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Development of a specific and reliable molecular marker to detect *Stachybrotyrs elegans*, a destructive mycoparasite of *Rhizoctonia solani*

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Short Title: Molecular marker of a biological control agent

ABSTRACT

M. Sc. **Plant Science**

Development of a specific and reliable molecular marker to detect *Stachybrotyrs elegans*, a destructive mycoparasite of *Rhizoctonia solani*

Xiben Wang

Stachybotrys elegans (Pidopl.) W. Gams is a destructive mycoparasite of the soilborne plant pathogen *Rhizoctonia solani*. It colonizes effectively all types of cells of *R. solani*, and is considered as an effective biological control agent (BCA). Monitoring the presence of this mycoparasite in the field trials requires the development of a reliable and sensitive diagnostic assay that is able to detect and differentiate the BCA from their target host. To achieve this, designed SCAR (sequenced characterized amplified regions) primers designated as SE-13F and SE-13R were generated from informative RAPD markers. They were tested in conventional PCR assays alone or in conjunction with the recently developed SCAR primers (SBU-177/336) designed for *Rhizoctonia solani* (Kühn) on several types of DNA. These included DNA extracted from pure cultures, co-cultures of the BCA and the pathogen, plant tissue and several types of soils inoculated with both the BCA and the pathogen. Irrespective of the type of the biological samples from which the DNA was extracted, the primers SE-13F/SE-13R successfully amplified only *S. elegans*. No cross-reaction was observed when the primers were used to amplify DNA of other fungi, bacteria and plant tissues. Likewise, the primer pair SBU-177/336 detected only its target organism, i.e., *R. solani*. The detection limit using these primers

on amplified DNA was as little as 1 pg DNA extracted from pure cultures of *S. elegans*, 100 pg DNA extracted from greenhouse soil and 33 pg DNA extracted from natural soil. This work is the first report on the development of SCAR markers for the BCA, *S. elegans*. These molecular markers offer not only an alternative diagnostic assay to conventional detection methods, but also the possibility of being used in ecological studies.

RÈSUMÈ

RÉSUMÉ

Développement de marqueur moléculaire spécifique et fiable pour la détection de *Stachybotrys elegans*, un mycoparasite destructeur de *Rhizoctonia solani*

Xiben Wang

Stachybotrys elegans est un mycoparasite destructeur du pathogène de sol *Rhizoctonia solani*. Il colonise efficacement tous les types de cellule de *R. solani* et est considéré comme un agent efficace de lutte biologique. La détection de la présence de ce mycoparasite en sol exige le développement d'un outil de diagnostic fiable et sensible qui serait capable de détecter et de différencier l'agent de biocontrôle de son hôte cible.

Pour réaliser cela, les amorces SE-13F et SE-13R de régions séquencées, caractérisées et amplifiées (RSCA) ont été conçues à partir de marqueurs repères RAPD. Ces amorces ont été testées seules dans des analyses conventionnelles de réaction de polymérase en chaîne et conjointement avec des amorces RSCA SBU-177/336 récemment développées pour *R. solani* sur plusieurs types d'ADN. Les ADNs ont été extrait de cultures pures, de cultures co-inoculées avec l'agent de biocontrôle et le pathogène, de tissu végétal et de plusieurs types de sol inoculés avec les deux microorganismes.

Indépendamment du type d'échantillon dont provenait l'ADN, les amorces SE-13F et SE-13R ont amplifié avec succès seulement *S. elegans*. Aucune réaction croisée n'a été observée lorsque les amorces ont été utilisées pour amplifier l'ADN de d'autres

champignons, bactéries et tissus végétaux. De même, les amorces SBU-177/336 ont détecté seulement le microorganisme cible, *R. solani*. La limite de détection en utilisant les amorces SE-13F et SE-13R sur de l'ADN amplifié était aussi petite que 1 pg à partir de culture pure de *S. elegans*, de 0.5 pg d'ADN /mg de sol de serre et de 0.33 pg d'ADN/mg de sol naturel.

Cette étude est le premier compte-rendu sur le développement d'amorces RSCA pour l'agent de biocontrôle *S. elegans*. Ces marqueurs moléculaires offrent non seulement une alternative aux méthodes de diagnostic conventionnelles mais aussi la possibilité d'être utilisées dans des études écologiques.

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INTRODUCTION

Development of a specific and reliable molecular marker to detect *Stachybrotyrs elegans*, a destructive mycoparasite of *Rhizoctonia solani*.

M. Sc. Xiben WANG Plant Science

Stachybotrys elegans (Pidopl.) W. Gams is a destructive mycoparasite of *R. solani* (Benyagoub *et al.* 1994). It colonizes and kills hyphae of *R. solani* (Kühn) and causes a drastic decrease in the germinability of sclerotia. Turhan (1990) reported that *S. elegans* has the strongest mycoparasitic ability to *R. solani* compared to other soil fungi such as *Trichoderma spp.*, *Coniothyrium spp.*, *Gliocladium spp.*, and *Verticillium spp.* Cell wall lytic enzymes excreted by *S. elegans* are believed to play an important role in the mycoparasitic process (Tweddell *et al.*, 1994 and 1996, Archambault *et al.*, 1998). All of the above evidences show that *S. elegans* is a promising candidate for the biological control of diseases caused by *R. solani*.

Our understanding of the diversity and function of fungal communities, particularly biological control agents in natural soils can be limited when only culture-dependent methods and morphological traits are used. The current method for isolation of *S. elegans* from soil mainly relies on axenic culture from selective media. (Turhan *et al.*, 1990). Identification of *S. elegans* is based on microscopic examination of morphological traits including phialide morphology and conidial shape (Barron *et al.*, 1964). These methods are relatively simple and inexpensive, but can be labor-intensive. Effective management practices in practical use of this biocontrol agent require the development of a rapid and accurate method for detecting *S. elegans* in soil. This is an essential approach to understand the distribution and population change of *S. elegans* in soil.

Molecular tools based on the extraction of nucleic acids from biological samples and amplification by polymerase chain reaction (PCR) of various sequences have been developed to overcome these limitations. Several of these molecular tools are being currently used to detect and monitor fungal and bacterial populations in natural environments (Forster *et al.*, 1994).

The Polymerase Chain Reaction (PCR) based techniques for species-specific detection assays have become very popular recently. Compared to other diagnostic methods, PCR based techniques have the advantages that they do not require the target organism to be cultured prior to detection and they are very sensitive, rapid and specific. These PCR-based diagnostic assays can be developed by exploiting sequence polymorphisms within internal transcribed spacer (ITS), unique sequences of mitochondrial DNA, cloned restriction fragments of genomic DNA, or the use of a sequenced fragments derived from randomly amplified polymorphic DNA (RAPD) (Edel, 1998).

The objectives of this research were to develop: (i) PCR primers using informative RAPD markers for specific amplification of *S. elegans* and (ii) a reliable and sensitive assay for the detection of *S. elegans* in soil and during interaction with its host, *R. solani*. The application of this technique will be useful for practical use of this biological control agent.

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CHAPTER 1.

GENERAL INTRODUCTION AND REVIEW OF LITERATURE

1. 1. The genus Stachybotrys:

The genus *Stachybotrys* Corda [teleomorph *Memnoniella echinata* (Rivolta) B. T. Galloway] which belongs to the Hyphomycetes was proposed by Corda in 1873, and includes 13 different species. Most members of *Stachybotrys* have a wide geographical distribution and can commonly be isolated from soil. They are capable of utilizing cellulose and damaging fabrics made of plant fibers as their carbon source. *S. chartarum* (Ehrenb.) S. J. Heghes is the most common species of this genus and has a worldwide distribution. Strains of *S. chartarum* are known to produce a mycotoxin called stachybotryotoxins, which have been reported to afflict animals and man (Nikulin *et al.*, 1994).

Another mycotoxin producer is *S. cylindrospora* C. N. Jensen. Hiratsuka *et al.* (1994) showed that this species produces two antifungal toxins, trichodermin and trichodermol which are effective against the *in vitro* growth of *Ophiostoma crassivaginatum* (H. D. Griffin) T. C. Harrington, a blue stain fungus. These results suggest that *S. cylindrospora* can be a potential candidate for the biological protection of wood chips and wood products of aspen from this stain-producing fungus.

S. elegans has been shown to be a destructive mycoparasite of the soilborne pathogen *Rhizoctonia solani* AG-3 with a great potential act as a biocontrol agent against the *Rhizoctonia* disease of potato (Tweddell *et al.*, 1994).

1. 2. Stachybotrys elegans: a destructive mycoparasite of R. solani AG-3

1. 2. 1. General introduction of S. elegans

S. elegans was first isolated from soil in Winnipeg, Canada in 1947. In other regions of the world, including Egypt, South Africa, Mozambique, Germany, Papua-New Guinea, the Society Islands, it has been recorded from soils and rhizospheres of many plants (Jong and Davis, 1976). Based on the size and shape of phialospores, Srinivasan and Barran (Barron, 1964) reported two new *Stachybotrys* species, *S. bisbyi* (Srinivasan) Barron and *S. sacchari* (Srinivasan) Barron. However in 1980, because of the similar morphological characteristics, isolates from *S. bisbyi* and *S. sacchari* have been regrouped into *S. elegans* (Domsch *et al.*, 1980).

