO	*Gliocladium*	0f
F93NO	*Penicillium*	35cde	F137SO	*Trichoderma*	0f
F132NO	*Gliocladium*	31cdef	F258NO	*Trichoderma*	0f
F5AT	*Penicillium*	28cdef	F139SO	*Gliocladium*	0f
F65	?	28cdef	F2AT	*Pestalotia*	0f
F53AT	*Penicillium*	23def	F42NO	*Verticillium*	0f
			Control	*R. solani*	100a

* Experiment was carried out at room temperature for 14 days of incubation. Three replicate plates were used for each isolate and 10 sclerotia were seeded per replicate.

** Each number represents the mean of 30 sclerotia. Data were log transformed and mean values followed by the same letter within the column do not vary significantly from each other according to student numan kuels (SNK) test ($P \geq 0.05$).

? = Unidentified isolate.

%SV = [(1-5)x1 + (6-10)x2 + (11-25)x3 + (>25)x4]/4 x 100/TS (TS = Total sclerotia).

2.4 Discussion

The results of this study represents to our knowledge the first report on the diversity of fungal populations naturally occuring on tuber-borne sclerotia isolated from Québec soils. The hyphal isolation method described by Warcup (1966) proved to be effective for studying the ecology of organisms associated with sclerotia. Our collection of microorganisms contained different fungal genera which were previously reported by others as antagonistic microorganisms including some mycoparasites which drastically affected the viability of *R. solani* sclerotia (Naiki, 1986). These fungi include *Trichoderma* spp., *Gliocladium* spp., and *Verticillium* spp. (Naiki and Ui, 1972).

Several methods assessing sclerotial germinability of antagonists have been described (Dos and Dhingra, 1980; Naiki, 1986), among which the sclerotial viability index method (Velvis *et al.*, 1988) has been successfully used to assess pathogenicity and aggressiveness of antagonists. Using this technique, we were able to show that most of the fungi exploited sclerotial tissue of *R. solani* and significantly decreased their germinability. One interesting finding of this collection of fungi is the similarity of fungi isolated from sclerotia originating from different potato cultivars, suggesting that potential antagonists might have the same efficiency in different potato fields.

Among the organisms tested, *Trichoderma* spp., *Gliocladium* spp., *Penicillium* spp., *Pestalotia* spp., and *Fusarium* spp. were more virulent than others. They were able to decompose and destroy the sclerotial cells and consequently, are effective mycoparasites. Mycoparasitic fungi require an intimate association between host and parasite before infection can be initiated. Such parasites may penetrate hosts with small pegs, coiling around or growing adjacent to the host mycelium (Kuter, 1984; Elad *et al.*, 1987). *Trichoderma* and *Gliocladium* species have been recorded as parasites of *R. solani* hyphae (Tu and Vaartaja 1981; Elad *et al.*, 1983b; Papavizas, 1985). There was a great reduction in the viability of *R. solani* hyphae when sclerotia were introduced to the PDA culture plates of *Trichoderma* or *Gliocladium*. In their presence, however, sclerotia failed to germinate.

They parasitized both mycelium and sclerotia of the host fungus and sporulated profusely. Lytic enzymes which may partially digest the host cell walls and make penetration easier, were probably responsible for the reduction in sclerotial viability. Many studies have demonstrated that destruction of sclerotial cells of *Rhizoctonia* and *Sclerotinia* by selective antagonists has been attributed to the action of ß-1,3-glucanases and chitinases (Tu, 1980; Dos and Dhingra, 1982; Elad *et al.*, 1983a; Papavizas, 1985; Zazzerini and Tosi, 1985), thereby causing soft rot, and penetration. Benhamou and Chet (1996) reported that ß-1,3-glucanases is the key enzyme involved in the destruction of sclerotial cell wall of *R. solani* and other pathogenic fungi by species of *Trichoderma* and *Gliocladium* (Tu 1980; Manocha, 1990) who had tested several Trichoderma isolates for their varied aggessiveness against sclerotia of *R. solani* and *Sclerotinia sclerotiorum* (Lib.) de Bary, respectively.

Although many isolates in this study manifested a killing potential near to that of those virulant antagonists, marked differences were also observed among isolates belonging to the same genus. These observations suggest that variation in the susceptibility of sclerotia may be attributed to difference in parasitic ability by the same genus. Our results are in agreement with those of Naiki (1986) and Dos and Dhingra (1980).

Since certain isolates of *Trichoderma*, *Fusarium*, *Pestalotia*, *Gliocladium*, *Pythium*, and *Verticillium* proved pathogenic to *R. solani* AG-3, these fungi will be further tested for their pathogenicity to *R. solani* on beet seedlings in order to screen for the best biocontrol agent.

CHAPTER 3.

THE EFFECT OF SELECTED ANTAGONISTS ON SEVERITY OF DISEASE CAUSED BY *RHIZOCTONIA SOLANI* ON TABLE BEET SEEDLINGS IN GREENHOUSE AND FIELD SOILS

3.1 Introduction

Fungal isolates which demonstrated great ability to colonize and kill sclerotia of *R. solani* (see Table 3) were retained for further testing. In the preceding chapter, we screened numerous fungal isolates of *Trichoderma* spp., *Gliocladium* spp., *Penicillium* spp., *Pestalotia* spp., *Pythium* spp., and *Fusarium* spp. for their ability to inhibit sclerotial germination of *Rhizoctonia solani.* In this section, we will investigate the use of these antagonists to control *R. solani* on seedlings under greenhouse and field soil.

Saprophytic and pathogenic activity of *R. solani* can be assayed using lettuce, carrot or table beet seeds (Papavizas *et al.*, 1975; Grisham and Anderson, 1985). In this study, initial experiments were conducted using seeds including carrot, lettuce, table beets and radish to evaluate the disease severity of our *R. solani* isolate (AG-3). Table beet seeds were the most susceptible to infection by AG-3 and therefore, they were used as the "host" seed to observe the pathogenic activity of *R. solani* grown in soils amended with the antagonists.

The purpose of this study was to evaluate the previously screened antagonists for their ability to protect Table beets against *R. solani* under greenhouse and using field soils, since the anticipated results from these experiments will more or less reflect natural conditions.

3.2 Materials and Methods

3.2.1 Selected antagonists

A total of 24 antagonists (Table 4) selected from the previous experiment were tested against *R. solani* on beet seedlings. The selection of these antagonists was based on their ability to significantly reduce or completely inhibit sclerotial germinability (Table 3).

3.2.2 Culture conditions

All antagonists were maintained on PDA medium except for isolate F216 (*Fusarium* spp.) which was grown on Synthetic Nutrient-poor Agar (SNA) media (Neirnberg, 1976; Appendices BI). All plates were incubated at 24^{0}C for 8 days. Sclerotia of *R. solani* were produced on PDA in petri-dishes for 5-8 weeks at room temperature. Uniform sized sclerotia (3-5 mm diam.) were cut from the agar surface layer using a sterile scalpel.

3.2.3 The effect of antagonists against *R. solani* on beet seedlings

(1) Greenhouse soil:

The composition of the green- house soil was 1/3 black earth, 1/3 peatmoss, and 1/3 sandy loam. The soil was sieved and autoclaved on 3 consecutive days for 45 minutes at 121^{0}C. Portions (10g) of the soil per replicate-plate (60 x 20 mm diam. petri plates) were amended with spore suspension of each of the 24 antagonists. Triplicate plates were used for each antagonist. Spores were gently scraped from the surface of the plate using a sterilized glass rod, suspended in sterile water, and added at a rate of 1 x 10^6 spores/10g of soil. After one week of incubation at room temperature, 10 laboratory-grown sclerotia were buried in the inoculated soil for one week at room temperature. Each petri plate recieved 10 beet seeds which were previously surface-sterilized with 20% sodium hypochlorite solution. All plates were incubated at room temperature. Soil treated with sterile double-distilled water and containing *R. solani* sclerotia alone served as controls. The effect of *R. solani* on table beet seedlings was assayed using the modified method described by Papavizas and Davey

(1962). After 5 days of incubation, the seedlings were recovered on a kitchen sieve, washed for 5 minutes in running tap water, transferred to petri plates (5 seedlings per plate) containing 10 ml of water agar (2% agar) and incubated for 48 hours. Seedlings grown in amended and non-amended soils, were examined for damping-off and rot symptoms caused by *R. solani.* The seedling's tap root length and the number of secondary roots were estimated on table beet seedlings grown in greenhouse soil only. Data were analyzed via a 1-way anova by the Duncan's multiple range test. Potential antagonists which demonstrated high activity were retained and the same experiment was repeated using field soil.

2) Field soil:

Sandy loam soil was collected from the Horticulture Farm in Macdonald Campus, Ste-Anne-de-Bellevue, Québec. Soil samples were collected at a 15 cm depth at different locations. The soil was sieved and thoroughly mixed. The experiment was conducted in petri plates (60 x 20 mm), each containing 20g of sieved field soil. Each treatment was carried out in four replicates. Each replicate plate was amended with spores of each of these 6 selected antagonists (F2, F11, F132, F158, F216, and F258) and added at a rate of $1x10^6$ spores/20g of soil. One week after incubation at room temperature, 10 uniform-sized sclerotia were buried in each plate and incubated for an additional one week. Non-amended soil infested with *R. solani* sclerotia served as a control. Ten (10) table beet seeds were then sown in each plate. The effect of the above six antagonists on *R. solani* survival was measured by determining % seed germination after 6 days of incubation. The data were subjected to log transformation and statistically analyzed via a 1-way anova by the Least significant difference (LSD) test.

3.3 Results

3.3.1 Effect of antagonists on disease severity in Table beet seedlings grown in greenhouse soil infested with *R. solani*

Of the 24 antagonists selected, only F2, F158, F216, and F258 were effective in increasing the number of secondary roots of table beet seedlings compared with the control (Table 4). F11 and F132 were generally less effective than the control, others had little or no effect. In the control treatment, *R. solani* significantly reduced the number of secondary roots. There were no significant differences observed in the root length of Table beet seedlings planted in soil amended with the antagonists and inoculated with *R. solani* compared with the control treatment. Amended soils with any one of the following six antagonists F2, F11, F132, F158, F216, and F258 slightly increased root length as compared with the control treatment. The above antagonists were further tested *in vivo* conditions using field soil.

3.3.2 Survival of antagonists in field soil

Although there was a slight decrease in population numbers of all antagonists except for F2, after 2 and 4 days of incubation, the antagonists were present at detectable population levels in non-treated and treated soils with sclerotia (Fig.1). Before the introduction of sclerotia to the soil at 0 day survival of antagonists of F2, F11, F132, F158, and F258 were determined to be $3.1x10^4$, $3.1x10^4$, $2.7x10^4$, $4.2x10^4$, and $3.2x10^4$, respectively. After the introduction of sclerotia to the soil at 6 days and further incubated for another 7 days, the population of antagonists of F2, F11, F132, F158, and F258 were estimated to be $4.7x10^4$, $4.5x10^4$, $4.2x10^4$, $4.1x10^4$, and $3.8x10^4$, respectively. There was a slight difference in survival among the 5 antagonists. Several factors, including experimental errors may have influenced in population numbers of antagonists before addition of sclerotia at 0-6 days of incubation. After this period there were no significant differences in population numbers of each antagonist at 6-14 days of incubation.

3.3.3 Effect of antagonists on beet seeds germination in field soil infested with *R. solani*

The ability of isolates F2, F158, F258, F11, and F132 to increase germination of table beet seeds in field soil was examined (Table 5). Applying the spores of each antagonist as a suspension to the soil that was incubated for one week prior to soil inoculation with sclerotia, significantly increased % seed germination (Table 5). In field soil infested with *R. solani* only (control), the germination rate remained at 30%, whereas, the germination rates in soils amended with F2, F158 and F258 increased 2- fold.

Table 4: Effect of antagonists on colonization of table beet seedling roots*

Antagonists	No. of secondary roots**	Tap root length (cm)**
F2AT	86a***	40a
F158 AT	76ab	37ab
F258NO	64abc	32abc
F216SO	64abc	34abc
F67NO	59abc	28abc
F231NO	44bcd	32abc
F138SO	524bcd	31abc
F5AT	52bcd	31abc
F147SO	54bcd	31abc
F148SO	54bcd	28abc
F130AT	53bcd	28abc
F20NO	47bcd	26abc
F234SO	49bcd	31abc
F4AT	55bcd	26abc
F146SO	424dc	31abc
F132NO	40dc	38ab
F11AT	38dc	36abc
F139SO	36dc	26abc
F76SO	42dc	26abc
F7SO	38dc	35abc
F137SO	41dc	24bc
F8SO	32dc	26abc
F218NO	30d	21c
F42NO	0e	0d
Control (AG-3)	43dc	27abc

* Experiment was conducted using greenhouse soils amended with inoculum of each antagonist ($1x10^6$ spores/10g of soil) and ten sclerotia of *R. solani*. Three replicates per treatment and ten beet seeds per replicate were planted.

** Beet seeds were recovered after 5 days of incubation at 24^0C and examined for the effect of *R. solani* on number of secondary roots produced and tap root length. Values are means of three replicates.

*** Mean values followed by the same letters within the same column do not vary significantly from each other according to Duncan's multiple range test ($P \geq 0.05$).

Figure legend

Figure 1. Survival of antagonists before and after addition of *R. solani* sclerotia in the field soils. Mean values of three replicates presented as a line graph. Original spore suspension was $1x10^6$/ml; propagule density assessed by dilution plating (dilution rate was 10^{-2}). Plates were incubated at room temperature for 3 days. Formula for colony-forming unit (c.f.u.)/ml is = No. of colonies x 1/aliquot x 1/dilution factor (aliquot was 0.5ml).