1. 2. 2. Mode of action of S. elegans

Mycoparasites are fungi that parasitize other fungi, growing on expense of nutrient from other fungal hosts. Two types of mycoparasites have been found. One type is necrophic mycoparasite which kills functional host cells rapidly and then derives nutrients from dead cells, such as *Pythium spp*, *Trichoderma spp.* and *S. elegans*. Another type is a biotrophic mycoparasite. This type of mycoparasite can feed on cells that remain alive for quiet long time while being parasitized. For example, *Verticillium biguttatum* Kleb is a biotrophic mycoparasite of *R. solani*. This obligate mycoparasite does not kill the *R. solani* hyphae when it penetrates, but feeds on living cells (Jager and Velvis, 1988).

In recent years several mycoparasites have been employed with some success in the control of plant diseases in the greenhouse or field. Some of these, such as *Trichoderma* spp. and *Pythium nunn* Drech have long been known as effective

antagonists against plant pathogenic fungi. The most prominent and extensive species studied are species of *Trichoderma* and *Gliocladium* (Baker, 1986) which are known to control *R. solani* and other fungal pathogens through their mycoparasitic action. Another promising biological control agent is the obligate mycoparasite *V. biguttatum* Kleb which has been used on large scales in the Netherlands to control Rhizoctonia disease of potato (Boogert and Deacon, 1994).

The antagonistic activity of *S. elegans* on the soil-borne plant pathogen *R. solani* was first assessed by Turhan (1990), and confirmed by Benyagoub and his co-workers (1994) who demonstrated that *S. elegans* was a destructive mycoparasite of *R. solani* AG-3. *In vitro* studies conducted by the latter authors revealed that *S. elegans* parasitised its host using several successive steps: (a) growth toward the host (b) contact and appressed growth, (c) coiling, (d) penetration of the host hyphae and internal growth, and (e) subsequent sporulation outside the host. When *S. elegans* encounters the host cells of *R. solani*, it is stimulated to overproduce an extracellular fibrillar matrix similar in chemical nature to the fimbriae of *Ustilago violacea* (Benyagoub *et al.*, 1996). These results strongly suggest that the fibrillar matrix play a role in the mycoparasitic process.

The recognition and attachment of most mycoparasites to their host surfaces involve the specific interaction of a proteinacous lectin with specific sugars on the surface (Manocha and Sahai, 1993). In the case of *S. elegans*, moderate amounts of galactose and glucose sugar residues were detected in the cell walls of *S. elegans* confirming that *R. solani* cell walls contain a D-galactose binding lectin (Archambault *et al.*, 1998).

Ultrastructural studies confirmed that partial degradation of *R. solani* mycelial and sclerotial cell walls at the sites of penetration by the mycoparsite occurred (Benyagoub *et al.* 1996). However, direct evidence implicating that *S. elegans* produces several extracellular cell wall enzymes during mycoparasitism and that some of them are responsible for cell lysis was established recently. Chitinases and endo-1,3-β-glucanases are produced in moderate amounts when *S. elegans* is grown on purified cell wall of *R. solani* as the sole carbon source (Tweddell *et al.*, 1995 and 1996). At least four extracellular 1,3-β-glucanases (75, 94, 110 and 180 kDa) are produced by *S. elegans* among which the 94 and 75 kDa 1,3-β-glucanases have been purified to homogeneity and tested for their biologiacl activity against living cells of *R. solani*. Exposure of growing hyphal tips of *R. solani* to either glucanases cause them to swell and lyse (Tweddell *et al.*, 1995; Archambault *et al.*, 1998). In addition, several extracellular chitinases, such as chitobiosidase and endochitinase, are also produced under the same conditions (Khan *et al.*, 1999).

All of the above evidences confirm that *S. elegans* has the potential to be used as biological control agent against the disease caused by *R. solani* AG-3.

1. 3. Different methods for the fungi detection

The efficient detection of mycoparasites and their target organism populations in soil is essential for monitoring the effects of treatments and for developing efficient control strategies. To date, various types of methods have been developed for the differentiation and detection of microorganisms including conventional cultural methods, chemodiagnostic methods, serological methods and nucleic acid-based methods.

1. 3. 1. Conventional cultural methods

Several techniques, involving baiting and culture plating on selective media have been developed for the detection of soil-borne fungal pathogens. Generally, fungi are isolated from the selective media, and visually identified based on specific morphological structures, cultural behavior or pathogenicity tests.

Different types of selective media are available for the isolation of *R. solani* from plant tissues and soil. Papavizas and Davis (1959) found that a mixture of fresh solution containing 50 ppm each of aneomycin hydrochloride, neomycin sulfate and streptomycin sulfate in water agar can increase the frequency of isolating *Rhizoctonia* spp. from soil. Latham and Linn (1961) reported that vancomycin and novobiocin did not inhibit the growth of *R. solani* while these two antibiotics can inhibit the growth of other contaminants. Yuen *et al.* (1994) used water agar media amended with metalaxyl and streptomycin sulfate to isolate *Rhizoctonia* spp. from soil. Vincelli and Beaupre. (1989) found that ethanol-potassium medium can be used to differentiate *R. solani* from other fungi.

For the detection of antagonists in soil, mainly mycoparasties, two common methods have been routinely used: 1) Direct plating of soil onto agar colonized by a host fungus. This method has been used to detect mycoparasites in soil such as *Pythium oligandrum* Drechs (Deacon and Henny 1978) and *S. elegans* (Turhan, 1990) and 2) Baiting of soil with mycelia, sclerotia or resting spores of the host, then isolating the mycoparasite from the baits on selective media. The baiting methods are used mostly for

biotrophic or host-specialized mycoparasites such as *Verticillium biguttatum* Kleb (Boogert and Jager, 1983).

These methods are simple, cheap and do not require complicated laboratory equipment. However they can be labor-intensive and results may not be obtained for weeks. In addition, considerable experience is needed for reliable diagnosis since visual inspection may be complicated by the similarity of morphological characteristics between different fungi existing in the same sample.

1. 3. 2. Chemodiagnostic methods

Fungi that are either slow growers or do not produce any spore bearing structures require an alternative identification tool instead of the conventional method. In these cases, biochemical properties such as specific chemical compounds and total protein profiles of fungi can be used as diagnostic tools for these fungi

1. 3. 2. 1. Biochemical test

Specific chemical compounds produced by certain fungi can be used to indicate the presence of fungi in samples. Fluorescein diacetate is a good indicater of lipase activity and has been used to detect the presence and viability of teliospores of *Tilletia controversa* Kühn on seeds (Chastain and King, 1990). Detection of β-glucronidase activity in infected tomato with *Cladosporium fulvum* (Cooke) was related to the presence and the amount of *C. fulvum* biomass in tomato (Oliver *et al.*, 1993). Sterols and fatty acid profiles have been used singly or in combination to differentiate among the species of *Aspergillus* (Nemec *et al.*, 1997).

1. 3. 2. 2. Protein profiles analysis

Gel electrohphoresis of fungal proteins has been shown useful in fungal taxonomy. This technique involves the use of the polyacrlymide gel to separate the total proteins extracted from fungal mycelia or conidia, followed by staining to visualize the separated proteins. It has been used for the species and subspecies identification of various soil fungi. Distinct electrophoretic patterns of soluble proteins revealed the variation between 5 different *R. solani* AGs (Reynolds *et al.*, 1983). The differentiation among six species of *Pythium* on winter cereals was achieved using the electrophoretic profile of the wall protein alanine-rich hygroxyproline (Takenaka and Kawasaki, 1994).

However this technique has several limitations. First, the initial set-up stage requires significant time and manipulation in order to produce reliable and reproducible protein profiles. Second, the electrophoretic protein profiles is difficult to interpret.

1. 3. 2. 3. Isozyme analysis

Isozymes are multiple molecular forms of a single enzyme that have similar enzymatic properties but slightly different amino acid sequences. Detectable isozymes can arise from three different genetic and biochemcial conditions: 1) multiple alleles at a single locus, 2) multiple loci coding for a single enzyme, and 3) secondary isozymes arising from post-translational processing (Bonde *et al.*, 1993). There are up to 90 enzymes that could be examined and compared in fungi.

Since isozymes are able to detect significant changes in enzyme structure which is often at the species level, isozyme analysis has been used to differentiate morphologically

similar or cloesly related species, varieties and formae speciales of several fungi. For example, Laroche *et al.* (1992) applied 7 isozymes to differentiate among 44 isolates of *R. solani* belonging to AG-3 and AG-9 into two dissimilar groups. The electrophoretic groups are in agreement with the anastomosis grouping concept. A similar technique was used by Damaj *et al.* (1993) to analyse the genetic variations in binucleate *Rhizoctonia* species. Four distinct isozyme groups were generated and these groups were consistent with prior groups determined by hyphal anastomosis and by DNA restriction pattern analysis.

Isozyme analysis has been also used to differentiate among biological control agents. For example, Leuchtmann *et al.* (1996) used isozyme analysis to distinguish among *Trichoderma longibrachiatum* Rifai, *T. citrinoviride* Bissett, and *T. parceramosum* Rifai, while Stasz *et al.* (1988) successfully used difffernet isozyme systems to differentiate *Trichoderma* from its closely related genus *Gliocladium*.