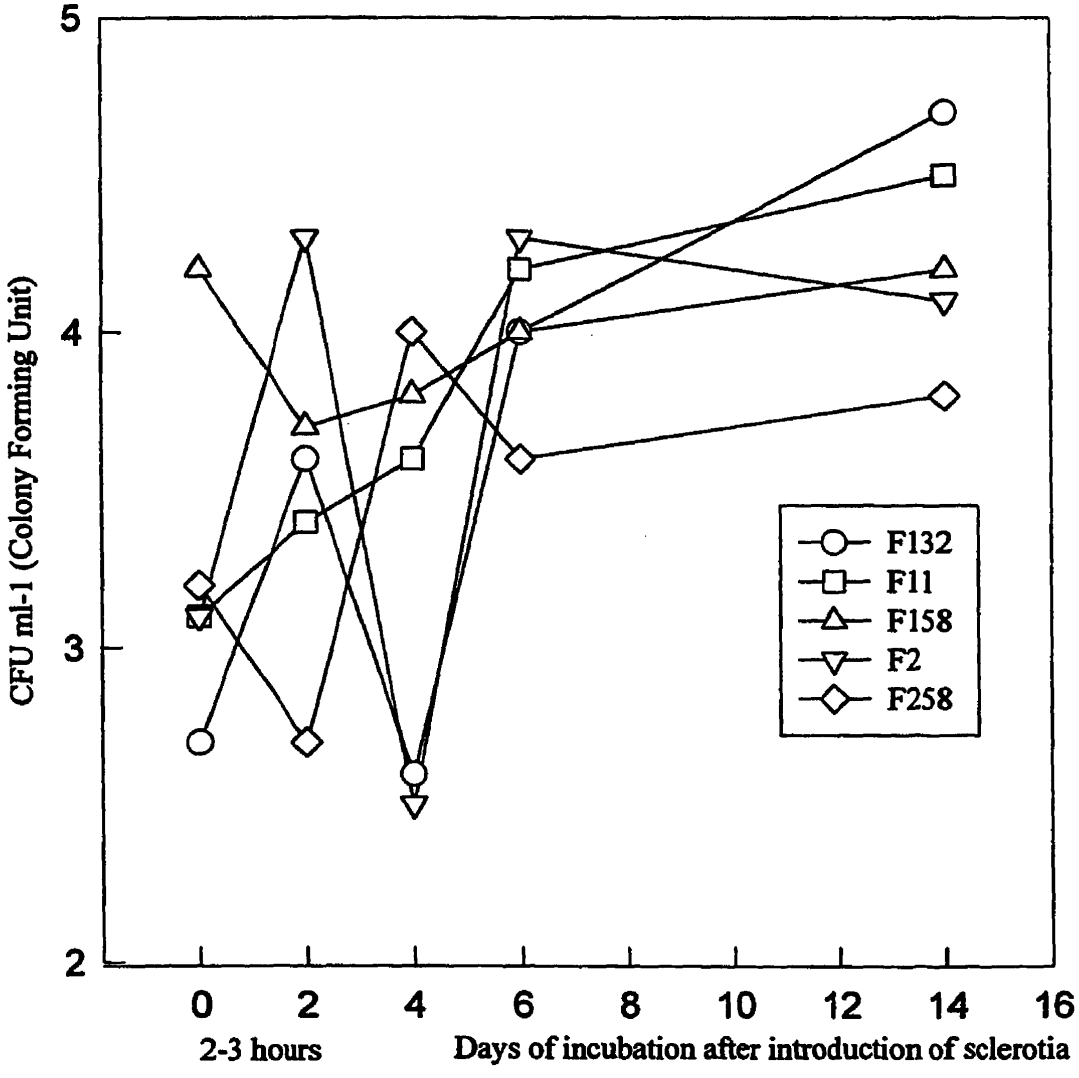

5
4
3
2
CFU ml-1 (Colony Forming Unit)
0
2
4
6
8
10
12
14
16
2-3 hours
Days of incubation after introduction of sclerotia
F132
F11
F158
F2
F258

Table 5: Effect of antagonists on % germination of table beet seeds grown in natural soils infested with *R. solani* (AG-3)*

Antagonists	% of seed germination
F258 + *R. solani*	67a**
F2 + *R. solani*	55ab
F158 + *R. solani*	60ab
F132 + *R. solani*	42abc
F11 + *R. solani*	30c
Control *R. solani* (AG-3)	30c

* Experiment was conducted using field soil amended with spores of the antagonists and sclerotia of *R. solani* incubated for 7 days at room temperature. Four replicate plates per treatment and ten Table beet seeds per replicate were sown.

** Data were log transformed for analysis; Mean values followed by the same letters within the column do not vary significantly from each other according to Least significant difference test ($P \geq 0.05$).

3.4 Discussion

We realize that *in vitro* screening in the laboratory for biological control agents effective against soil borne plant pathogen is a simplistic approach to understanding a small sector of a biological system in disease control. The soil screening procedure used in this study with natural soil provided an effective means of determining which antagonist was most suitable for biocontrol of *R. solani*. Since tuber-borne inoculum is responsible for the stem canker phase of the Rhizoctonia disease, isolates that reduced sclerotium viability (Table 3) might be expected to reduce disease incidence and severity of *R. solani* on table beet seedlings as well. In our first screening test using greenhouse soil, only some of the antagonists (total 24) that caused a significant reduction in sclerotial viability, increased number of secondary roots and tap root length of seedlings. When the same antagonists were tested in natural field soils, only isolate F258 provided better control of Rhizoctonia damping off than isolates F2, F132 and F11 (Table 5). This variation is expected, because biological entities are influenced by many physical and biological factors of the soil such as texture, pH, and moisture (Elad *et al.*, 1980; Marshall, 1982), and competition.

It is often assumed that a biological control agent should be able to establish itself in the soil and only then could interact with the pathogen in such a way that disease is reduced. For example, the number and height of stunted barley plants caused by *R. solani* infections increased significantly when placed in soil preincubated for 24 hours with the mycoparasite *Verticillium biguttatum* (Morris *et al.*, 1993). The time at which the inoculum of a biocontrol agent was added to soil in relation to the time *R. solani* was added is also important in establishing biocontrol effectiveness. Elad *et al.* (1980) showed that the longer the pathogen was in contact with the biocontrol fungus before planting a susceptible crop, the more successful was the biocontrol. In our study, preincubation of the biocontrol agents one week prior to the application of *R. solani* was sufficiently long to allow germination of spores, hyphal elongation and production of conidia and as well, securing good control of *R. solani*. Reduction in the ability of *Rhizoctonia solani* to colonize table

beet seedlings may be attributed to antagonism. In our experiments, we believe that any reduction that the biocontrol agents produced was may be due to some type of parasitism. These selected biocontrol agents were able to degrade and parasitize sclerotial cells (Table 3). Parasitism relies on lytic enzymes for the degradation of cell walls of pathogenic fungi. For example, chitinases and ß-1,3-glucanases are important fungus-controlling enzymes due to their ability to degrade the fungal cell wall components, chitin and ß-1,3-glucan (Henis and Chet, 1975; Schroth and Hancock, 1981). Microorganisms capable of producing and excreting chitinases (Ordentlich *et al.*, 1988; Sivan and Chet, 1989; Inber and Chet, 1991) and glucanases (Fridlender *et al.*, 1993) have been shown to be efficient biocontrol agents. In biocontrol experiments carried out under greenhouse conditions, ß-1,3-glucanases and chitinases-producing bacteria identified as *Pseudomonas cepacia* Burkhoder (Fridlender *et al.*, 1993) decreased the incidence of diseases caused by *R. solani*, *Sclerotium rolfsii* Sacc. and *Pythium ultimum* Trow. by 85%, 48% and 71% respectively. This bacteria caused partial degradation of the pathogenic fungal hyphae.

CHAPTER 4.

EVALUATION OF ANTAGONISTS FOR THEIR ABILITY TO PARASITISE *RHIZOCTONIA SOLANI*

4.1 Introduction

A biocontrol agent may act against pathogens by using one or more of the following mechanisms: competition, antibiosis, parasitism as well as activating host defense mechanisms (Papavizas and Lumsden, 1980; Sequeria, 1983). Several fungi and bacteria have been reported to be effective antagonists of *Rhizoctonia solani* (Roy, 1989). Among these, are species of *Trichoderma* (Chet, 1987), *Verticillium* (Boogert *et al.*, 1989; Morris *et al.*, 1992), *Laetisaria* (Lewis and Papavizas, 1992), *Stachybotrys elegans* (Benyagoub *et al.*, 1994), *Acrophialophora* and *Neucosmospora* (Turhan and Turhan, 1989). The above mentioned antagonists are known to control *R. solani* through their mycoparasitic action. This is characterized by (i) appressed growth, (ii) contact, (iii) coiling of antagonist hyphae around the host hyphae and (iv) penetration of host cells through mechanical and enzymatic action and subsequent utilization of the host cytoplasm (Sivan and Chet, 1989; Benyagoub *et al.*, 1994).

In this section, the course of parasitism by the antagonists F2, F11, F132, and F258 of hyphal cells of *R. solani* was studied using light microscopy. The choice of these antagonists was based on the results obtained from previous experiments. These four isolates were shown to have a strong antagonistic activity against sclerotia of *R. solani in vitro*.

4.2 Materials and Methods

4.2.1 Organisms culture conditions

The four antagonists, *Pestalotia* spp. (isolate F2), *Trichoderma* spp. (F258), *Penicillium* spp. (F11), *Gliocladium* spp. (F132), and their host *R. solani* (AG-3) were maintained on PDA at 24^{0}C for one week.

4.2.2 Hyphal-Hyphal interaction

To obtain interaction sites of hyphae, the dual-culture slide technique (Benygoub *et al.*, 1994) was used. Briefly, a cellophane membrane was autoclaved, and placed on the surface of microscope slide (2.5 x 7.5 cm) coated with 2% PDA. Two one-week-old agar disks, one bearing the antagonist and the other with mycelium of *R. solani* were placed on the cellophane membrane, at opposite ends. Cellophane with 2% PDA culture slides inoculated with either the host or the mycoparasites alone served as control. All culture slides were incubated at 24^{0}C for 7-14 days. The experiment was repeated once. The mycoparasites and their host grew towards each other and their hyphae intermingled. For light microscopy, strips of cellophane (1.5 cm^2) cut from the zone of the intermingled hyphae of both the parasite and its host 7-14 days after contact, and from the growing margin of pure cultures of either host or mycoparasites (controls) were stained with 1% (v/v) lactophenol cotton blue. Slides were examined and representative ones were photographed with Zeiss phase-contrast and Nomarski interference-contrast light microscopy.

4.3 Results

4.3.1 Light microscopy observations

In pure or dual cultures, cells of *Pestalotia* (Fig. 2A), *Gliocladium* (Fig. 3A), and *Penicillium* (Fig. 4A-4B) could be easily distinguished from those of *R. solani* (Fig. 2B) by the diameter of their hyphae and profuse production of conidia which arise from phialides. After 7 days of contact, mycelial samples taken from the interaction zones of dual cultures of all antagonists, except for *Trichoderma* spp. (F258), showed directed growth towards *R. solani* hyphae, close contact and coiling around *R. solani* cells (Figs. 2C, 3B, 4D). In the case of *Trichoderma* spp. (F258), hyphal-hyphal interaction did not take place. The hyphae of the antagonists as well as those of *R. solani* overgrew and crossed each other (Data not shown). Upon contact all three antagonists were able to penetrate the hyphae of *R. solani* at different locations on the cell wall (Figs. 2C, 3C, and 4C). Only mycoparasite F2 produced short globular structure resembling appresoria. These were formed at the end of side branches (Fig. 2C arrow) and made contact with the surface of the cells. In the case of F132 and F11, penetration of host cells was observed in unspecialized hyphal branches (Figs. 3C and 4C-D). In some instances, dissolution of *R. solani* cell walls at the point of contact with the hyphae of the mycoparasite was noticeable (Fig. 3C, arrow). After penetration, the hyphae of all mycoparasites grew inside the host hyphae and colonized other adjoining cells through dolipore septa (Fig. 2D, 3D, 4C). Colonized cells of *R. solani* appeared empty and devoid of cytoplasm (Fig. 4C) compared with non-colonized cells (Fig. 2B). In all of the host-mycoparasite combinations no visible inhibition zone was observed; confirming that antibiosis is not involved but parasitism.

Fig. 2. Light microscopy of *Rhizoctonia solani* and *Pestalotia* spp. interaction sites. Materials stained with lactophenol cotton blue.

A. Showing hyphae of mycoparasite (arrow HY), dark brown conidia (c) on PDA with 2-3 appendages (A) inset .(Bar = 50μm). B. 7 days-old culture of *R. solani* hyphae (H) with cytoplasm, served as control. (Bar = 50μm). C. Showing invasion of a host hyphae (H) by hyphae of mycoparasite (M). Growth of mycoparasite towards its host, producing short or peg-like structure (P) and penetrate the host hyphae (Bar=100μm). D. Internal colonization of mycoparasite from one septum (S) to another observed. (Bar = 50μm).

A
B
C
D

Fig. 3. Light micrograph of *Rhizoctonia solani-Gliocladium* spp. interaction sites.