Recenlty, the technique based on the detection of various pectinases has also been found useful in identifying groups within *Rhizoctonia* spp. Using this technique, Sweetingham (1990) established 5 distinct pectic zymograms-pattern groups (ZGs) within *R. solani* AG-8. These ZGs are found to correspond with grouping based on random amplified polymorphic DNA markers. A similar technique was also used by Ballali *et al* (1995). In their research, field isolates of *R. solani* AG-3 were divided into three distinct ZGs.

Isozyme analysis is much faster and far less expensive than either immunological tests or tests involving the polymerase chain reaction (PCR) and species-specific primers (Bonde *et al.*, 1993). However, this technique also has some drawbacks. First, in isozyme

analysis large amounts of organism must be present compared with that required for immunological or PCR techniques. Second, since there are many facts which can affect the mobility and detection of isozymes, including conditions under which an organism is grown, pH, electric current, buffer system, age and strength of staining solution, much time is needed to set up a system that yields consistent results.

1. 3. 3. Serological diagnostic methods

Serological detection is based on the well-known capacity of mammals to develop antibodies in the blood that react specifically to the antigens which are foreign proteins or carbohydrates. Various serological techniques have been developed in the last ten years for the detection of fungal or bacterial pathogen. Among them, enzyme linked immunoabsorbent assay (ELISA) is mostly used. Because of its sensitivity, rapid identification and capacity of handling large number of samples, ELISA has become a preferred serological diagnostic method to detect soil fungi such as species of *Phytophthora* (Miller *et al.*, 1997), *Pythium* (Macdonald *et al.*, 1990), *Trichoderma* (Thronton *et al.*, 1996a) and *Gliocladium* (Breuil *et al.*, 1992) and *Rhizoctonia* (Thronton *et al.*, 1996b)

Many other types of serological methods have also been developed for diagnostic purposes such as radio-immunoassay (Savage and Shall, 1988), dip stick immunoassay (Dewey, 1988), dot immunobinding assay (Gleason *et al.*, 1987), immunofluorescence assay (Gabor *et al.*, 1993.) and immunogold labelling (Day *et al.*, 1986) and PCR-ELISA (Leblanc, *et al.*, 1999)

1. 3. 3. 1. Immunoreagents

Antibodies are serum proteins known as immunoglobulins and are produced from β-lymphocytes in response to the presence of foreign materials. Different biological materials have been developed for the preparation of antigens, which range from whole cells, thalli and/or spores to broken cells, culture filtrates, surface washings, purified proteins, enzymes, toxins and polysaccharides (Werres and Stettens, 1994). The antibodies commonly used in immunoassays include polyclonal antibodies and monoclonal antibodies.

1. 3. 3. 2. Polyclonal antibodies

Polyclonal antibodies (PABs) have been used routinely in immunoassays for plant pathogen diagnosis. PABs are relatively easy and inexpensive to prepare and require only a short time for their development. However, PABs contain a population of antibodies that may react with determinants of different immunogens which can lead to undesired cross-reactivity.

Wakeham and White (1996) used whole and sonicated resting spores of *Plasmodiophora brassicae* Woronin to develop specific polyclonal antisera. The antisera showed a low cross-reactivity with a range of other soil fungi. Lu *et al.* (1994) used rabbit antiserum against exoantigens of *Penicillium aurantiogriseum* Thom in an indirect competitive ELISA. The exoantigens showed a very low cross-reactivity with the exoantigens from other species and genera of fungi and had no cross-reactivity with water-soluble plant protein. Matthew and Brooker (1991) developed polyclonal antibodies which were raised against the secreted protein from *R. solani* AG-8. They

found that different *R. solani* AG groups can be differentiated based on the immunoblot pattern using these polyclonal antibodies. However because of cross-reactivity, the antisera can not be used directly to detect certain individual isolate in contaminated samples.

1. 3. 3. 3. Monoclonal antibodies

Because monoclonal antibodies (MABs) react with a single determinant of an antigen, they exhibit high specificity useful to detect organisms at the genus, or species level. Until now, MABs have been used in different serological diagnostic methods to detect fungi in different types of samples, such as wood, mycorrhizal plants, soil, plant and food (Burge *et al.*, 1996). Thornton and Dewey (1996a) developed an ELISA assay for the detection of *Trichoderma harizanum* (Bonord.) Bainier using a monoclonal antibodies raised against phialoconidia of this fungus. The same author also developed a monoclonal antibody using the surface washing of slant cultures of *R. solani* (AG-2) isolates (Thornton, 1996b). The antibody showed high specificity and reacted with the surface washing from *R. solani* isolates and did not recognize any of the other soil fungi tested.

Several commercial diagnostic kits based on monoclonal antibodies have been developed for the detection of some fungal pathogens. They have proved to be useful where there is a need for a rapid and accurate diagnosis of the disease. An ELISA kit for *Phytophthora* spp. based on monoclonal antibodies has been developed by Agri-Diagnosis Associates (Cinnaminson, New Jersey, USA). It can readily detect *Phytophthora* spp. in irrigation water, diseased soybean root and soybean field soil

(Timmer *et al.* 1993). Also, A ELISA kit based on monoclonal antibody has also been developed by Agri-Diagnostics Associates for the detection of fungi belonging to the genus *Rhizoctonia*. The assay has been used to detect *R. solani* and binucleate *Rhizoctonia* spp. in container-grown ornamentals (Macdonald *et al.*, 1990).

1. 3. 4. Nucleic acid-based diagnostic methods

Recently because of the rapid development of recombinant DNA technology, the application of molecular techniques in the diagnosis of plant pathogens has become popular. Compared to conventional cultural methods and biochemical methods, nucleic acid-based assays have several advantages. Nucleic acid-based diagnostic methods are more stable and accurate compared to other methods since its target is nucleic acid. The sensitivity of nucleic acid-based diagnostic methods can be very high when hybridization and polymerase chain reaction techniques are used. Relatively small amounts of raw materials are required for the nucleic acid base assays. Different molecular approaches have been developed recenlty for the genetic identification of fungal strains and species.

1. 3. 4. 1. Nucleic acid-based detection techniques.

1. 3. 4. 1. 1. Nucleic acid hybridization techniques

A DNA probe is a section of single-strand DNA having a sequence homologous to a specific portion of the genome of a particular organism. The probes may be radioactively or biotin-labeled and used in different types of hybridization assays (e.g., filter, solution and *in situ* hybridizations). Depending on the applications, all these assays have been successfully used to detect microorganisms.

A species-specific repetitive DNA probe of *Phytophthora parasitica* Kleb was developed and used in slot blot and colony hybridization assays to detect *P. parasitica* in tomato plants, infested soil, plant leaf samples (Goodwin *et al.*, 1989). Whisson and Francis (1995) developed a high copy DNA probe, pRAG12, that shows high specificity to the bare patch pathogen *R. solani* AG-8. As little as 50 pg of AG-8 DNA was detected in 1ng of total organic matter DNA obtained. Ballali *et al.* (1996) reported a highly repeated DNA fingerprinting probe specific to isolated of *R. solani* AG-3. This probe hybridized with all AG-3 isolates in southern blot, but had no or very weak reactions with isolates from other anastomosis groups.

Within the various *in situ* hybridization techniques, fluorescence in situ hybridization (FISH) is a relatively new and powerful technique. It was originally used in medicine and developmental biology. FISH combines the specificity inherent in nucleic acid sequences with the sensitivity detection systems based on fluochromes. With FISH, it is possible to observe the precise location of target sequence at organelle, cellular or tissue level (Uzawa and Yanagida 1992). To date a few applications of this techniques in fungal biology have been published. Li *et al.* (1997), labelled a 21-mer oligonucleotide probe directed at the 18s RNA of *Aerobasidium pullulans* (de Bary) Arnaud with fluorescein isothiocyanate, and applied it *in situ* hybridization to detect the presence of *A. pullulans* on the surface of apple leaves. Similarly, Nonomura *et al.* (1996) developed an assay for the detection of *Fusarium oxysporum* Schlechtend:Fr. in soil and in its host.

Since these methods can detect traces amount of DNA in small amount of cells, they have the potential to be used for detection of the latent infections, tissue or organ-restricted diseases and seed-borne pathogens (Chu *et al.*, 1989).

1. 3. 4. 1. 2 Restriction fragment length polymorphism

Restriction fragment length polymorphism (RFLP) analysis has been found useful to determine the taxonomic relationships among fungi. This technique makes use of restriction enzymes to digest DNA, which is then separated by agarose-gel electrophoresis. Difference in the size and number of restriction fragments can be detected by southern blot analysis or be observed directly by staining gels with ethidium bromide. These differences can result from loss or gain of restriction endonuclease recognition sites. Depending on the restriction enzyme used, digestion of target DNA may produce a set of specific fragments that can be considered as a fingerprint for a given strain.

Total, mitochondrial and ribosomal DNA have been used for RFLP analysis (Manicom *et al.*, 1987, Kim *et al.*, 1993, Muthumeenakshi *et al.*, 1994, Vilgalys and Gonzale, 1990). Because mitochondiral DNA is relatively small, and upon restriction enzyme digestion it can generate 10-20 distinct fragments on agarose gel, it has been used regularly as the target in the RFLP analysis. It is known that mtDNA fragment patterns are very similar within species but dissimilar among the different species (Brown *et al.*, 1994). In the studies of Kouvelis *et al.* (1999), mitochondrial DNA (mtDNA) restriction fragment length polymorphisms (RFLPs) were obtained from 54 fungal isolates identified as *Verticillium lecanii* (A. zimmerm.) Viegas, *V. psalliotae* Treschow. Analysis of the mtDNA RFLPs showed 20 different patterns, indicating considerable genetic variation within the V. *lecanii* species complex. Similar studies are also

performed in *Colletotrichum* spp. (Buddie *et al.*, 1999), *Phytophthora* spp. (Hall, 1997). *R. solani* (Banniza *et al.*, 1996).

In addition to mt DNA, rDNA is another type of DNA which has been used in RFLP assays. RFLP analysis of rDNA of various AG groups of *R. solani* revealed the distinct differences among AGs (Vilgalys and Gonzales 1990). Anastomosis groups 3, 4, 7 and 8 each had unique rDNA RFLPs and all isolates within an AG were homogeneous with respect to RFLPs. Jabaji-Hare *et al.* (1990) differentiated among 38 isolates of *R. solani*, representing 10 AGs using RFLPs. Their result indicated that strains belonging to AG-1, 2, 3 and 6 possessed one or more unique RFLP patterns which are not found in other groups. Intragroup RFLP variation was present in all AGs. A similar technique was also used to analyse the intraspecific molecular variation among *Trichoderma harzianum* isolates (Muthumeenakshi *et al.*, 1994). Three RFLPs groups were determined based on the RFLP patterns and furthermore these RFLP groups were conformed by random amplified polymorphic DNA (RAPD) analysis.

To date, the RFLP data is mainly used for assessing the genetic diversity of the pathogen population as well as for determining the extent of relatedness among the pathogen groups. However, it also can help to identify regions of DNA that could be used for the development of species- or race-specific probes. A few papers have been published in which RFLP was used to develop diagnostic probes. Yao *et al.* (1991) developed a specific DNA probe generated from mt DNA of *Peronosclerospora sacchari* with the help of RFLP analysis of mtDNA of *P. sacchari*. Jabaji-Hare *et al.* (1990) generated five random nuclear DNA recombinant clones *R. solani* AG-3 that were used in Southern hybridization to probe the DNA of *R. solani* isolates. All of the probes

hybridized only to AG-3 isolates indicating that they are group specific. The specificity of the hybridization patterns suggests these probes have the potential to be used in the diagnosis of *R. solani* AG-3 isolates.

In the past few years, a few articles have been published in which polymerase chain reaction (PCR) and RFLP were combined to detect specific microorganisms in complex biological samples. Liu and Sinclair (1993), used a PCR-based restriction mapping method to detect DNA

polymorphism in the 18S nuclear rRNA gene region intraspecific groups (ISGs) representing 11 anastomosis groups (AGs) of *R. solani.* Four types of DNA restriction maps of this region were constructed for these 25 ISGs. Bounou *et al.* (1999) combined restriction enzyme analysis and PCR to distinguish *R. solani* AG-3 from other anastomosis groups. An amplicon of 179 bp, produced by primer SBU177 and SBL336, was disgested with Xho I endonuclease which generated two fragments of 52 and 127 bp unique to *R. solani* AG-3 isolates.

1. 3. 4. 1. 3. Polymerase chain reaction based diagnostic methods

1. 3. 4. 1. 3. 1. Overview of polymerase chain reaction (PCR)

The polymerase chain reaction allows the exponential amplification of specific DNA fragments by *in vitro* DNA synthesis. This enzymatic reaction can amplify the specific DNA fragment from complex DNA samples. It provides a method for increasing the number of copies of a target sequence without having to culture the microorganisms, thereby allowing increased sensitivity in detecting trace amount of DNA sequence in mix samples.

The standard PCR requires a DNA template containing the region to be amplified and two oligonucleotide primers flanking this target region. The amplification is accomplished by using a thermostable DNA polymerase isolated from *Thermus aquaticus* called *Taq* polymerase. (Saiki *et al.*, 1988). PCR includes three essential steps: 1. Melting of the target DNA, 2. Annealing of two oligonucleotide primers to the denatured DNA strands, and 3. Primer extension by a thermostable DNA polymerase.

Compared to other diagnostic methods, PCR offers several advantages. First, the organism needs not be cultured prior to their detection by PCR. Second, this technology provides enhanced sensitivity and specificity. With PCR it is possible to detect a single or many members of a group of related microorganisms. There have been lots of examples of PCR-based techniques developed for detection of fungal strains, species or higher taxa in plant pathology. The different PCR-base assays have been developed to detect the presence of *Fusarium poae* (Peck) Wollenweb in infected wheat seeds (Parry and Nicholson, 1996), *Verticillum tricorpus* (I. Isaac.) in potato root and soil (Moukhamedov *et al.*, 1993), *Tapesia yallundae* Cash in cereal (Nicholson *et al.*, 1997), *R. oryzae* Ryker & Gooch in plant tissue (Mazzola *et al.* 1996), *R. cerealis* Vander Hoeven in wheat (Nicholson and parry *et al.*, 1996), *R. solani* AG-1 in rice (Johanson *et al.* 1998), *R. solani* AG-3 in soil and potato (Bounou *et al.*, 1999), *Trichoderma virens* Tode in soil (Baek and Kenerley, 1998) and *Monosporascus* spp. in muskmelon (Lovic *et al.*, 1995).

1. 3. 4. 1. 3. 2. Application of repetitive DNA in PCR-based assays

Different regions in fungal DNA have been used in PCR for the differentiation and detection of fungi. One of them is repetitive DNA element such as different types of rDNA and satellite DNAs.

The internal transcribed spacers (ITS) are located within the rDNA repeats. ITS regions of fungi have several characteristics. They are relatively short and have multicopy in fungal genome. They are highly variable among morphological distinctly species (Bridge and Arora, 1998). All of these characteristics make the ITS species-specific primers for fungi that can be generated very quickly by PCR without the need to construct a chromosomal library. rDNA ITS regions have been examined from a wide range of fungi such as *Ophiosphaerella herpotricha* (Fr.:Fr.) J. C. Walker (Tisserat *et al.* 1994), *Fusarium oxysporum* Schlechtend (Appel and Gordon, 1996, Pavanen-Huhtala *et al.*, 1999), *R. solani* AG-1 (Matsumoto *et al.*, 1997), AG-4 (Boysen *et al.* 1996), AG-8 (Mazzola *et al.*, 1996), *Trichoderma harzianum* Rifai (Bulat *et al.*, 1998 and Lubeck *et al.*, 1999)

Satellite DNAs are groups of identical or similar sequences, which are organized in blocks of tandemly repeated monomers. Oligonulcleotides corresponding to satellite DNA, mainly microsatellite DNA, has been widely used as fingerprinting probes to identify RFLP polymorphism among DNA samples from different individuals. Schonian *et al.* (1993) used DNA fingerprinting originated from Satellite DNA to identification the strains of *Candida albicans* (C. P. Robin) Berkout.

1. 3. 4. 1. 3. 3. PCR-derived methods

Several modifications of the original PCR procedures have been developed in order to increase the sensitivity or the specificity of the standard procedure, including nested PCR, random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), and *in situ* PCR.

1. 3. 4. 1. 3. 3. 1. Nested PCR

This is a simple modification of standard PCR, in which sensitivity and specificity of detection are enhanced by performing a second round of PCR with amplified DNA from the first round of PCR as the template and primers internal to the first round primer. Schesser *et al.* (1991) used single round PCR and nested PCR to detect *Gaeumannomyces graminis* (Sacc.) Arx&D. olivier var. avenaein (E. M. Turner) Dennis infected wheat plants. It was found that the sensitivity of nested PCR has an increase about 1,000 fold compared to single round PCR. The increase of the sensitivity is believed partially due to the dilution of plant-derived inhibitors of PCR and the non-specific DNA present in reaction. Also in nested PCR, primers may bind more efficiently to the target DNA. Nested PCR can also increase the specificity of the PCR since the inner set of primers will discriminate against any background or non-specific amplification from first round. This technique has been used to detect *Helminthosporium solani* Durieu & Mont. in soil (Oliver and Loria, 1998), *Glomus mosseae* Nicol & Gred. in soybean root and soil (Tuinen *et al.*, 1998), *Phytophthora fragariae* C. J. Hickman in strawberry (Lacourt *et al.*, 1997) and *Polymyxa betae* Keskin in sugar beet roots (Mutasa *et al.*, 1995).

However, nested PCR requires the transfer of amplified products from first round into a second round reaction tube. This increases the risks of cross-contamination among

the samples. To minimize the risk of cross contamination and save the time and PCR reagent, a one-tube nested PCR has been developed by Mutasa *et al.* (1996), in which first and second round of PCR were added simultaneously to a single reaction tube.