A. Showing hyphae (HY), phialides (arrow PH) of mycoparasite and conidia (C) on PDA, served as control. (Bar = 50μm). B. Hyphae of mycoparasite (arrow M) adjacent to the host hyphae (H) and growing along with it. (Bar = 50μm). C. In response to the penetration by mycoparasite (M), cytoplasm of *R. solani* hyphae digested. (Bar = 50μm). D. The hyphae of the mycoparasite which grew inside (arrow) the host hyphae and colonized the neighboring cells through the septa (S). (Bar = 50μm)

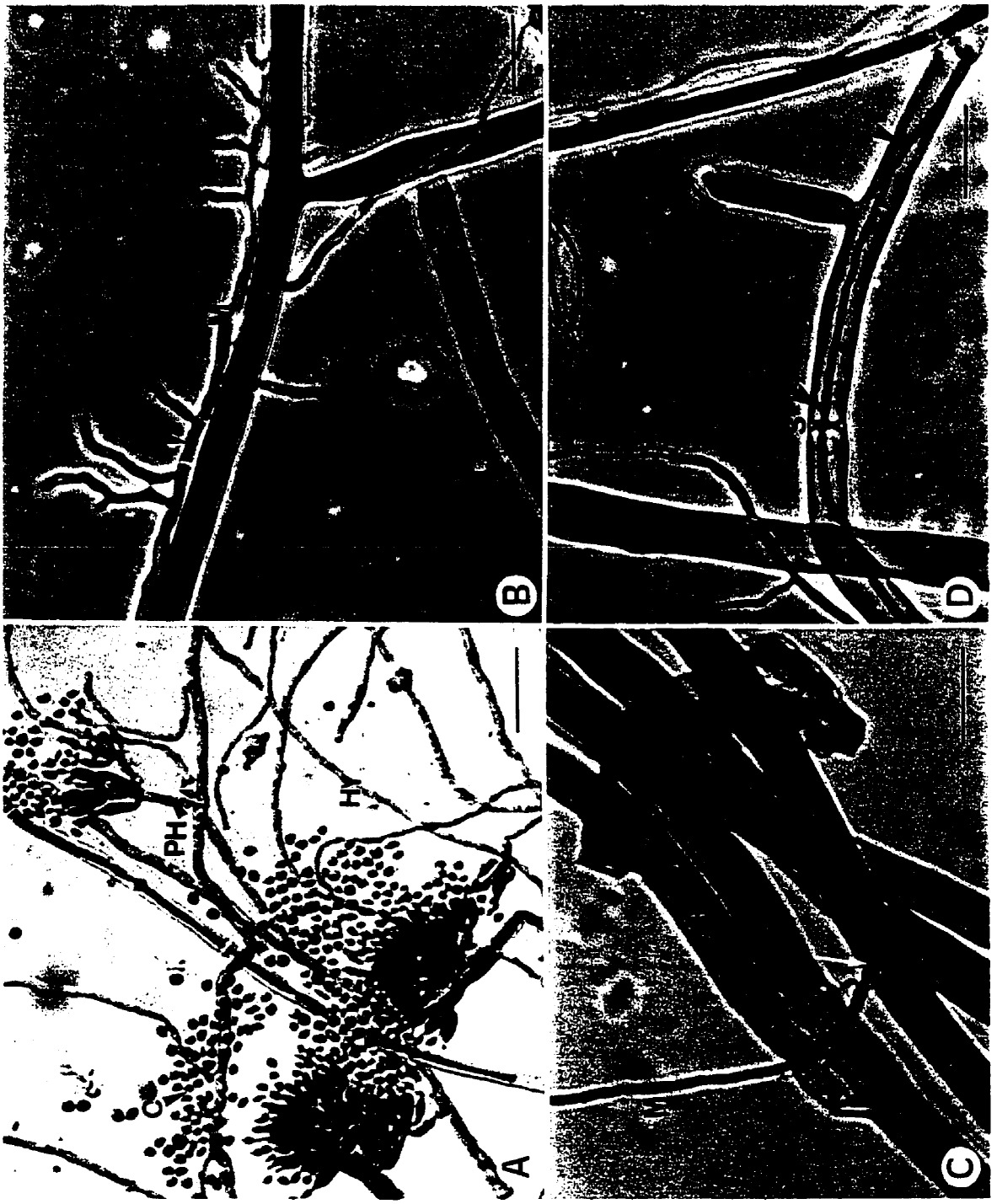

A
B
C
D
PH
H

Fig. 4. Light micrograph of *Rhizoctonia solani-Penicillium* spp. interaction sites.

A. Host hyphae (H) cytoplasm became irregular by the invasion of mycoparasite hyphae (M) (Bar = 50μm). B. Hyphae of mycoparasite alone (HY) and phialides (PH) indicating as control. C. Penetration and intercellular parasitism (arrow) by mycoparasite on a hyphae of *R. solani* as seen in the light microscope. (Bar = 50μm).

D. Invasion of a host hyphae (H) by short lateral branches or penetration peg (P) of mycoparasite. (Bar = 50μm).

A
B
C
D
PF
H
P

4.4 Discussion

Antagonists F2, F11, and F132 are destructive mycoparasites of *R. solani*. The interaction between these 3 mycoparasites and *Rhizoctonia solani* is characterized by (a) contact and appressed growth, (b) loose coiling, (c) penetration of the host hyphae and internal growth *in vitro*. These observations suggest the involvement of parasitism followed by lysis rather than antibiotic production. This mode of parasitism is similar to previously described necrotrophic mycoparasites of *R. solani* such as *Trichoderma harzianum* Rifi (Elad *et al.*, 1981 a,b; Elad *et al.*, 1987;), *T. hamatum* (Chet *et al.*, 1981) *Pythium nunn* (Lifshitz *et al.*, 1985), *Verticillium biguttatum* (Boogert *et al.*, 1989), and *Stachybotrys elegans* (Benyagoub, 1993).

For a mycoparasite to be effective and to be considered a successful biological control agent, it should be effective against resistant structures of plant pathogens (Baker and Cook, 1974). Our previous results (Table 3) clearly demonstrated that F2, F11, and F132 were able to exploit sclerotial tissues of *R. solani* and significantly decreased their germinability as indicated by the decreased viability of parasitized sclerotia. In addition, when they were tested against *R. solani* on table beet seeds, an increased number of secondary roots and length of tap roots of beet seedlings as well as increased % of seed germination were also observed. After directed growth and contact, the mycoparasites dissolved the cell wall, through which infection peg-like structures entered the host cell wall. Physical contact followed by penetration of infection pegs through pores has also been described for *Trichoderma* spp. (Elad *et al.*, 1983a, b), *Verticillium biguttatum* (Boogert *et al.*, 1989) on *R. solani*, and *S. elegans* (Benyagoub et al., 1993).

Obviously, wall thickness of melanized sclerotial cells did not provide an effective barriers to these mycoparasites. Evidence obtained from light microscopy suggest that enzymatic processes are involved and presumably play the major role in the mycoparasitism of *R. solani*. Apparently, a variety of extracellular, inducible wall lytic enzymes might play an important role for the parasite to initiate infection. In our results, dissolution of the cell

walls at the penetration sites suggest that presumed enzymatic activity of the mycoparasites was limited to these sites rather than generalized, leaving the host cell walls intact with the cell contents destroyed. A similar mechanism is also reported in parasitism of *R. solani* by *Trichoderma* spp. (Elad *et al.*, 1983 a; b) and of *R. solani* by *Stachybotrys elegans* (Benyagoub *et al.*, 1994). The penetration of *R. solani* cells by *S. elegans* causes a disruption and eventual disintegration of the host cytoplasm. The host's disintegrated cytoplasm provides evidence that the cytoplasm has been chemically altered due to the intracellular colonization of hyphae of *S. elegans*. Based on these results, it seems plausible to assume that a similar process takes place in the parasitic action of *R. solani* by *Pestalotia* (F2), *Gliocladium* (F132), and *Penicillium* (F11).

CHAPTER 5.

DETECTION OF EXTRACELLULAR ENZYMES (ECE) PRODUCED BY SELECTED ANTAGONISTS, ON SOLID MEDIA

5.1 Introduction

Previously, isolate F2, F11, and F132 were shown by light microscopy to be destructive mycoparasites of *Rhizoctonia solani in vitro*. The invasion eventually resulted in degradation, disintegration and lysis of the host hyphae and implicated the involvement of enzymatic activity. However, the mode of action of mycoparasites is not yet completely elucidated, but extracellular cell wall degrading enzymes more specifically chitinases, and glucanases, (Tweddell *et al.*, 1994; Di Pietro *et al.*, 1993), lipases (Chet, 1987), and proteases (Chiu and Tzean, 1995) have been implicated. These hydrolytic enzymes are important in host infection and breakdown of organic matter (Hoitink and Fahy, 1986). It has been demonstrated that *Trichoderma* spp. and other mycoparasites for example, *Pythium nunn, Serratia* sp., *Stachybotrys elegans, and Streptomyces* spp. possess one or more of the above enzymes which are necessary to attack the major fungal cell wall components, chitin and glucan (Lilley and Bull, 1974; Elad *et al.*, 1985; Benhamou and Chet, 1996; Benyagoub *et al.*, 1996).

Comparisons of fungi on the basis of enzyme production are very time consuming. They require growing the fungus in a culture medium, assaying the mycelium as well as the culture filtrates for enzyme production. Alternatively, the use of solid media permits the rapid screening of large populations of fungi for the absence or presence of specific extracellular enzymes (ECE) (Hankin and Anagnostakin, 1975; Donly and Day, 1984). When fungi are grown on solid media, extracellular enzymes diffuse into the agar around the colony. If appropriate substrate is incorporated in the medium, presence of the enzyme

activity can be seen as a lysis zone of degraded substrate around the fungal colony which can be easily measured (Hankin and Anagnostakin, 1975).

In this section, we investigated the production of several ECE by six selected antagonists for biocontrol of *R. solani*, using a relatively easy and rapid agar plate screening method for individual ECE.

5.2 Materials and Methods

5.2.1 Selected antagonists: The fungal isolates, F2 (*Pestalotia* spp.), F11 (*Penicillium* spp.), F132 (*Gliocladium* spp.), F216 (*Fusarium* spp.), F158 (*Gliocladium* spp.), and F258 (*Trichoderma* spp.) were retained to study their production of extracellular enzymes when grown under specific substrate. To compare the ECE production of the above fungi with another established mycoparasite, *S. elegans* has been chosen in this study.

5.2.2 Media: All tests were made on prepoured plates (7-10ml) containing a specific substrate. Plates were inoculated with a PDA plug containing mycelial growth of one of the above antagonists. Plates were incubated at room temperature for 4 to 6 days. The determination of enzymatic activity under optimized conditions is described below and is also summarized in Table (6).

5.2.3 Pectolytic activity: This medium contained 500ml of mineral salts solution, 1g of yeast extract, 15g of agar, 5g of pectin (citrus or apple), and 500ml of distilled water; pH 7.0. The mineral salts solution contained per liter: $(NH_4)_2SO_4$, 2g; KH_2PO_4, 4g; Na_2HPO_4, 6g; $FeSO_4.7H_2O$, 0.2g; $CaCl_2$, 1mg; H_3BO_3, 10μg; $MnSO_4$, 10μg; $ZnSO_4$, 70μg; $CuSO_4$, 50μg; MoO_3, 10μg, pH 7 or 5 as needed. To detect pectate lyase production, the pH of the medium was adjusted to 7.0, while for the production of polygalacturonase activity, the pH was adjusted to 5.0. Plates were incubated for 4-5 days and then flooded with a 1% (w/v) aqueous solution of hexadecyltrimethylammonium bromide (Fisher Scientific Nepean, Ontario). This reagent precipitates intact pectin in the medium and clear zones developing around a colony indicates degradation of the pectin.

5.2.4 Amylolytic activity: This medium contained Difco Nutrient Agar plus 0.2% of soluble starch, pH 6. After 4 days of inoculation the plates were flooded with an iodine solution (Appendices CI) and a yellow zone around a colony indicated amylolytic activity.

5.2.5 Lipolytic activity: The medium described by Sierra (1957) was used to detect production of lipolytic enzymes. Sorbitan monolaurate (Tween 80, Fisher Scientific Co.) was used as the lipid substrate. This medium contained per liter: Difco peptone, 10g; NaCl, 5g; $CaCl_2.2H_2O$, 0.1g; agar, 20g, pH 6. The Tween 80 was sterilized separately by autoclaving for 15 min. and 1ml was added per 100ml of sterile and cooled agar medium. Lipase degradation of Tween 80 results in the deposition and formation of insoluble crystals of the calcium salt of lauric acid liberated by the enzyme, visible as white particulate concentric rings of white flakes.

5.2.6 Proteolytic activity: This enzyme was detected using a medium containing gelatin as the protein substrate (*Manual of methods for pure culture study of bacteria* , 1951). The medium consisted of Difco Nutrient Agar plus 0.4% gelatin, pH 6. An 8% solution of gelatin in water sterilized separately and added to the nutrient agar at rate of 5ml per 100ml of medium. After 4-5 days of incubation, the plates were flooded with an aqueous saturated solution of ammonium sulfate. Large clear halos around the colonies indicated degradation of the substrate.

5.3 Determination of chitinases, glucanases, cellulases and mannases activity

5.3.1 Media

Chitinases activity: Chitinase activity was determined with a medium composed of 500ml of mineral solution (described under pectolytic activity), 500ml of distilled water, 0.02% of yeast extract, 15g of agar, and 2.4% of purified chitin (poly-N-acetyglucosamine from crab shells, Sigma Chemical Co., St. Louis, Mo.) as described by Campbell and Williams (1951).

Glucanases activity: Plate assay for determining the activity of glucanases using the same medium described under chitinase activity but substituting 0.5% (w/v) laminarin for chitin was prepared.

Cellulases activity: 0.25% w/v of Carboxymethylcellulose (CMC) containing plates were prepared using the same medium for chitinase but substituting CMC for chitin.

Mannases activity: Yeast α-mannan 0.5 (w/v) containing plates were prepared using the same medium for chitinase but substituting yeast α-mannan for the chitin.