1. 3. 4. 1. 3. 3. 2. Random amplified polymorphic DNA (RAPD) and sequenced characterized amplified regions (SCARs)

A set of primers flanking the target DNA region is required in the reaction, which means that certain sequence information of the target DNA to be amplified is required. When sequence information for the primer design in classical PCR assays is not always available, random amplified polymorphic DNA (RAPD) provides a solution by using a single short primer with an arbitrary nucleotide sequence (Williams *et al.*, 1990). This method has also been called arbitrary primed PCR (AP-PCR) (Welsh and McClelland, 1990) and DNA amplification fingerprinting (DAF) (Caetano-Annolles *et al.*, 1991).

RAPD has been proved to be powerful in detecting intra-specific polymorphisms among organisms. It has the advantage of analysing a large number of loci in a relative short time. It has been used to produce markers to differentiate races of *Fusarium f. sp. Vasinfectum* (Assigbetse *et al.*, 1994 and Manulis *et al.*, 1994) and other fungal species such as *Alternaria linicola* Groves & Skolko (McKay *et al.*, 1999), *Gliocladium virens* J. H. Miller (Lexova *et al*, 1998), *R. solani* AG-1 (Toda *et al.*, 1998), AG-2 (Zens and Dehne, 1997), AG-3 (Bounou *et al.*, 1999), and AG-8 (Yang *et al.*, 1995)

In RAPD assays, the short and non-specific primers are used and the amplification reaction is performed under low stringency conditions. The results of

RAPD-PCR can be easily affected by the quality of template mixture, the brand of Taq DNA polymerase and thermocycler used. This decides that random PCR can not be used directly for the diagnostic purpose. However, taxon-specific markers generated by RAPD or other fingerprinting methods can be cloned and sequenced, and these sequence-characterized amplified regions (SCARs) can be used to design specific primers for detection assays. This techniques was originally used in plant breeding to develop genetic markers linked to disease resistance genes in lettuce (Paran and Michelmore, 1993). Now, several primers has been developed by this approach and used for the detection of fungi. RAPD amplicons specific to the non-pathogenic bionucleate *Rhizoctonia* isolates (AG-G) were sequenced and specific primers were designed (Leclerc-Potvin *et al.*, 1999). These SCAR markers were used in PCR assays to detect binucleate *Rhizoctonias* in soil and plant samples. A Similar technique was also used to design primers specific to *F. culmorum* (Wm. G. Sm.) Sacc (Chelkowski *et al.*, 1999), *Erysiphe graminis f.sp. hordei* (McDermott *et al.*, 1994), *R. solani* AG-3 (Bounou *et al.*, 1999) and *S. elegans* (Wang *et al.*, 1999). All these primers have been proved to be reliable and specific to the target organism.

1. 3. 4. 1. 3. 3. 3. Amplified Fragment Length Polymorphism

In this technique, genomic DNA is first digested with restriction enzyme and the oligonucleotide adapters are ligated to the digestion product. Then a PCR is performed in which primers containing the sequence of adapters the part of restriction enzyme recognition sequence and several additional nucleotides, usually 1-5 nucleotides, are used to selectively amplify the restriction fragments. Genetic variation among different

individuals leading to differences in restriction digestion as well as differences in lengths of amplified fragments can be identified.

In contrast toRAPD techniques, AFLP uses sequence-specific primers, which eliminates the variablility associated with nonspecific primers. The AFLP is powerful because it generates numerous DNA fragments from minute quantities of DNA and the reaction is performed under stringent conditions which improve reproducibility.

AFLP technique was developed for mapping plant and bacterial genomes. Just recently, it has has been applied to plant pathogenic fungi. Rosendahl and Taylor (1997) used AFLP technique to develop multiple genetic markers and study genetic variation in the mycorrhizal fungus *Glomus* species. Similarly Bonants et al. (1997) developed a race-specific marker for *Phytophthora fragariae* Corda. AFLP technqiue has also been used to reveal the genetic relationship and/or genetic variability in various fungi including *Collectrotrichum* pathogens of alfalfa (O'Neil *et al.*, 1997), *Leptosphaeria maculanFs* (Pongam *et al.*, 1999), *Colletotrichum lindemuthianum* (Gonzalez *et al.*, 1998), *Cladosporium fulvum* (Majer *et al.*, 1996).

1. 3. 4. 1. 3. 3. 4. In situ PCR

In situ PCR is a relatively new technique. It combines PCR and in situ hybridization. Generally, in situ PCR technique includes the following steps: sample fixation, sample permeabilization which allows the penetration of PCR reagent, in situ amplification and visualization. The detection of the signal can be achieved either by hybridization with labeled probe or by using labeled primer in the amplification step.

Until now, in situ PCR technique has been used successfully in both prokaryotic and eukaryotic cells. There have been many applications of using this techniques for the identification and localization of specific DNA and RNA sequence in intact cells (Hodson *et al.*, 1995, Gressens and Martin, 1994). Although the majority of the application of in situ PCR has been concerned with biomedical purpose, this technique has begun to be used for the identification and localization of the expression of specific plant genes.

Recently Bago *et al.* (1998) have used this technique to detect mycorrizhal fungi belong to *Glomales* spp. in soil and its host. In their studies, fluorescent, glomalean-specific primers were used in *in situ* amplification to amplify the ribosomal SSU genes within AM fungal spore sections. Then the amplified product was directly detected by means of fluorescence microscopy.

CHAPTER 2.

MATERIALS AND METHODS

2. 1. *Fungal and bacterial cultures*

Strains of *Stachybotrys* species and other genera were obtained from international culture collections, previous studies in our lab or collaborating researchers (Table 1). Fungal isolates were grown on potato dextrose agar (Difco Laboratories, Detroit, MI) at 25°C. For long-term storage, fungi were maintained on sterile kernel at 4°C. Isolates of *Streptomyces scabies* were grown in tryptic soy broth (TSB) and maintained at –80°C in 20% (v/v) glycerol.

2. 2. Genomic DNA extraction

For fungal DNA preparation, agar plugs cut from the margin of actively growing fungal cultures were used to inoculate aseptically 100 ml potato dextrose broth (PDB). Cultures were incubated on an orbital shaker at room temperature for 1 week and agitated at low speed (100 rpm). Mycelia were harvested by filtering through Whatman No. 1 filter paper and then freeze-dried. The mycelium mats were ground in liquid nitrogen to produce a fine powder and stored in -80°C until DNA extraction. Total fungal genomic DNA was isolated from 20-30 mg of freeze-dried mycelium according to the protocol of Lee and Taylor (1990). Genomic DNA from bacterial isolates was extracted using the method in Current Protocols (Schatz *et al.*, 1992). For plant materials, DNA was extracted from 500 mg of fresh potato tubers or beet roots following the method of Doyle and Doyle, (1987).

Total DNA was extracted from sieved greenhouse soil (1/3 black earth, 1/3 peatmoss and 1/3 sandy loam) and natural soil samples essentially as described by Leclerc-Potvin *et al.* (1999). Briefly, soil (200-300 mg) was mixed with 160 mg of 0.1 mm zirconia/silica beads (Xymotech Biosystems Inc.), 500 µl extraction buffer (100mM sodium EDTA [pH 8.0], 100 mM Tris-HCl [pH 7.0], 1.5M NaCl) and vortexed for 1 minute. Then a volume of 50 µl of 20% SDS and 1 µl of RNAse (10 mg/ml) were added and the mixture was incubated at 65°C for 30 minutes. After centrifugation, a 0.5 volume of 65% polyethylene glycol (Grade-8000) was added to the supernatant and incubated at room temperature for 2 hours. The DNA pellet was collected by centrifugation and dissolved in 300 µl of TE (100 mM Tris-HCl, 1 mM EDTA, [pH 8.0]). Cold potassium acetate (7.5 M) was added and the mixture was incubated on ice for 10 minutes. The supernatant was collected by centrifugation, and further purified by two rounds of choloroform/iosamyl alcohol extraction. A 0.6 volume of pre-cold isopropanol was added to the supernatant to precipitate DNA. The DNA pellet was washed twice using 500 µl washing buffer (10mM ammonium acetate, 76% ethanol), vacuum dried and then resuspended in 100 µl TE (pH 8.0). In order to get rid of contaminating organic matter, such as humic acid which may inhibit the activity of *Taq* DNA polymerase, DNA was further purified on Sepharose 4B spin column (Sigma) according to the method of Jackson *et al.* (1997). Briefly, 100 µl DNA was loaded onto Sepharose column and centrifuged for 5 minutes at11, 00 x g, then 100 µl of TE (pH 8.0) was loaded onto the top of column and centrifuged at same speed (1,100 x g) for another 5 minutes. The eluting liquid (100 µl) was collected and dilutions of 50x or 100x dilutions were used for PCR amplification.

Genomic DNA was quantified by comparison of DNA standard after agarose gel electrophoresis. All stated DNA amounts were based on gel estimates.