5.3.2 Assay procedure

All plates (contain chitin, glucan, CMC, and yeast α-mannan) inoculated with antagonists were incubated at room temperature for 4-6 days and then flooded with 1% congo red solution in water. The stain was removed after 30 min. and plates were destained with 1M NaCl in buffer A (Appendices CII) solution for 15 min. (Hagerman *et al.*, 1985). Clear zones developing in the opaque agar around the colonies indicated the degradation of the substrate. Plates which were not flooded with any of the stains described above, served as the control.

5.3.3 Measurement of the lytic zone diameter

Triplicate plates were used for each enzyme. The actual diameter of the lytic zone was mesured and mean values of the lysis zone for each fungus were recorded. The experiment was repeated twice and the data of two separate experiments were pooled and statistically analyzed using the student numan kuels (SNK) test.

5.4 Results and Discussion

In this study, seven potential biocontrol fungi were grown on solid media to induce ECE production. The term enzyme production here is intended to mean both synthesis of the enzyme by the fungus and activity of the enzyme in the medium after it is produced. The data presented in Table 7 showed that various enzymes produced by fungi can easily be detected and measured by the lysis zone on solid media and as well it demonstrates the usefulness of such media in surveys for fungi able to produce specific enzymes (Hankin and Anagnostakis, 1975). Our results showed that some fungi such as, isolates F2, F11, F132, and F216 produced 7-8 enzymes out of 9, while others produced few or none under our test conditions (*S. elegans*, F158, and F258).

The CMC-containing plates were used to detect cellulases. CMC is cellulose modified to a soluble forms by the addition of carboxymethyl groups. Congo red binds CMC (Wood and Fulcher, 1978; Beguin, 1983) and forms a complex which is red at acidic pH. F2, F132, and F216 were the only isolates which produced cellulases. Among them, F132 produced significant amounts of cellulases (Table 8). High cellulase production by soil fungi is an important attribute of a biocontrol agent. In a study conducted by Ahmad and Baker (1987) and Foster *et al.* (1983), the production of cellulases by *Trichoderma harzianum* isolate was shown to be positively correlated with their competitive saprophytic ability and their ability to be successful rhizosphere competent.

The detection of chitinases, ß-1,3-glucanases and α-1,3-mannases were favored by media containing solid chitin, laminarin and yeast α- mannan respectively. In our study, F2, F11, and F132 produced significant amounts of ß-1,3-glucanases, chitinases, and mannases. All isolates except for F258 produced amylases. Isolate F2 produced the largest halos on amylase medium (Table 8).

The various grades of Tween are suitable for measuring lipase activity of microorganisms (Sierra, 1957). In this study, the formation of lipase by 6 out of 7 fungal isolates was demonstrated by adding water soluble Tween 80 to nutrient medium. Around

the colonies with lipolytic activity, there appears a very visible halo which is due to crystallization of the calcium salt of the fatty acid liberated by lipolysis. Lipase production was not detected in isolate F216 (Table 7 & 8).

In the absence of proteolytic activity, the plates are uniformly colored white. Proteases degrade the substrate to peptides which are not precipitated, so the activity is usually indicated by clearing. The only isolate which did not produce protease is F258 (Table 7 & 8). Since both enzyme synthesis and activity are pH dependent, media for the detection of pectinases were tested at pH values from 5 to 7. Microorganisms produce two major types of pectolytic enzymes, pectate lyases and polygalactorunases (Bateman and Miller, 1966). Lyases are usually characterized by alkaline pH optima, whereas polygalactorunases have more acid optima. The extracellular pectic enzymes, polygalactorunase and pectate lyase are active at pH 5 and pH 7, respectively (Hankin and Anagnostakis, 1975). The high activity at pH 5 and the lack of, or decrease in pectolytic activity, at pH 7 suggest that polygalactorunase are the major pectolytic enzymes produced by our test fungi on solid media (Table 8). Media at pH 7 will not detect galactorunase and those at pH 5 will not detect the lyase.

Of the seven antagonists, five were shown to be producers of polygalactorunase but not pectate lyase. Four isolate (F2, F11, F216, and F158) were weak producers, but isolate F132 produced the largest halos (Table 8). Isolate F258 and *S. elegans* did not produce any pectic enzymes. None of the fungi tested except for F216, were able to produce pectate lyase under our conditions (Table 8).

In this study, the mineral medium containing pectin and yeast extract was used to detect a wide variety of pectolytic microorganisms (Hankin and Sands, 1975). They provide useful tools in screening potential fungi or pectolytic soil organisms. In all these connections, the mineral medium with pectin and yeast extract is especially useful in enumerating such organisms.

Most of our test fungi were able to produce cellulases, amylases, pectinases, lipases, and mannases suggesting that they have good saprophytic competitive ability in soil. In addition, they were good producers of cell wall degrading enzymes specifically, chitinases and ß-1,3-glucanases. Based on our results, selection of biocontrol agent candidates based on their ECE activities would thus favor F2, F11 and F132 showing high enzyme activity and most importantly, for cell wall lytic enzyme production.

Although, the substrate-agar plate screening method is semi-quantitative, it is relatively easy and rapid. This method would allow rapid surveys of the enzymatic capabilities of soil fungi and facilitate recognition of individual isolates that produce specific extracellular enzymes. For quantitative estimation of the above ECE, colorimetric assays are essential. In the proceeding section, we have estimated quantitatively the amounts of chitinases, glucanases, cellulases, and proteases secreted by antagonists F2, F11, and F132 in synthetic media supplemented with the appropriate carbon source.

Table 6: Screening conditions for the detection of extracellular enzymes (ECE) on substrate-agar plate*

Enzyme	Substrate (%w/v)	Incubation conditions	Reagents for visualization
α 1,3-Mannases	Yeast α-mannan (0.5)	1	Congo red**
ß-Glucanases	Laminarin (0.5)	2	Congo red
ß-1,3-Glucanases			
Chitinases	Chitin (2.4)	3	Congo red
ß-1,4-endoglucanases	Carboxymethylcellul-ose CMC (0.25)	1	Congo red
Proteases	Gelatin (0.4)	1	1% Hexadecyltrimethyla-mmonium bromide (30 min.)
Pectinases	Apple pectin (0.5)	3	Saturated ammonium sulphate soln. (2hrs.)
Lipases	Sorbitan monolaurate (v/v Tween 80)	2	Precipitates of calsium crystal salt
Amylases	Soluble stearch (0.2)	1	Iodine solution (15min.)

* Experiment was conducted at room temperature (RT).

** Congo red staining at room temperature for 30 min. and destanined for 15 min. with buffer A solution.

1- Incubated at RT for 4-6 days.

2- Incubated at RT for 4 days.

3- Incubated at RT For 4-5 days.

Table 7: Enzyme production by fungal antagonists on solid media *

			Enzyme detected									
Isolate No.	Genus		α 1,3-Mannases	ß 1,3-Glucanases	Chitinases	Cellulases	Lipases	Polygalactorunases	Pectate lyases	Proteases	Amylases
F2 **	*Pestalotia*	spp.	(+)	(+)	(+)	(+)	(+)	(+)	(-)	(+)	(+)
F11	*Penicillium*	spp.	(+)	(+)	(+)	(-)	(+)	(+)	(-)	(+)	(+)
F132	*Gliocladium*	spp.	(+)	(+)	(+)	(+)	(+)	(+)	(-)	(+)	(+)
F158	*Gliocladium*	spp.	(+)	(-)	(+)	(-)	(+)	(+)	(-)	(+)	(+)
F216	*Fusarium*	spp.	(+)	(+)	(+)	(+)	(-)	(+)	(+)	(+)	(+)
F258	*Trchoderma*	spp.	(-)	(-)	(-)	(-)	(+)	(-)	(-)	(-)	(-)
Stachybotrys	*elegans*		(-)	(+)	(+)	(-)	(+)	(-)	(-)	(+)	(+)

* Incubation at room temperature . Media contained no antibiotics.

** Designated fungal isolate number. Triplicate plates were used for each enzyme.

(+) indicates that the antagonist produced enzyme.

(-) indicates that the antagonists did not produce enzyme.

Table 8: Detection of lytic zones produced by extracellular enxymes (ECE) of selected antagonists on substrate+Agar plate*

	Enzymes detected								
Antagonists	Mannase	Glucanase	Chitinase	Cellulase	Lipase	Polygalctorunase	Pectate lyase	Protease	Amylase
Pestalotia spp. (F2)**	2.3b***	2.3bc	2.5d	2.2b	1.8ab	1.0dc	0.0b	2.1c	4.8a
Penicillium spp. (F11)	1.6b	11a	3.6c	0.0c	1.7ab	3.3b	0.0b	5.5b	2.5bc
Gliocladium spp. (F132)	5.1a	3.0b	4.6b	7.4a	1.5bc	5.2a	0.0b	7.4a	2.7bc
Gliocladium spp. (F158)	2.6b	0.0c	2.6d	0.0c	1.3dc	3.2b	0.0b	6.1b	1.7dc
Fusarium spp. (F216)	2.4b	2.6bc	3.3c	1.8b	0.0e	1.5c	1.2a	8.8a	3.3abc
Trichoderma spp. (F258)	0.0c	0.0c	0.0e	0.0c	1.9a	0.0d	0.0b	0.0d	0.0d
S. elegans	0.0e	4.6b	5.7a	0.0d	1.2b	0.0e	0.0b	9.1a	1.9c

* Experiment was conducted at room temperature. Numbers represent the lysis zone diameter measured in cm. Triplicate plates were used for each enzyme.

** Number of designated fungal isolate.

*** Data of two seperate experiments were pooled and mean values followed by the same letter within the column do not vary significantly from each other according to SNK test ($P \leq 0.05$).

CHAPTER 6.

QUANTITATIVE MEASUREMENT OF CHITINASES, ß-1,3-GLUCANASES, CELLULASES, AND PROTEASES PRODUCED BY SELECTED ANTAGONISTS

6.1 Introduction

Microorganisms capable of lysing other organisms are widespread in natural ecosystems (Whipps, 1992). An ideal biocontrol agent would be the one that employs multiple defense strategies against pathogens. One defense strategy is mycoparasitism which denotes the parasitation of one fungus by another (Butler, 1957). Mycoparasites usually attack and invade their hosts by short lateral branches via appressoria and concomitantly produce cell wall-lytic enzymes, such as chitinases, cellulases, glucanases, and proteases (Elad *et al.*, 1985; Chet, 1987;). The chitinolytic (Lorito *et al.*, 1993) and glucanolytic (Rapp, 1989; Lorito *et al.*, 1994; Tweddell *et al.*, 1995; Chiu and Tzean, 1995) enzymes or combination of these produced by the mycoparasites usually act synergistically rather than alone. The invaded fungal hosts hyphae are usually lysed and destroyed (Tweddell *et al.*, 1994; Chiu and Tzean, 1995; Benyagoub *et al.*, 1996). Thus, lytic enzymes are suggested to be a major mechanism responsible for biocontrol (Di Pietro *et al.*, 1993). Due to devastating effects, some mycoparasites for example, *Trichoderma* (Chet, 1987) and *Gliocladium* (Howell, 1982) have been extensively and successfully used for biocontrol of a wide range of plant pathogens including *Rhizoctonia solani* by their capabilities of producing hydrolytic enzymes (Howell, 1982).

In the previous section, we tested selected antagonists for the production of a range of extracellular enzymes including chitinases, cellulases, ß-1,3-glucanases and proteases on solid media. The agar plate screening method showed that the fungal isolates, F2, F11, and F132 produced pronounced chitinases, cellulases, glucanases, and proteases activities.

These results lead us to assume that these enzymes are responsible for the mycoparasitic events in our previous study. In this section, the detection and quantitative production of these enzymes by F2, F11, and F132 which were previously grown on purified cell walls of *R. solani*, were confirmed using well established colorimetric methods.

6.2 Materials and Methods

6.2.1 Organisms and culture conditions

The antagonists F2, F132, F11 were selected from agar plate assay experiment. They were maintained on PDA agar slants, while *R. solani* (AG-3) was maintained on sterile oat seeds. The oat seeds and agar slants served as stock cultures.

6.2.2 Preparation of *R. solani* (AG-3) cell wall

R. solani cell wall fragments were prepared by the method of Chet *et al.* (1967) with some modifications. Erlenmeyer flasks (250ml) containing 100ml of potato dextrose broth (Difco) were incubated with one plate of homogenized agar containing *R. solani* mycelia previously grown on PDA for 7 days. The inoculated flasks were incubated at 24^{0}C for 14 days and shaken at 110 rpm. The mycelium was then collected by filtration through Whatman no. 1 filter paper, washed with sterile water. Freeze-dried mycelium was ground into powder using liquid nitrogen and suspended in 0.05M of acetate buffer pH 4.5. The suspended solution was centrifuged at 20,000 x g for 30 min. at 4^{0}C; then sonicated 3 times at 4^{0}C for 10 min. in order to break down the cell walls, and recentrifuged for 30 min. Ten ml (10) of borate buffer (0.05M, pH 8.8) was added to the centrifuged cell walls and homogenized for 1 min, then autoclaved at 121^{0}C for 10 min. and centrifuged at 15,000 x g for 30 min at 4^{0}C. The supernatant was collected through 0.45μm millipore membrane and discarded. The *R. solani* purified cell walls were freeze-dried and stored it at -20^{0}C until further use. Protein concentration was determined by the method of Bradford (1976) using bovine serum albumin (BSA; Sigma, Chemical Co. St-louis Mo. USA) as the standard curve; protocol provided by the Bio-Rad assay (BioRad Laboratories Richmond, CA, USA). Glucanases, proteases, chitinases activities were estimated with glucose oxidase reagent (Sigma, Chemical Co.), the Azocoll method (CalBiochem, San Diego, CA) and N-acetyl glucosamine (Ressig *et al.*, 1955) methods respectively.