2. 3. *RAPD and PCR amplifications*

Decamer primer kits OPN and OPB (Operon Technologies, Alameda, CA) were used for initial RAPD screening. RAPD amplification reactions were carried out in 25 µl of 1 × PCR buffer (50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl, pH 9.0) containing 5-10 ng template DNA, 0.2 uM primers, 0.1 uM dNTPs (Pharmacia Biotech.), 1.0 U Taq DNA polymerase (Pharmacia Biotech.). PCR amplification using SCAR primers SE-13F/SE13-R was performed using the exact parameters as that of RAPD except that 10-20 ng of template DNA was used. One drop of mineral oil was used to overlay every reaction mixture prior to PCR. Amplifications were performed in a Perkin Elmer Cetus 480 DNA Thermal Cycler. The program for RAPD amplification was designed for an initial denaturation step of 94°C for 1 min, followed by 40 cycles of 1 min at 94°C, 1 min at 35°C and 2 min at 72° C, then a final extension step of 10 min at 72°C was used to complete the reaction. The thermal cycler parameters for SCAR primers were: denaturation for 3 min at 94°C and followed by 33 cycles of one minute at 94°C, 3 minutes at 72°C. After the 33rd cycle, the extension reaction was continued at 72°C for a further 10 minutes and products were held at 4C until analysis. PCR reactions using the primers SBU-177 and SBL-336 (Table 2) to amplify *R. solani* AG-3 were performed using the published parameters of Bounou *et al.* (1999).

An aliquot of 5 µl PCR products were resolved by electrophoresis on agarose gel (a 0.8%) at 80 V using 1 × TAE (40 mM Tris acetate, 2 mM EDTA) as running buffer.

1Kb or 100bp ladder (BRL) were used as molecular size standards. Gel images were recorded with a video copy processor P40U camera system connected to a gel print 2000i-documentation system (Genomic Solutions).

2. 4. Probe labelling and southern and slot blot a hybridization

The 951bp amplicon, produced by primer OPN-16, which is specific to all isolates of *S. elegans* was used as a probe for hybridization experiments. The amplicon was purified after electrophoriesis using Geneclean II (Bio101 Inc.). A total of 25 ng of 951 bp amplicon was denatured by heating for 10 min at 100°C and radioactively labelled with P^{32} dCTP using the random primed DNA labelling kit (Boehringer Mannheim). Unincorporated P^{32} dCTP was removed from the labelled DNA using Sephadex^{R} G-50 DNA grade columns (Pharmacia Biotech.)

For slot blot hybridizations, 10 ng RAPD-PCR product was transferred onto the nylon (Hybond N^{+}, Amersham-Buchler Inc.) membrane by Bio-Dot SF blotting apparatus (BioRad) according to the manufacturer's recommendation. For Southern hybridization, the electrophoresed RAPD products were transferred onto nylon membranes using vacuum blotter (model 785, BioRad) following the manufacturer's protocol.

The Southern/blot membranes were prehybridized for 18 hours 42°C in a solution containing 3 × SSC, 5 × Denhart solution, 50% formamide, 0.06 M NaHPO4, 0.2 % sodium dodecyl sulfate (SDS) and 0.1 mg/ml of salmon sperm DNA. After prehybridization, 20 µl probe was added and hybridization was proceeded overnight at 42°C. The membranes were washed with 5 × SSC, 0.1% SDS at 55°C for 1 hour followed by a stringency wash (0.2 × SSC, 0.1% SDS at 60°C) for 1 hour. For signal detection,

membranes were blotted dry and baked 1 hour at 80°C, and mounted for autoradiography for 30 minutes at room temperature.

2. 5. Cloning and sequencing of the RAPD fragment

The protruding 3' termini of 951 bp amplicon were polished with cloned *Pfu* DNA polymerase and inserted into pCR-Script Amp SK (+) cloning vector (Stratagene). Ligated plasmids were used to transform *E. coli* XL1-Blue MRF' Kan supercompetent cells according the manufacturer's protocol (Stratagene). Plasmids were extracted from positive clones using the standard protocol (Sambrooke *et al.* 1989) and purified using QUIAGEN plasmid kit (Quiagen). The cloned fragment was cycle-sequenced by the University of Calgary Core DNA facilities from both ends using the Perkin-Elmer 377 cycle sequencer (Applied Biosystems). The complete sequence was submitted to Genebank and EMBL database for nucleotide similarity and analyzed using Blast @ncbi.nlm.nih.gov (Altschul *et al.*, 1990, Gish and States, 1993) and FASTA @embl-heidelburg.de (Pearson and Lipman, 1988 and Pearson, 1990).

2. 6. Primer design, PCR and electrophoresis

Based on the full-length sequence of the 951 bp amplicon, a pair of SCAR primers was designed with the aid of computer program DNAMAN (Lynnon Biosoft, 1994) and designated as SE-13F/SE-13R (Table 2). One oligonucleotide primer (SE-13F) contained the complete sequence of the original arbitrary primer (OPN-16). Both primers had overall G+C content of 50-60%. The primers were custom synthesized by Alpha DNA (Montreal, Canada).

2. 7. S. elegans and R. solani in dual culture assays

Intermingeled hyphae from the interaction zone between *S. elegans* and *R. solani* were obtained using the dual-culture plate technique (Benygoub *et al.*, 1994). Two agar plugs, one bearing *S. elegans* and the other *R. solani,* were placed at opposite ends on top of a cellophane membrane overlaying a thin layer of PDA culture. Culture slides inoculated with *S. elegans* or *R. solani* only served as controls. The slides were incubated at 24°C for one week. Genomic DNA from the intermingled hyphae of the interaction zone and from control culture slides was extracted as previously described and used for PCR amplification.

2. 8. Soil inoculation

Inoculum source for *S. elegans* and *R. solani* consisted of conidial and uniform-sized sclerotia, respectively. The experiments were conducted in sterile and non-sterile greenhouse soil, and natural soil samples. Natural soil samples were obtained from three different potato grown regions in Quebec which have a history of *Rhizoctonia* disease. Inoculation of all soil samples consisted of incorporating 1 ml of *S. elegans* conidial suspension (2×10^6 spores/ml of sterile distilled water) with 10 grams of soil or mixing the soil with 10 laboratory-grown sclerotia of *R. solani* and incubating it in petri plates (15 × 20 mm diam.) at 24°C for 7 days. Several treatments each consisting of three replicates were established: (i) soil inoculated with *S. elegans* after which sclerotia of *R.. solani* were added; (ii) soil inoculated with *S. elegans* (iii) soil inoculated with *R. solani*, and (iv) soil treated inoculated with sterile water. After one-week of incubation in the dark at 24°C, 10 beet seeds (Green Top Bunching) seeds, that were previously surface

sterilized with 1 % sodium hypochlorite solution for 30 minutes, were sown into each plate and incubated for 5 more days. Emerging seedlings were harvested and transferred to water agar (2% agar) plates to observe root rot symptoms on beet seedlings. Disease severity, % seed germination and length of seedling's taproot were estimated using the method described by Papavizas and Davis (1962). The disease severity was measured according to diseased area in the root. Four scales (1, 2, 3, 4) were used to indicate 0-25%, 25-50%, 50-75%, 75-100% diseased area in the root.

The experiments were repeated twice and each treatment consisted of three replicates. Root length and seed germination data were analysed via a 1-way ANOVA non-parametric test. Means were seperated by the Duncan's mutiple range test. Disease severity data of beet seedlings were analysed using Kruskal-Wallis non-parametric test.

2. 9. Detection and quantification of amplified products of S. elegans and R. solani in dual culture assays, soil and plant samples

PCR assays, using the SCAR primers SE-13F/SE-13R and SBU-177/336 for *S. elegans* and *R. solani* respectively, were performed on DNA extracted from dual cultures of *S. elegans* and *R. solani*, beet roots, artificially inoculated sterile, non-sterile green house soil and natural soil samples from the places which have the history of Rhizoctonia disease. All PCR experiments were repeated at least three times. Dilutions of 10^2 and 10^4 of DNA were routinely used in PCR assays to detect *S. elegans* in soil and axenic cultures, respectively.

The amount of amplified DNA of *S. elegans* in one gram of soil was quantified based on gel estimation, and correlated with the total number of colonies of *S. elegans* present in the same amount of soil. Estimates of the number of *S. elegans* colonies in all soils were deduced using a standard serial soil dilution method, and plating a 200 µl aliquot of the soil suspension on PDA containing 0.1 µg/ml of chloramphenicol and novobiocin. The number of colonies forming units of *S. elegans* (using 10^4 dilution for counting) was estimated after 5 days of incubation at 25°C. Since *R. solani* is a non-sporulating fungus, estimates of its amplified DNA from soils was correlated to disease severity on beet seedlings

CHAPTER 3

RESULTS

RAPD assays

Forty RAPD primers (Operon®) were used in PCR screening of *Stachybotrys* species and other heterogeneous organisms. Primers that gave no bands or inconsistent results were not considered further. The remaining primers (total of 10) tested gave a clear DNA amplification and revealed polymorphism among the 11 species of *Stachybotrys*. These primers were tested several times in order to confirm reproducibility and consistency (Appendix I-III). Only primer OPN-16 amplified a 951bp DNA fragment that was unique to all isolates of *S. elegans* and *S. bisbyi* except for isolate 399.65 (electrophoresis no.199; Fig. 1 A). This amplicon was absent in other species of *Stachybotrys*, as well as in other fungi, bacteria and plant tissue (Figs 1B and C).