6.3 Conditions for enzyme production

Fungal growth was carried out on minimal synthetic medium (MSM; see appendices DI) supplemented with the appropriate carbon (0.5mg cell wall of *R. solani*/ml of MSM) and nitrogen sources (NH_4NO_3; 1mg/ml of MSM). For cellulase activity, the nitrogen (NH_4NO_3) and carbon (cellulose: Sigmacell type 20 from Sigma) sources were incorporated at a rate of 1 mg/ml. Flasks (250ml) containing 40 ml of culture media were inoculated with 1ml of $1x10^6$ conidial suspension of each of the above mentioned antagonists. All inoculated flasks were placed on a rotary shaker (110rpm/min.) at room temperature for 2, 4, 6, and 8 days. Flasks containing culture media without carbon source served as control. Triplicate flasks per enzyme were harvested after 2, 4, 6 and 8 days of growth of each antagonists for each enzyme assay. Culture filtrate from each flask was collected by filtration through a nylon mesh cloth and freeze-dried and stored at -80°C.

6.3.1 Estimation of ß-1,3-glucanases and chitinases activity

ß-1,3-glucanases and chitinases activities were estimated with slight modifications according to the method of Elad *et al* (1982). ß-1,3-glucanases activity was assayed by monitoring the release of free glucose, using glucose oxidase reagent (Sigma) according to the manufacture's recommendations. The culture filtrates were rehydrated with 0.1M citrate buffer, pH 4.7. The reaction mixture, which contained 2ml of rehydrated culture filtrate and 1.6mg of soluble laminarin. All tubes were incubated at 40°C for 1h and the reaction was stopped by boiling 3 min. Five ml of color reagent was added to 0.5 ml of reaction mixture and incubated at 37°C for 30 min. ß-1,3-glucanases activity was read by Beckman spectophotometer (DU® 640) at 425 wavelength and specific activity was expressed as μmoles / milligram of protein / hour.

Chitinases activity was assayed according to the method of Ressing *et al.* (1955). The culture filtrates were rehydrated with 0.2M phosphate buffer, pH 5.6 and the reaction mixture contained 2ml of rehydrated culture filtrate and 10mg of chitin. The reaction

mixture was incubated at 37^{0}C for 1hour, and residual chitin was removed by low speed centrifuged for 3 min (Rotar type 4180). One-hundred microliter (100μl) of borate buffer was added to 0.5ml of supernatant of reaction mixture and the reaction was stopped by 3 min boiling. Finally, 2ml of dimethyl-amino-benzaldehyde (DMAB; appendices DII) color reagent was added. All tubes were incubated for 20 min. at 37^{0}C and activity was read by spectophotometer at 585 wave length. Chitinase activity was expressed as μmoles/ milligram of protein / hour.

6.3.2 Estimation of Proteases activity

Quantitative proteases activity was assayed by the Azocoll method (CalBiochem). The culture filtrates were rehydrated with 2.5ml of Azocoll solution. The reaction mixture was incubated at 37^{0}C for 15 min. After cooled down in ice, the reaction mixture was collected by filtration through Whatman no.1 paper and the activity was read at 520 wave length using Beckman spectophotometer. Proteases specific activity was expressed as μmoles / milligram of protein / hour.

6.3.3 Estimation of Cellulases activity

Cellulases activity was estimated, with slight modification, according to the method of Ghose (1987). The activity was assayed by the release of reducing sugars estimated by the dinitrosalicylic (DNS; appendices DIII) acid method (Miller, 1959) using glucose as the standard. The culture filtrates were rehydrated with 0.05M citrate buffer, pH 4.8. The reaction mixture, which contained 0.5 ml of rehydrated culture filtrate and 0.5 ml of a 2% (w/v) carboxymethylcellulose solution was incubated at 37^{0}C for two hours and the reaction was stopped by boiling. Cellulase specific activity was expressed as μmoles / mg protein / hour.

6.4 Results

6.4.1 Enzymatic activity in control treatment

The addition of purified cell walls of *R. solani* as carbon source caused a progressive increase of chitinases, ß-1,3-glucanases, cellulases, and proteases activities in the culture media than in control treatment containing no carbon source. Irrespective of the mycoparasites tested, the production of extracellular enzymes significantly varied with time (Fig. 5-8).

6.4.1.1 Production of chitinases

When mycoparasites F2, F11, F132, and F258 were grown in liquid culture enriched with *R. solani* cell wall as carbon source, they all produced various amounts of chitinases. F11 produced significantly high amounts of chitinases followed by F2, F132, and F258 (Fig.5). The yield of chitinases produced by F11 and F2 in the medium significantly increased up to 11.66 and 8.25 (μmoles/mg of protein /hour) after 6 days of growth, respectively. Significantly lower activities of chitinases produced by F132 and F258 were detected after 6 days of growth. No significant overall difference in enzyme activity was detected after 2, 4, 6, and 8 days of growth.

6.4.1.2 Production of ß-1,3-glucanases

The time course of ß-1,3-glucanases is shown in Figure (6). The highest activity of glucanases was produced by F11 followed by F132 and F258. Maximum amounts of ß-1,3-glucanases were produced by F11, F132, and F2 after 6 days of growth on cell walls of *R. solani*. On the other hand, F258 showed high activity of glucanases production at 4 days of incubation.

6.4.1.3 Production of proteases

There was a significant increase in proteases activities produced by F2, F11, and F132 after 4 days of growth (Fig. 7). F258 had high activity at 4 days but it decreased sharply at 6 and 8 days of incubation. The secretion of proteases by F2 and F132 was more or less constant after 2, 4, 6, and 8 days of growth. Similar and higher amounts of proteases were produced by F2, and F11 followed by F132.

6.4.1.4 Production of cellulases

In the case of F11 and F132, the period of incubation had no effect on the production of cellulases. F2 had significantly higher activity of cellulases at 8 days of growth compared to that produced at 2, 4, and 6 days. F132 was the highest producer of cellulases in all of the incubation periods followed by F11 and F2 (Fig. 8).

In summary, among the four antagonists tested, F2, F11, and F132 produced appreciable amounts of one or more extracellular enzymes. Although isolate F258 was able to produce chitinases, glucanases, and proteases under induced conditions, the amounts were minimal compared with those detected in F2, F11, and F132.

Figure legend

Figure 5. Time course of chitinases production by antagonists F2, F132, F11, and F258 on MSM media containing *Rhizoctonia solani* cell wall as carbon source (0.5 mg/ml). Specific activity values represent the mean of net specific activity of 3 replicates. Net values were obtained by subtracting specific activity values of control from treatment values. Mean values followed by the same letter within the column do not vary significantly from each other according to Duncan's multiple rang test ($P \leq 0.05$).

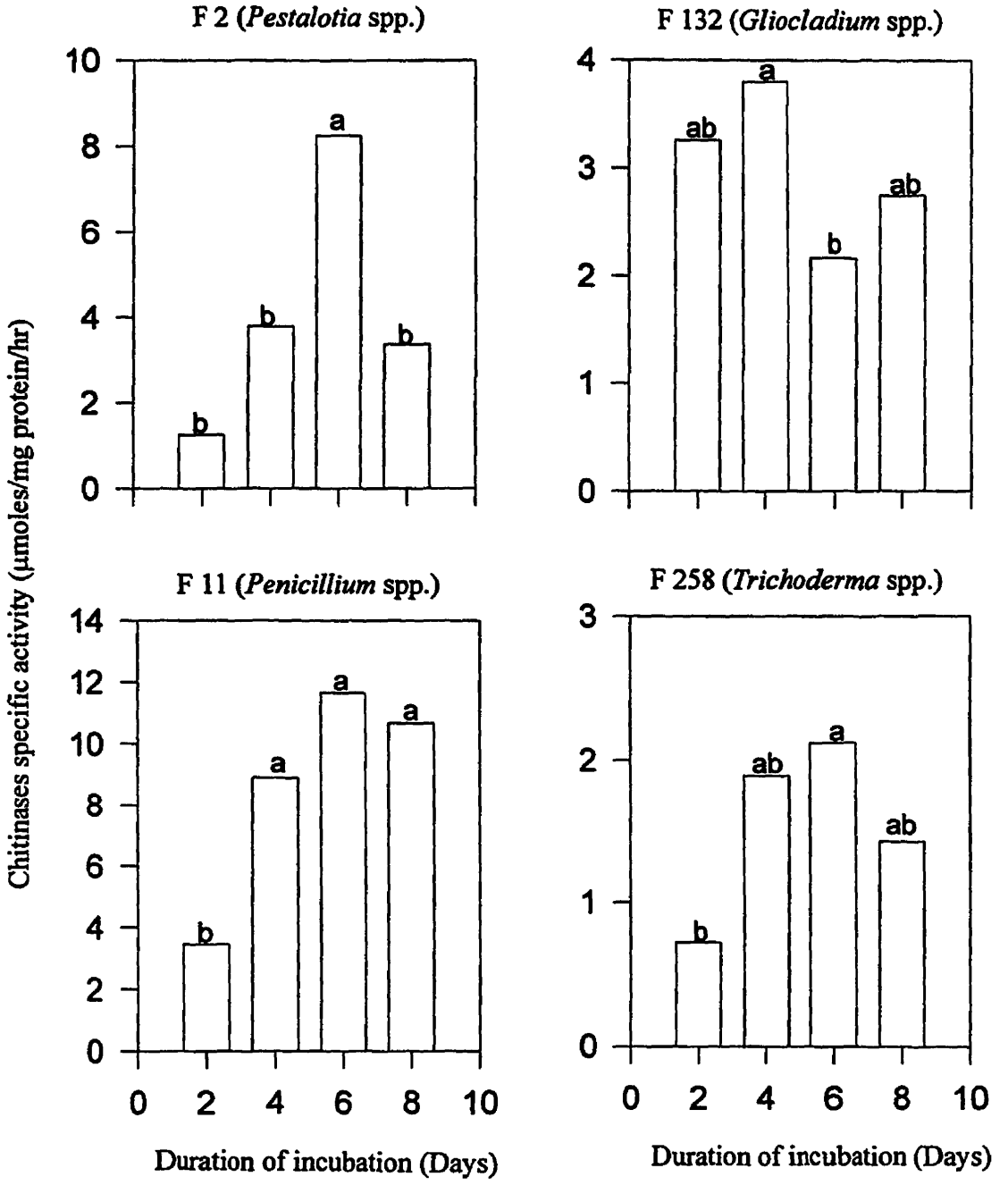

F 2 (*Pestalotia* spp.)
F 132 (*Gliocladium* spp.)
F 11 (*Penicillium* spp.)
F 258 (*Trichoderma* spp.)
Chitinases specific activity (µmoles/mg protein/hr)
Duration of incubation (Days)
Duration of incubation (Days)
a
b
ab

Figure legend

Figure 6. Time course of 1,3-ß-glucanases production by antagonists F2, F132, F11, and F258 on MSM media containing *R. solani* cell wall (0.5 mg/ml) as carbon source. Specific activity values represent the mean of net specific activity of 3 replicates. Net values were obtained by subtracting specific activity values of control from treatment values. Mean values followed by the same letter within the column do not vary significantly from each other according to Duncan's multiple range test ($P \leq 0.05$).

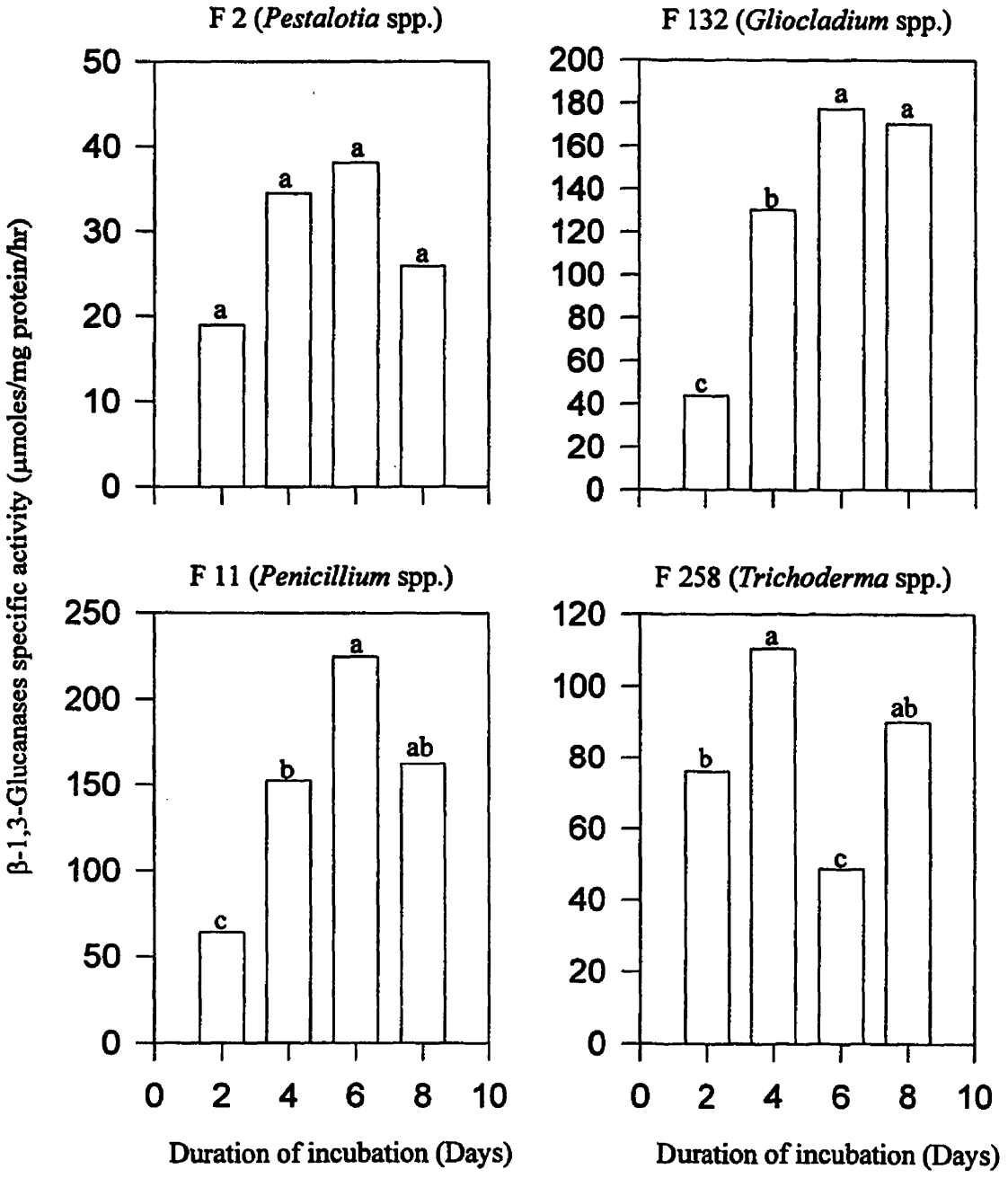

F 2 (*Pestalotia* spp.)
F 132 (*Gliocladium* spp.)
F 11 (*Penicillium* spp.)
F 258 (*Trichoderma* spp.)
β-1,3-Glucanases specific activity (µmoles/mg protein/hr)
Duration of incubation (Days)
Duration of incubation (Days)

Figure legend

Figure 7. Time course of proteases activity by F2, F132, F11, and F258 on MSM media containing *R. solani* cell wall (0.5 mg/ml) as carbon source. Specific activity values represent the mean of net specific activity of 3 replicates. Net values were obtained by subtracting specific activity values of control from treatment values. Mean values followed by the same letter within the column do not vary significantly from each other according to Duncan's multiple range test ($P \leq 0.05$).