Southern and slot blot hybridization

To determine the possible homology of the 951bp amplicon derived from the OPN-16 to *S. elegans* isolates, the resulting 951 bp RAPD fragment (thereafter designated OPN-16-951) was radiolabeled and hybridized to membranes containing respective PCR products. The OPN-16-951 produced a strong hybridization signal in slot blot hybridization that was common to all *S. elegans* isolates and to one isolate of *S. bisbyi* (Fig. 2, electrophoresis no. 207). A very faint signal was detected in *S. bisbyi* isolate 198 when the membrane was exposed at room temperature for three days. No signal was observed in other fungal, bacterial isolates and plant tissue (Fig. 2).

When OPN-16-951 was used as a probe in southern blot hybridization, a strong signal was observed in all *S. elegans* isolates and in only one isolate of *S. bisbyi* (Fig. 3A, electrophoresis no. 207). The probe hybridized intensely with two RAPD fragments. One strong band, corresponding in size to the progenitor 951 bp amplicon, was common to all *S. elegans* isolates and to *S. bisbyi* (electrophoresis no. 207, Fig. 3A). Another band, corresponding to 1.6 Kb in size, was detected in two isolates of *S. elegans* (electrophoresis nos. 48 and 47) and in *S. bisbyi* (no. 207). Fainter bands (1.4 kb and 3.5 kb), detected with OPN-16-951, were observed in *S. bisbyi* isolates no. 207 and in only two isolates of *S. elegans* (Fig. 3A, electroporesis nos. 48 and 49). Interestingly these isolates had a similar hybridization pattern. No hybridization signal was detected on RAPD profiles of isolates belonging to other *Stachybotrys* species, other fungal and bacterial species as well as plant tissue (Figs 3B, C). A very faint band, detected not at the position corresponding to 951 bp fragment, was observed for *S. scabies* (electrophoreiss no. 202; Fig. 3C).

Cloning, sequencing and primer design

The 951 bp amplicon from *S. elegans* isolate 13 was cloned, completely sequenced and analyzed. The 951 bp amplicon had an overall G+C content of 64% (data not shown). Data base searches for nucleotide similarity using Blastn (Gish & States, 1993) and FASTA (Pearson, 1990) revealed homology of 64.5% in 341 bp overlap to the sequence of the Sur2p and Syr2 genes of *Saccharomyces cerevisiae*. Both genes are involved in the regulation of syringomycin growth *of S. cerevisiae* which is required for the growth of yeast. (Cliften *et al.*, 1996).

On the basis of OPN-16-951 sequence information, specific SCAR primers (24 mers) were designed and designated SE-13F and SE-13R (Table 2). The specificity of the SCAR primers was tested against all *Stachybotrys* species, other fungal and bacterial pathogens including pathogens that are commonly associated with potato tubers. PCR conditions were optimized to amplify a single fragment of 880 bp from total genomic DNA (Fig. 4). The 880 bp amplicon was detected in only *S. elegans* isolates and in two of *S. bisbyi* isolates 198 and 207 (Fig. 4A). No cross-reaction or DNA amplification was observed in other species of *Stachybotrys,* heterogeneous organisms, and plant tissue (Fig. 4A, B). The homology of the 880 bp amplicon from *S. elegans* and *S. bisbyi* isolates was confirmed by Southern blot hybridization.

Amplification of DNA of S. elegans and R. solani during dual culture

In mixed or dual culture assays, *S. elegans* SCAR primers amplified a single fragment of 880 bp from DNA purified from intermingled hyphae of *S. elegans* and *R. solani* growing at the interaction zone (Fig. 5, Zone 2) and from hyphae of *S. elegans* located in regions away from the interaction zone (Zone 1). Likewise, *R. solani* specific primers SBU-177/SBU-336 (Bounou *et al.*, 1999) amplified a single fragment of 179 bp from DNA extracted from intermingled hyphae and from hyphae of *R. solani* growing in regions away from the interaction zone (Fig. 5, Zone 3). The primers SE-13F/R detected a minimum of 1 pg of *S. elegans* DNA, while primer SBU-336 /177 detected as little as 0.01 pg *R. solani* DNA.

Detection of S. elegans and R. solani in different types of soil and plant tissue

The sensitivity of the SCAR primers was tested in the presence and absence of target DNA extracted from a minimum amount of inoculated sterile and non-sterile greenhouse soils (200 mg), and natural soils (300 mg) with either *S. elegans*, *R. solani* or both. Following amplification with primers SE-13R/F, the 880 bp amplicon specific to *S. elegans* was only observed in all types of soils inoculated with *S. elegans* only or inoculated with both *S. elegans* and *R. solani*. No product was amplified from control soil, soil inoculated with *R. solani* alone, or from beet seedlings growing in these soils (Figs. 6 and 7). Soil samples inoculated with *R. solani* and from beet seedlings growing in these soils produced a 179 bp fragment following amplification with the primer pair SBU-177/336 (Figs. 6, 7). The179 bp amplicon was not observed in soil inoculated with *S. elegans* alone or in soil treated with water (Figs. 6A and 7A). In non-inoculated natural soil samples, the PCR assay using primers specific to *S. elegans* and *R. solani* AG-3 failed to amplify *S. elegans* and *R. solani* DNA which may indicate the absence of *S. elegans* and *R. solani* AG-3 in these soil samples (Fig. 7A).

In the case of greenhouse soil, both SCAR markers successfully detected a minimum of 0.5 pg soil DNA/mg of soil. When using these primers in natural soil, the detection limit of PCR using both primers was 0.33 pg of soil DNA/mg of soil.

Effect of S. elegans on Rhizoctonia disease of beet seedlings

In sterile and non-sterile greenhouse soils, a 100% survival rate was observed in soils containing *S. elegans* alone as well as in soils containing both *R. solani* and *S. elegans* (Table 3). A low but significant decrease in % germination (7.5% and 5%) was observed in both sterile and non-sterile infested with *R. solani* (Table 3). The presence *of*

S. elegans in soils affected the ability of *R. solani* to cause disease on beet seedlings, as assessed by % incidence and disease severity. Compared to soils inoculated with *R. solani* alone, disease incidence and severity of root rot decreased significantly in sterile and non-sterile soils when *S. elegans* was present (Table 3). In addition, tap root length of beet seedlings growing in soils pre-inoculated with the mycoparasite was significantly longer than those of seedlings growing in soils infested with *R. solani* or controls (Duncan, $P = 0.05$).

Interestingly, the addition of *S. elegans* to non-sterile soil that have been verified by PCR assays to be free of *R. solani* (AG-3) and *S. elegans*, caused a significant decrease in disease incidence of beet seedlings compared to that of seedlings grown in control non-sterile soils. These results suggest that *S. elegans* may be an effective biocontrol agent against either other AG groups of *R. solani* or other soilborne pathogens that may cause seedling root rot.

Similar experiments were also conducted in three different natural soil samples originating from different fields with history of *Rhizoctonia* and potato scab disease (Leclerc-Potvin *et al.*, 1999). The results showed that a comparable decrease in disease incidence and disease severity was observed in all three soil types when *S. elegans* was introduced to these soil samples (Table 5). In two out of three soil types, tap root length of beet seedlings was significantly longer in soil pre-inoculated with the mycoparasite than in the same soil infested with *R. solani* alone or controls (Table 5).

Quantification of amplified products of *S. elegans* and *R. solani* in soil

The relationship between the amount of *S. elegans* as estimated by PCR and conventional conidial count in the soil was determined. In both sterile and non-sterile greenhouse soils, the number of *S. elegans* spores in 1 g of soil represents an amount $7x10^4$ ng of amplified *S. elegans* DNA (0.7 ng 880 bp amplicon/spore). This amount was similar in soils containing *S. elegans* alone or in soil containing both the mycoparsite and the pathogen (Table 4). However, a significant decrease in the amount of *R. solani*, as estimated by disease severity and by amplified DNA was observed in soils containing both the pathogen and the mycoparasite.

In the case of inoculated natural soils, there was no significant change of spore number of *S. elegans* and total amount of the 880 bp amplicon from 1 g of soil containing *S. elegans* alone or both the mycoparasite and pathogen (Table 6). In natural soil, the number of *S. elegans* spores in 1 g soil represents $1x10^5$ ng amplified *S. elegans* DNA (0.55 ng 880 bp amplicon/spore). However, significant decrease in total amount of 179 bp amplicon specific to *R. solani* was observed when *S. elegans* was introduced to the soil.

CHAPTER 4

DISCUSSION

RAPD markers have been used as a useful tool in studies of the genetic variation of many fungi (Stanoz *et al.*, 1999, Caligiorne *et al.*, 1999 and Bardin *et al.*, 1999). In the present study, RAPD analysis of a large number of *Stachybotrys* isolates belonging to several species revealed that there is a high level of heterogeneity within most species. In the case of *S. elegans*, isolates were separated into two distinct genetic groups (group a 48, 49, and group b: 13, 92 and 93) based on their RAPD amplification profile that was generated by the molecular marker OPN-16 (Fig. 1) and by OPN-11, OPN-12, OPN-15, OPB-7 and OPB-12 (Appendix I-III). These two groups reflected the geographic distribution (North America and south eastern Europe) of the isolates. This result may be indicative of lack of movement of *S. elegans* isolates across continents. The results of this study also showed that the amplified profile of one of the *S. bisbyi* isolates (isolate 207) was identical to that of the Canadian *S. elegans* isolates (Fig.1A). Interestingly, isolate 207 is also originated from Canada.