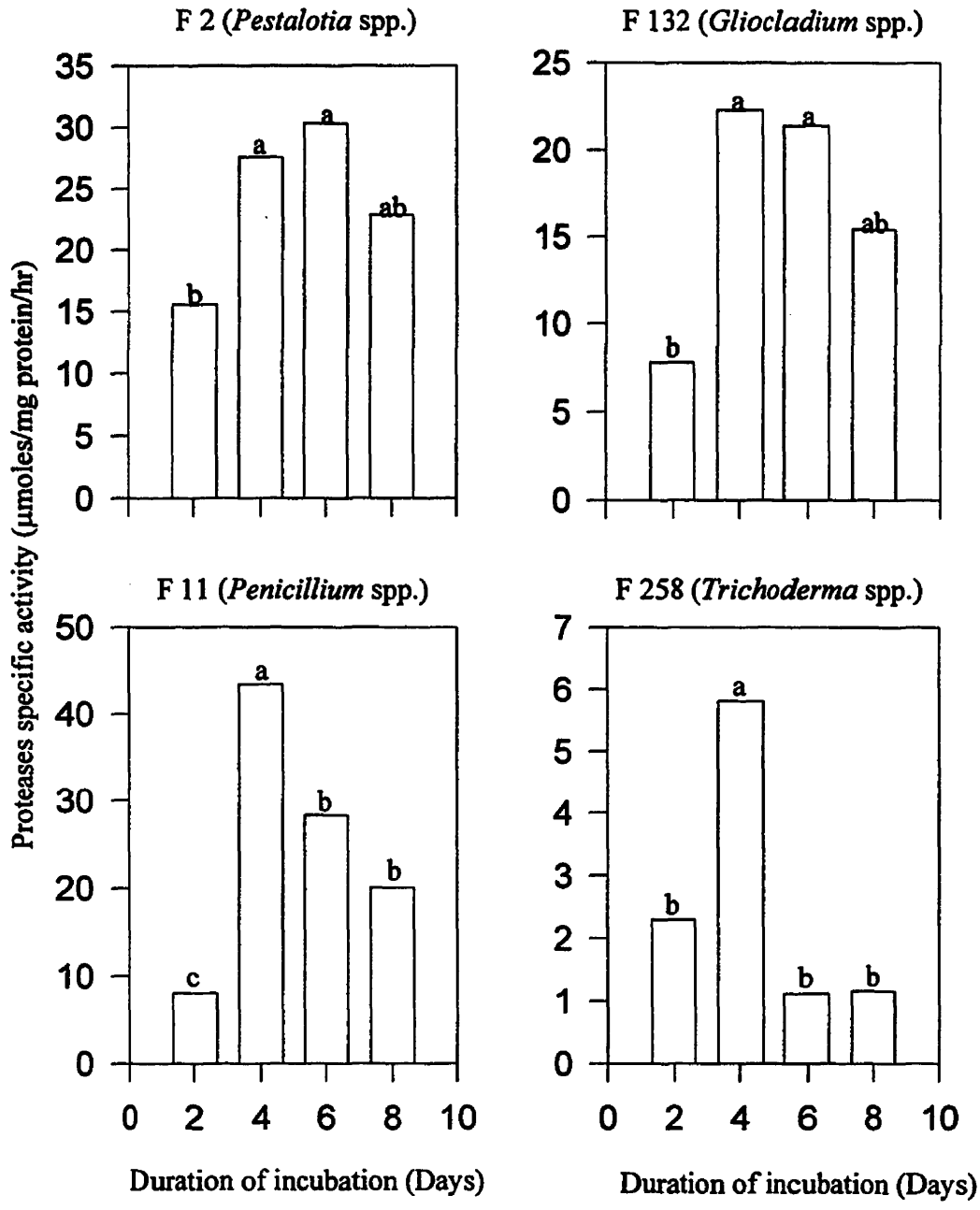
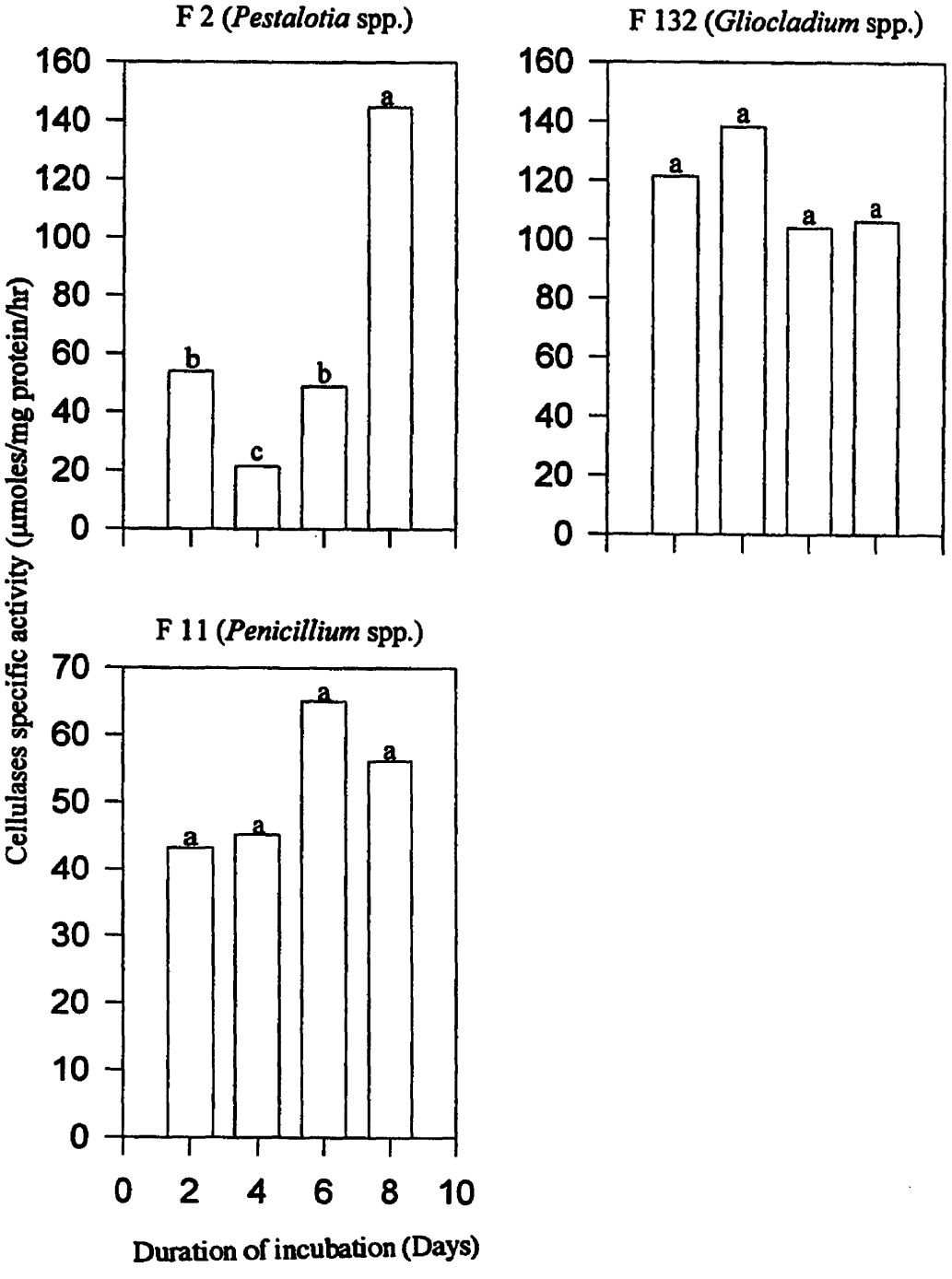

F 2 (*Pestalotia* spp.)
F 132 (*Gliocladium* spp.)
F 11 (*Penicillium* spp.)
F 258 (*Trichoderma* spp.)
Proteases specific activity (µmoles/mg protein/hr)
Duration of incubation (Days)
Duration of incubation (Days)

Figure legend

Figure 8. Time course of cellulases (endo-1,3-ß-endoglucanase) production by F2, F132, and F11 on MSM media containing cellulose (Sigma cell Type 20) as carbon source (1 mg/ml). Specific activity values represent the mean of net specific activity of 3 replicates. Net values were obtained by subtracting specific activity values of control from treatment values. Mean values followed by the same letter within the column do not vary significantly from each other according to Duncan's multiple rang test ($P \leq 0.05$).


F 2 (*Pestalotia* spp.)
F 132 (*Gliocladium* spp.)
F 11 (*Penicillium* spp.)
Cellulases specific activity (µmoles/mg protein/hr)
Duration of incubation (Days)
0
20
40
60
80
100
120
140
160
0
10
20
30
40
50
60
70
0
2
4
6
8
10
a
b
c

6.5 Discussion

Lysis by hydrolytic enzymes excreted by microorganisms is a well-known feature of mycoparasitism. Chitinases, and ß-1,3-glucanases are especially important fungus-controlling enzymes due to their ability to degrade the fungal cell wall components (Henis and Chet, 1975; Schroth and Hancock, 1981; Elad *et al.*, 1985; Ordentlich, 1988; Tweddell *et al.*, 1994; Benyagoub *et al.*, 1996). In this study, all mycoparasites (F2, F11, and F132) except F258 were able to produce significant amounts of cell-wall degrading enzymes in the presence of *R. solani* cell wall fragments than in medium containing no carbon source (control).The enzymes production was favoured by acidic pH and the presence of ammonium nitrate as the nitrogen source incorporated into the medium.This result suggests that, prior to infection, hydrolytic enzymes are constitutively present in the fungus in small amounts. The accumulation of these enzymes have been shown to appear following invasion of the pathogen (Ye *et al.*, 1989). Acidic pH was also reported to be an important growth parameter in the production of chitinases and ß-1,3-glucanases by fungal mycoparasites such as, *Trichoderma harzianum* (Elad *et al.*, 1982) and *S. elegans* (Tweddell *et al.*, 1994) and by thermophilic actinomycete *Streptomyces* sp. (Lilly and Bull, 1974).

Chitinases and ß-1,3-glucanases are the most investigated and well established lytic enzymes in the biological control of plant diseases (Lilly and Bull, 1974; Elad *et al.*, 1982; Tweddell *et al.*, 1994; Chiu and Tzean, 1995). *Trichoderma* harzianum (Elad *et al.*, 1982), *S. elegans* (Benyagoub *et al.*, 1994) and *Schizophyllum commune* Fr. (Chiu and Tzean, 1995) actively lysed *R. solani* mycelium. They were found to liberate chitinases and ß-1,3-glucanases for the hydrolysis of chitin and glucan, the major structural element of the cell walls of many fungi (Wessels and Sietsma, 1981).

Several studies have shown that chitinolytic enzymes together with ß-glucanases or cellulases, are the enzymes most frequently considered in biocontrol (Chet, 1987; Elad *et al.*, 1985; Lorito *et al.*, 1993). For example, a combination of chitinases and ß-1,3-

glucanases strongly inhibited fungal growth and lysed the mycelium of the pea pathogen *Fusarium oxysporum* Schechtend. f. sp. *pisi* (J.C. Hall) (Mauch *et al.*, 1988). Microscopic observations showed that growth inhibition by these hydrolases is due to swelling and lysis of the hyphal tips. Thus, the two hydrolases (chitinases and glucanases) clearly have an antifungal potential when adminsted together.

Cellulases, may play a major role in mycoparasitic and saprophytic ability. Chiu and Tzean (1995) showed that cellulases (endo-ß-1,4-glucanase) produced by *Schizophyllum commune* Fr. were capable of hydrolyzing cell walls of *R. solani*, *Fusarium moniliformae* J. Sheld. and other fungi. This group of enzymes may have a synergistic effect with chitinases and glucanases in the dissolution of *R. solani* cell walls. The combination of these enzymes were also shown to induce a high release of protoplasts from young mycelium of *R. solani* (Hashiba and Yamada, 1982). On the other hand, the amount of cellulase production was found directly correlated with competitive saprophytic ability and rhizosphere- competent (Ahmad and Baker, 1987).

Some other lytic enzymes such as proteases and lipases have been claimed to play some roles in fungal cell wall degradation or mycoparasitic interactions. Kobayashi and Barrad (1996) isolated a bacterial isolate N4-7 which produced lipases and proteases, and inhibited the growth of *Magnaporthe poae in vitro*, the causal agent of summer patch disease of turfgrass. In another study, proteases purified from *T. harzianum* caused inhibition of spore germination as well as hyphal elongation of *Botrytis cinera* (Schirmbock *et al.*, 1994).

In this experiment, compared with other isolates, F258 (*Trichoderma* spp.) did not perform as well in culture filtrates. In both substrate + agar plate assay and culture filtrates, the enzymatic activities of F258 detected was the same as in the control treatments. We have found that isolate F2 (*Pestalotia* spp.), F11 (*Penicillium* spp.), and F132 (*Gliocladium* spp.) produced appreciable amounts of extracellular enzymes and parasitized *R. solani* hyphae. In previous sections, they were also found to be inhibit sclerotial

germination (0%, 12%, and 32% sclerotial viability occured respectively; see Table 3) and increased beet seed germination (Table 5). Therefore, we can assume that, the availability of a large supply of extracellular enzymes by our test fungi (F2, F11, and F132), which are responsible for mycoparasitic action and may provide or consequently will increase Rhizoctonia disease control efficacy of potato by combining lytic enzymes with Rhizosphere competence.

To date, no work have been done on antagonism between *Pestalotia* spp. (F2), *Penicillium* spp. (F11) and with *R. solani*. *Pestalotia ramulosa* was shown to produce the compound ramulosin which has antimycotic properties (Benjamin and Stodola, 1960) and was also found to inhibit *Pythium ultimum*. However, the mode of action of this compound is not known (Domsch and Gams, 1968). Although *Penicillium* is one of the most thoroughly investigated genera of fungi, very little is known of the interaction between *Penicillium* species and other soil fungi. In one study, *Penicillium* spp. have been isolated from sclerotia of *Sclerotium cepivorum* Berk. the pathogen of onions and were shown to be antagonistic to mycelial growth of the pathogen (Moubasher *et al.*, 1970). It was found that they frequently produce metabolites and cause inhibitory effects upon *S. cepivorum*.

All of the above findings suggest that our isolates (F2, F11, and F132) seem to belong to a group of biocontrol agents which possess high chitinases, ß-1,3-glucanases, cellulases and proteases activity. Therefore, F2, F11, and F132 were selected as effective biocontrol agents of *R. solani* in potato under controlled conditions.

CHAPTER 7.

EVALUATION OF THE EFFICACY OF SELECTED MYCOPARASITES IN CONTROLLING THE DEVELOPMENT OF RHIZOCTONIA DISEASE OF POTATO UNDER CONTROLLED CONDITIONS

7.1 Introduction

Rhizoctonia disease limits profitable production of potato (*Solanum tuberosum* L.), throughout the world (Hide *et al.*, 1992). The pathogen forms sclerotia which are resistant to drought, heat, and fungicides and may remain viable in the soil for many years (Coley-Smith and Cooke, 1971; Coley-Smith, 1979). These sclerotia can act as inocula for the infection of the underground shoots, producing a stem canker phase of the disease in which stems may be girdled by dark, sunken lesions. The surfaces of newly produced potato tubers can be heavily contaminated by sclerotia (blackscurf) (Otrysko *et al.*, 1988; Banville, 1989). Black scurf is more of a problem where potatoes are grown repeatedly on the same land. *R. solani* itself do not reduce the yield of potato but decrease the quality by formation of sclerotia (Hide *et al.*, 1973).

Biocontrol of *R. solani* by treatment of seeds or soils with antagonistic fungi or bacteria has sometimes been successful. Merriman and co-workers (1974) were able to reduce symptoms of *Rhizoctonia solani* in wheat, substantially by inoculating the seed with *Streptomyces griseus* or *Bacillus subtilis*. In cotton, Howell and Stipanovic (1979) obtained effective protection against *R. solani* by treating the seeds with *Pseudomonas fluorescens*. In laboratory studies, Harman *et al.* (1980) successfully controlled Rhizoctonia infection of pea and radish by inoculating seeds with *Trichoderma hamatum*. In the Netherlands (Jager and Velvis, 1985) and Great Britain (Morris *et al.*, 1992), the newly discovered sclerotium-inhibiting mycoparasite *Verticillium biguttatum* was shown to

suppress potato blackscurf disease. They found that inoculation of seed with *V. biguttatum* was effective in reducing the amount of sclerotia on tubers harvested.

In this section, we investigate the efficacy of the fungal mycoparasites F2, F132, and F11 to control and reduce Rhizoctonia disease of potato under controlled conditions.

7.2 Materials and Methods

7.2.1 Inoculum production

Rhizoctonia solani (AG-3)

R. solani (AG-3) was maintained on sterilized oat-kernels, and the sclerotia of *R. solani* were produced on PDA plates as described previously (see section 2.2.3.).

Mycoparasites

Isolates F2, F132, and F11 were revived from PDA slants and grown on plates of PDA at 24^{0}C for 3 days (primary plates). Mycelial discs from primary plates were then used to inoculate new plates of PDA from which conidial suspensions were prepared after 7 days of growth at 24^{0}C. Suspensions were prepared by gently detaching the conidia from the surface of agar into sterile distilled water (DW) using a glass hockey stick. The crude suspensions from the plates were first sieved through two layers of cheese- cloth, centrifuged at 20000 g for 5 min. and re-suspended in sterile DW. This procedure was repeated 3 times. The concentration of the conidial suspension was measured with a hemocytometer and adjusted to 1x10^6 conidia/ml according to Boogert (1989).

7.2.2 Collection of Soil

Sandy loam soil was obtained from the experimental Horticultural Farm of Macdonald campus. The procedure of soil sampling is described in section 3.2.5. Samples of soils were sieved (5 mm mesh screen) and stored in paper bags for 3-4 weeks at 4^{0}C until further use.

7.2.3 Source of potato seed tuber

Clean potato seed tubers were supplied by Mr. David Jennings, Agriculture Canada, Corner Brook, NewFoundland. These seed tubers were produced from tissue cultured potato plants of the variety Mirton Pearl. The tubers were left to sprout in daylight for 4 weeks.

7.2.4 Experimental design

The experiment was conducted in four replicate pots (15cm diam.) containing approximately 650g soils. Each pot was inoculated with conidial suspension at the rate of $1x10^6$ conidia/ml of each fungus. Fungal inocula had been thoroughly incorporated by hand mixing according to the procedure of Morris *et al.* (1993). All pots were incubated in a growth chamber for 2 days at 25°C. After 2 days of pre-incubation, each pot was inoculated with *R. solani*. The inoculum consisted of a homogenized 9 cm-PDA-agar plate containing 7-days old colony of *R. solani* plus 80 sclerotia (3-5mm diam.). Mixing was done in order to achieve maximum contact between the target fungus and the mycoparasite. Two control treatments were included: (i) diseased control consisted of soil amended with *R. solani* (AG-3) only and (ii) healthy control consisted of soil amended with water only. Two previously sclerotia- free sprouted potato seed tubers were planted in each pot. Treatments were arranged in a randomized complete block design, pots were placed in a growth chamber for 5 weeks and watered daily to maintain sufficient soil moisture. The growth chamber was maintained at 25°C, with a 15-hours light time. To maintain nutrient status in each pot, soil fertilizers of N, P, K (20:20:20) were applied 2 weeks after planting potato seeds.

7.2.5 Sampling procedure

The plants were harvested by gently shaking off the adhering soil of each plants per pot. Roots were recovered by repeated washing under running tap water. Root and shoot dry weights of each plant were determined after drying at 65°C for 2 days. Weight of healthy and newly formed tubers were also determined.

7.2.6 Assay procedure

Disease severity was estimated 5 weeks after planting. The severity of stem and stolon canker was estimated by evaluating each underground stem and stolon on a scale of 0-4,

where 0 = healthy, no lesion, 1 = cankers on one stolon, 2 = cankers on two or more stolons but less than half of them, 3 = cankers on more than half of the stem and stolon but not affecting all, and 4 = lesions on all stolons and stems (Escande and Echandi, 1991). The number of sclerotia on the newly formed tubers and mother tubers were estimated separately. At harvest time, plant height, root and shoot dry weights were also measured for each plant.

7.2.7 Statistical analysis

The growth chamber experiment was conducted in a complete block design. Each treatment combination was replicated four times, and the entire experiment was a total of 20 observations. The data for number of sclerotia were subjected to log transformation and the mean values for dry weight of roots and shoots, plant height, and fresh weight of new tubers were seperated with Least significant difference (LSD) test ($P \geq 0.05$).

7.3.1 Rhizoctonia disease severity index on stems and stolons

Compared with the control (AG-3), isolate F11, and F132 proved to be more effective in protecting the plant from stolon and stem canker than isolate F2 (Table 9). Healthy control (water only) showed no disease development whereas, high disease severity and higher number of aerial stolons (a typical symptoms of Rhizoctonia disease) were detected in the control treatment in which soil was amended with *R. solani* (AG-3).

7.3.2 Sclerotium indices of the harvest

The effect of the mycoparasites F2, F132, and F11 on blackscurf is shown in Table 9. The occurrence of black scurf on mother tubers caused by *R. solani* was reduced by 100%, 99%, and 93% by F2, F132 and F11, respectively. On daughter tubers, 93%, 80%, and 100% disease reduction occurred in soils amended with F2, F132, and F11, respectively. In diseased control treatments, black scurf was present on both mother and daughter tubers. In healthy controls that were amended with water only, sclerotia were absent in mother and daughter tubers.

7.3.3 Effects of mycoparasites on Rhizoctonia disease development of potato

When tubers were planted in soil amended with mycoparasites and *R. solani*, there was a significant increase in dry weights of shoots and roots (Table 10). However, there was no significant difference in plant height and fresh weight of new tubers. Although statistically not significant, the fresh weight of tubers recovered from soil amended with F11 and *R. solani* were high. This is not surprising since these tubers were sclerotia free (Table 10).

Table 9: Effects of mycoparasites on disease severity and sclerotial formation on potato tubers under growth chamber conditions*

Treatment	Disease index (DI)**		Aerial stolon	No. of sclerotia***	
	Stolon	Stem		Mother	Daughter
F2 + AG-3	0	1	0	0.0b	0.25b
F132 + AG-3	0	0	0	0.125b	0.75b
F11 + AG-3	0	0	0	0.75b	0.0b
Control (AG-3)	2	2	3	10.0a	3.75a
Control (water)	0	0	0	0.0b	0.0b

* Experiment was conducted under controlled conditions with field soil inoculated with spore suspension ($1x10^6$ spores/ml) of each mycoparasite and with sclerotia and mycelium of *R. solani*. Four replicate pots were used per treatment and two potato seed tubers planted in each pot. All pots were incubated at 25^0C for 5 weeks.

** The data represent the severity of the disease expressed on a scale 0-4, where, 0 = no disease and 4 = Killed.

*** Data were subjected to log transformation and mean values within the column followed by the same letter do not vary significantly from each other according to LSD test ($P \geq 0.05$).

Table 10: Effects of mycoparasites on dry weight of roots and shoots, plant height and fresh weight of new tubers*

Treatment	Dry weight (g)		Plant height	Fresh weight of new tubers
	Root	Shoot		
F2 + AG-3	0.39a**	2.2ab	10.06a	11.8b
F132 + AG-3	0.39a	3.0a	10.81a	11.1b
F11 + AG-3	0.40a	2.4ab	9.75a	21.7a
Control (AG-3)	0.25b	1.4c	10.12a	18.3ab
Control (water)	0.38a	2.0bc	11.18a	13.1b

* Experiment was conducted under controlled conditions with field soils inoculated with spore suspension ($1x10^6$ spores/ml) of each mycoparasite and with sclerotia and mycelium of *R. solani*. Four replicate pots were used per treatment and two tubers planted in each pot. All pots were incubated at 25^0C for 5 weeks.

** Mean values followed by the same letter within the column do not vary significantly each other according to Least significant difference test ($P \geq 0.05$).

7.4 Discussion

To assess the ability to control and protect potato plants from Rhizoctonia stem and stolon canker, the three mycoparasites, F2, F132, and F11 were chosen on the basis of their efficacy of sclerotial inhibition, production of a range of extracellular enzymes of their saprophytic and mycoparasitic ability, mode of parasitism, and increased germinability of table beet seeds. Significant level of protection of potato plants from Rhizoctonia disease were achieved with mycoparasites isolates F2, F132, and F11 in field soils artificially infested with *R. solani* (AG-3).

When applied as a spore suspension (10^6 conidia/ml), each mycoparasite reduced Rhizoctonia canker severity compared with diseased control (AG-3) treatment. Data in Table (9) indicated that the inocula of the 3 mycoparasites depressed sclerotial formation significantly. The number of sclerotia in the control was much higher than other treatments when potato seeds were planted in soil. In addition, these mycoparasites were also found to be effective in reducing sclerotial viability as shown in table 3. These results agree with those of Velvis and Jager (1983) who demonstrated that liberal seeding of natural soil with *Verticillium biguttatum* inhibited formation of sclerotia.

In some biocontrol systems, using *Trichoderma* and *Gliocladium* species to control Rhizoctonia diseases in lettuce, growth stimulation of lettuce has been recorded (Coley-Smith *et al.*, 1991; Lynch *et al.*, 1991 and Maplestone *et al.*, 1991). In our system, none of the mycoparasites demonstrated an increase plant height. However, mycoparasite F11 showed increased fresh weight of new tubers compared to both controls. Similarly, neither the UK *V. biguttatum* isolate Vb1 nor the Dutch isolate M73 stimulated an increase in healthy plant height. On the other hand, there was a significant increase in dry weights of shoots and roots compared with controls (AG-3). Our findings indicate that the growth chamber evaluation method was useful to identify isolates with potential in controlling *R. solani* in agricultural practice. Other than being able to protect plants against disease, biocontrol agents should provide a long-lasting protection similar to that of chemical

treatments and should be non-pathogenic to other crops (Heikki and Lynch, 1995). In this study, the success of the mycoparasites against *R. solani* is believed to be based upon : (i) reduction of sclerotial formation on tubers (ii) prevent canker severity on stolons and stems (iii) increase shoots and roots dry weight and (iv) increase fresh weight of new tubers.

CHAPTER 8.

GENERAL DISCUSSION AND CONCLUSION

In the eastern provinces of Canada, Rhizoctonia disease of potato is the most common disease (Platt *et al.*, 1993). The saprophytic stage of the pathogen *Rhizoctonia solani* (AG-3) occurs when the fungus overwinters as sclerotia on potato tubers or as mycelium in the soil and infect the plant. The disease is controlled by fungicides, but concerns about the risks that synthetic fungicides pose to the environment and human health have increased the urgency for more research into non-chemical methods of crop production. Therefore, other approaches of disease control have to be studied. Cultural control for example, crop rotation, flooding, fertilization are alternative methods but they are not sufficient alone to control Rhizoctonia disease of potatoes. Biological control is attractive as a potentially powerful tool which can be used in combinations with other methods to control the disease. This study was undertaken to screen and select antagonists indigenous to Québec soil in the hope to find some that can be used as effective biocontrol agents of *R. solani*.

Effectiveness of screening criteria

Concepts and approaches for screening or developing biocontrol agents appear to vary greatly *in vitro* and *in vivo*. Depending on the objectives and whether biological relevancy has been considered in experimental designs, the screening procedures have to be well studied. Our first objective was to obtain a collection of a wide number of species which were in close contact with the pathogen in order to increase the probability of finding potential biological control agents. This collection of fungi was the basis of our future studies, in the development of biocontrol agents for *R. solani*. The organisms were identified to the genus level only. Those that have demonstrated excellent biocontrol abilities will be identified in the future to the species level.

Our overall experimental set up or screening criteria involved a methodical approach. The primary screening test of antagonists was directed towards sclerotial viability which was successful since inhibition of sclerotial germination is considered the basic criteria of biological control (Papavizas and Lumsden, 1980). The results of this revealed that more than 50% of the tested fungi significantly inhibited sclerotial germination, bringing the number of fungi down to 24 potential antagonists. Among these, 6 isolates protected table beet seedlings from Rhizoctonia damping-off and root rot, and some of them even increased seed germination. Another criterion that we tested was the survival of these antagonists in natural soils. Our results from population studies suggest that the introduced antagonists survived and can be maintained at a certain carrying capacity when applied at a population density of $1x10^6$spores/ml in field soils.

Another criteria that we investigated was the mechanisms of control of selected isolates F2, F11, and F132 which were identified as species of *Pestalotia*, *Penicillium*, and *Gliocladium* respectively, as well as their ability to produce cell wall hydrolytic enzymes. We have found that the correlation between sclerotial inhibition and mode of action can be partly explained by the ability of fungal isolates to produce hydrolytic enzymes. Using light microscopy studies, antagonists were proved to be mycoparasites of *R. solani*, and the following sequence of events were observed (i) contact, (ii) formation of appresoria, (ii) penetration, and (iv) intracellular colonization of host hyphae. This led us to believe that mycoparasitism is the principal mode of action between the antagonists and their host *Rhizoctonia solani*. To further substantiate our hypothesis, our best pathogen-suppressing isolates, F2, F11, and F132 produced significant amounts of cell wall degrading enzymes such as chitinases, ß-1,3-glucanases, cellulases, proteases, and in turn, they effectively inhibited *in vitro* sclerotial germination of *R. solani*.

Finally, the efficacy of these mycoparasites to control Rhizoctonia disease was carried out in field soils artificially infested with *R. solani*. As expected, the mycoparasites reduced the severity of Rhizoctonia symptoms in potato which strongly suggests that F2,

F11, and F132 may be promising biological control agents for Rhizoctonia disease of potatoes.

In summary, the sequence of our operations resulted in selecting suitable antagonists which were able to control the target disease. However, the last resort would be to add other criteria, such as their temperature growth range, their sensitivity to the environment, and their pathogenicity to the other crops.

Further perspective

Extending our biocontrol studies from controlled environmental conditions to natural condition is essential in order to enhance our understanding of the interaction between the antagonists and the pathogen. Detailed field studies will not only provide us with realistic information but also identify how best we can integrate these biocontrol agents into cultural practices. Success in the future will probably result from use of a combination of antagonists with cultural practices that could effectively inhibit the target organism.

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10. APPENDICES

AI a. Medium to isolate fungi:

Half-strength of Potato Dextrose Agar (PDA) supplemented with the antibiotics, chloramphenicol and novobiocin at the rate of 100ppm /L.

AI b. Medium to isolate bacteria:

Half-strength of Nutrient Agar (NA) with the antibiotic, cyclohexamide at the rate of 100ppm /L.

AII. Selective media of *Trichoderma* and *Gliocladium*

Chemical	G/L
$MgSO_4.7H_20$	0.2
K_2HPO_4	0.9
KCL	0.15
NH_4NO_3	1.0
Glucose	3.0
Chloramphenicol	0.25
PCNB	0.20
Rose bengal	0.15
Agar	20.0
H_2O	1000ml

Autoclave at 121^{0}C for 20 min. Pour into plates when culture medium is cooled to 45-55^{0}C. This medium can be stored for 1-2 months at 4^{0}C.

AIII a. Oat-soil technique:

Sieved pasteurized soil was used to store fungi. The soil was kept for 24 hours to dry. Grounded oat meal (w/w) was added to the soil. Half of a glass test tube was filled. Pour 6 ml distilled water in each tube. Use cotton plug to close mouth of tube. Autoclave all tubes for 45 min. on a 3 consecutive days. A small agar piece of fungal culture (0.5-0.8 cm diam.) placed on top of the soil in the tube using sterile scalpel. Copper sulphate sealant (Appendix AIV) was used to seal cotton plug of each test tubes. All tubes were stored at 4^0C.

AIII b. PDA slant

Potato dextrose agar (PDA) was poured into test tubes in slanting position. A fungi bearing plug was inoculated onto the slanting agar area surface in the tube. After growing the whole area of PDA slant, sterile mineral oil was poured in the tube, close cap and parafilm it. All tubes were stored at 4^0C.

AIV. Copper sulphate sealant

Ingredients:

Copper sulphate (Cu_2SO_4)	2g
Gelatin	10g
Distilled water	100ml

Procedure:

- Heat distilled water
- add Cu_2SO_4
- add gelatin
- pour into petri plates
- flame cotton in tubes
- dip into sealant

- apply cigarette paper

- flame extra paper

- wipe with kimwipes

Store copper sulphate sealant plates at 4^0C and use it.

AV. Sodium-glycerol (NaGly) media to store bacteria at -80^0C:

1000 ml distilled water

8 g nutrient broth

20% glycerol in H_2O (v/v)

Procedure: Placed enough of this medium (about 1.25 ml) in a bacterial storage vial to fill it 60% full. Autoclave it for 20 min. Each vials was inoculated with a loop of bacterial colony. The vial was vortexed for 30 seconds and keep stored at -80^0C. To use the stored cultures, touch (rub) an autoclaved Q-tips to the top of the frozen medium and streak normally on nutrient agar plate.

BI. Synthetic Nutrient-poor Agar (SNA) media

Chemical	G/L
KH_2PO_4	1.0
KNO_3	1.0
$MgSO_4.7H_2O$	0.5
KCL	0.5
Glucose	0.2
Sucrose	0.2
Bacto Agar (Difco)	20.0
H_2O	1000ml

Autoclave at 121^0C for 20 min. Place pieces (1 cm^2) of sterile filter paper (Whatman no. 1) onto the agar surface since this helps to induce more spores of *Fusarium* spp.

CI. Iodine solution

Iodine	0.3g
Potassium iodide (KI)	1.5g
Water	100ml

Dissolve on hot plate with magnetic stirrer. Store at room temperature.

CII. Buffer A

Ingredients: 0.05 M Na_2HPO_4 solution (add 13.40 gm /L. of double distilled water)

0.01 M citric acid solution (add 1.25 gm/500 ml of DDW)

Procedure: To make buffer A at pH 6.3

Na_2HPO_4	200 ml
citric acid	220 ml

Adjust the pH at 6.3 and store at 4^0C.

1 M Nacl in buffer A :

Buffer A	100 ml
NaCl	5.844 gm (molecular weight of NaCL is 58.44)

DI. Minimal synthetic media (MSM)

Chemical	G/L
$MgSO_4.7H_20$	0.2
K_2HPO_4	0.9
KCL	0.2
$FeSO_4.7H_20$	0.002
$MnSO_4$	0.002
H_2O	1000ml

Adjust the pH at 6.3. For nitrogen source add NH_4NO_3 1 mg/ml.

DII. Dimethylaminobenzaldehyde (DMAB) color reagent

Stock solution :

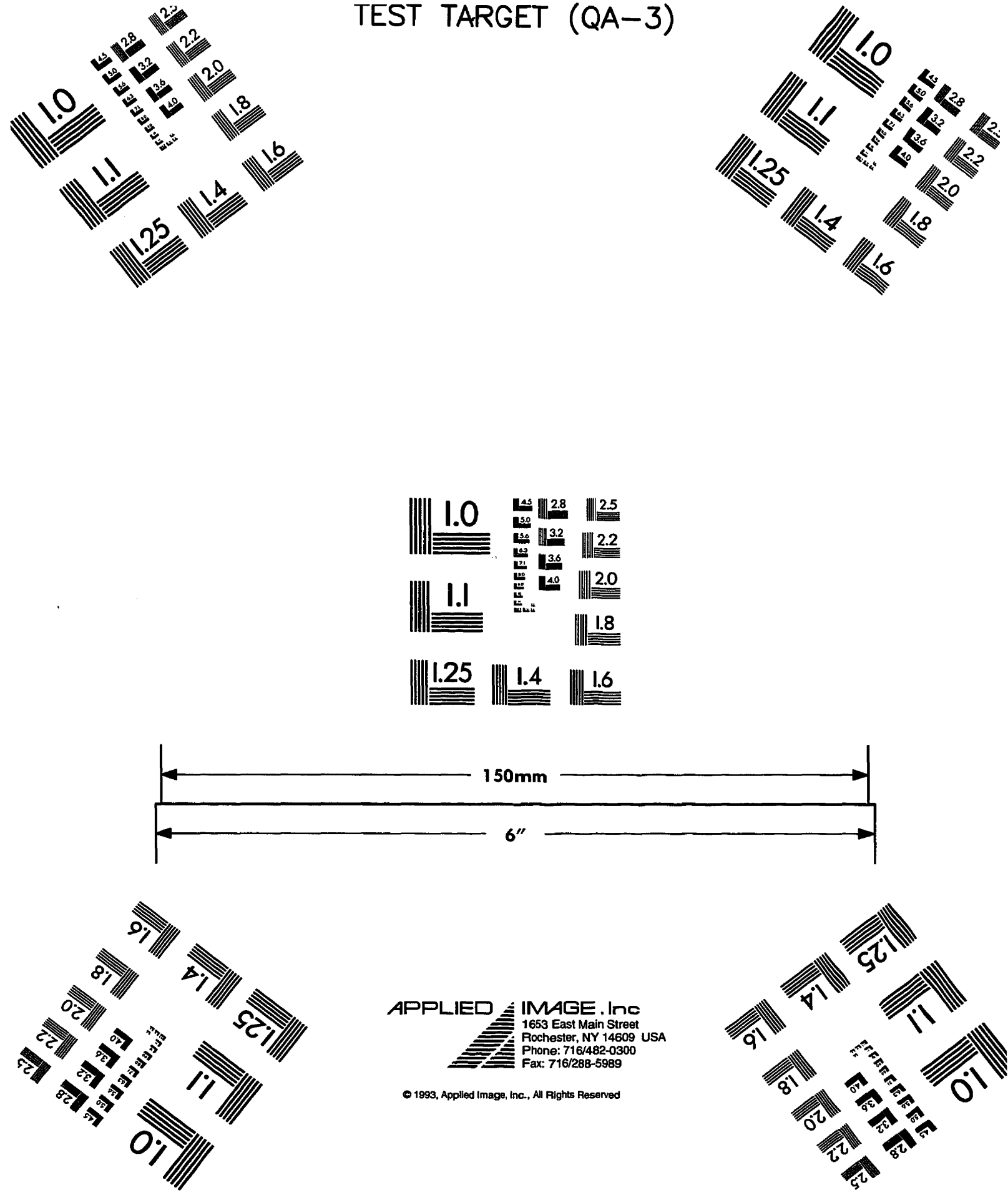
p-dimethlaminobenzaldehyde	8g
glacial acetic acid	70 ml
concentrated HCL	10 ml

Mix 1 vol. of stock solution with 9 vol. of glacial acetic acid immediately before use. Stock solution can be stored at 4^0C.

DIII. DNS reagent

3,5 Dinitro salicylic acid	7.49 gm
NaOH	13.98 gm
Na-K tartarate	216.10gm
Phenol	5.37ml
Na meta bisulphate	5.86gm

Dissolve 3,5 Dinitro salicylic acid and NaOH, then add Na-K tartarate, phenol and Na meta bisulphate.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