In addition to the similar RAPD profile, OPN-16 has amplified a unique fragment of 951 bp that was only present in *S. elegans* and in two isolates of *S. bisbyi* (198 and 207). Slot blot and southern hybridization further verified these results and confirmed that the 951 bp fragment is present in all *S. elegans* isolates and in the designated *S. bisbyi* isolates 198 and 207. These results are not surprising since both species are taxonomically very similar, and it has been proposed that both species should be combined to form one species (Jong and Davis, 1976). Although the amplified gel banding pattern of *S. bisbyi* isolate 198 was similar to that of *S. elegans* isolates 48 and

49, no hybridization signal for *S. bisbyi* isolate 198 was oberved in southern blot when 951 bp amplicon was used as the probe (Fig. 3A). However, when the 880 bp amplicon derived from amplification of *S. elegans* (isolate 13) using SCAR primers (SE-13F/R) as the probe, a strong signal was detected in isolate 198 (data not shown). This may indicate that *S. bisbyi* isolate 198 and other *S. elegans* isolates share homolgy in the 880 bp region. Based on our finding, a re-evaluation of the taxonomic characteristics of these two species is essential. This can be done by complete sequencing of the 951 amplicon of all *S. elegans* and *S. bisbyi* isolates and complimented with conventional taxonomic criteria is required.

The ability to detect and identify microorganisms using PCR technology has great potential for enhancing diagnostic capabilities. This technology is particularly suited to simultaneous detection of soil borne plant pathogens and mycoparsites in biological control systems. To achieve this, specific primers can be designed from specific cloned DNA fragment or from PCR amplified specific fragment (Ersek *et al.*, 1994). The taxon-specific markers generated by RAPD or other fingerprint methods can be cloned and sequenced. These sequence-characterized amplified regions can be used to design specific primers for detection assay. Based on methods used in our recent studies (Bounou *et al.*, 1999 and Leclerc-Potvin *et al.*, 1999), we successfully designed distinct SCAR-RAPD marker for establishing diagnostic PCR assays for the mycoparasite *S. elegans*. The 951 bp amplicon was fully sequenced and a pair of primers (SE-13F/SE-13R) was designed to amplify a 880 bp fragment from pure *S. elegans* cultures, co-cultures containing *R. solani*, or from contaminated soil samples containing multiple types of DNA. The designed SCAR primers allowed the unequivocal detection of 1 pg of

DNA from pure cultures of *S. elegans* and 0.5 pg soil DNA/mg of green house soil and 0.33 pg soil DNA/ mg of natural soil.

This contention is supported by the lack of detectable cross-reaction with pure cultures DNA of a range of taxonomically diverse fungal and bacterial strains as well as with total microbial community DNA obtained from several natural soils.

These results clearly demonstrate that the SCAR primers SE-13F/SE-13R are specific and sensitive, and can be used to detect *S. elegans* in the presence of contaminating organisms. Based on these findings and on recently designed SCAR primers for *R. solani* (Bounou *et al.*, 1999), we can determine whether any type of soil contains both organisms by extracting DNA from soil and doing PCR with both primers. The presence of 880-bp and 179 bp products indicates that *S. elegans* and *R. solani* are present, respectively. We also have routinely used this technology to detect the presence of *S. elegans* and its effect on *R. solani.*

A major problem in molecular investigation of soil microbial biology and diversity has been the extraction of pure DNA from soil. Humic substances are usually co-extracted and precipitated with the target DNA. They can inhibit the activity of Taq DNA polymorase, therefore affecting the sensitivity of polymerase chain reaction (Ogram *et al.*, 1988). To circumvent this problem, several methods have been developed in order to remove humic substances (Hilgar and Myrold, 1991). One such method is the use of Sepharose columns (Whisson and McClelland, 1995, Jackson *et al.* 1997). In this study, target DNA extracted from different types of soil of varying degrees of organic matter was further cleaned using Sepharose-4B spin columns (Jackson *et al.*, 1997). This step significantly ameliorated the quality of the amplified. With the primer pair SE-

13F/SE-13R, we were able to detect *S. elegans* from as little as 0.5 pg soil DNA/mg of green house soil and 0.33 pg soil DNA/ mg of natural soil.

Although the PCR assay is sensitive, one of its limitations is the inability to differentiate dead cells from living cells. Schaad *et al.* (1995) developed a combined biological and enzymatic amplification technique (Bio-PCR) in which a plate bioassay and PCR were combined. Bio-PCR has the advantage compared to conventional PCR that it can eliminate the false positive signal resulting from the presence of dead cells that may present in soil. In our study, living cells of *S. elegans* were detected by combining the PCR assay with direct plating of inoculated soil on antibiotic- amended culture medium.

Although the amount of 880 bp and 179 bp amplicon specific to *S. elegans* and *R. solani,* respectively were correlated to spore number of *S. elegans* in soil and to *Rhizoctonia* disease severity on beet seedlings, the amount of *S. elegans* and *R. solani* DNA in soil can not be accurately quantified using this method. Because of the exponential nature of PCR, it is difficult to estimate the amount of target DNA in a PCR reaction. Recently, several quantitative methods have been developed for the quantification of the amount of initial target DNA in PCR reaction (Forster 1994). One of them is competitive PCR (Hu *et al.*, 1993, Elsas *et al.*, 1998 and Nicholson *et al.*, 1998). In the case of our research, internal competitor can be developed for the 880 bp amplicon and the 179 bp amplicon specific to *S. elegans* and *R. solani,* respectively. Then quantitative information of *S. elegans* and *R. solani* DNA in soil can be obtained by co-amplification of the internal competitors for *S. elegans* and *R. solani* with soil DNA.

A new quatitative strategy, Real-time PCR has been recenlty developed to quantify the amount of a specific amplicon on-line with a fluorogenic detection system (Orlando *et al.*, 1998). This technique has recently been used by Böhm *et al.* (1999) to monitor the growth of *Phytophthora infestans* (Mont.) *and Phytophthora citricola* Sawada in their respective hosts. In the case of *S. elegans*, a specifc probe specific to the 880 bp can be developed and labeled with two fluorogenic dyes at both ends. This procedure will allow us to quantify accurately the amount of *S. elegans* in the soil.

This type of PCR assay can be valuable in epidemiological studies, especially for analyzing competition between different fungi species in soil during seedling. This assay can also be applied to monitor the growth of *S. elegans* in soil and how *S. elegans* affect *R. solani* over its various developmental stages of infection.

In conclusion, we have successfully developed reliable and sensitive SCAR primers to detect a biological control agent in different contaminated biological material. We also have used this marker with conjunction with another recently developed marker (Bounou *et al.*, 1999) to simultaneously detect both the biocontrol and the pathogen at the same time in *planta* and in soil samples.

CHAPTER 5

CONCLUSION AND SUMMARY

We have successfully designed and developed a pair of SCAR primers (SE-13F/R) and used them in PCR-based assays to routinely detect the biological control agent (BCA), *S. elegans*, in pure and co-cultures as well as in contaminated natural soil samples and plant tissue. No cross-reaction was observed when the designed primers were used to amplify DNA of other common soil fungi, bacteria and plant tissue. The assay is reliable and sensitive with a detection level of 0.5 pg soil DNA/mg of green house soil and 0.33 pg soil DNA/mg of natural soil. This PCR-based assay can be routinely applied to detect the presence of the BCA in natural conditions. In addition, we have shown that these primers can be used in conjunction with other SCAR primers to simultaneously monitor the presence of more than one organism not only *in vitro* conditions but also under natural conditions.

Based on our knowledge, this work is the first report on the development of molecular marker for the identification and detection of *S. elegans*. These primers can be useful in the taxonomic and ecological study of *S. elegans*. Furthermore, they also have the potential to be used as a diagnostic tool in large-scale biocontrol field experiment to follow the fate of the introduced *S. elegans* under natural conditions. This might facilitate the use of this biocontrol agent in the control of disease caused by *R. solani*.

CHAPTER 6

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Table 1. Fungal and bacterial isolates used in this study

Strains	Electrophoresis number	Original isolate designation	Source	Host or Habitat	Geographic Origin
Stachybotrys species					
elegans	48	1526	3	soil mixed wood	Ontario, Canada.
elegans	49	22215	1	Roots of grasses	Canada
elegans	13	No.1	2	Unknown	Unknown
elegans	92	No. 2	2	Unknown	Unknown
elegans	93	No. 3	2	Unknown	Unknown
bisbyi	198	363.58	1	soil from mangrove swamp	Inhaca Island, Mozambique
bisbyi	199	399.65	1	Zea mays roots	BernburgGermany
bisbyi	210	142.97	1	leaf litter of *Bambusa vulgaris*	Spain
bisbyi	211	268.76	1	*Saccharum officinarum roots*	Taiwan
bisbyi	207	598.69	1	Maize field soil	Ontario, Canada
albipes	173	7750	3	*Ulmus* sp.	England
albipes	212	325.90	1	*Fagus sylvatica*	Bari, Italy

Parvispora	215	253.75	1	Soil	Wageningen, Netherlands
Parvispora	179	7749	3	Soil	Congo
Dichora	176	7748	3	*Senecio jacobaca*	England
Longispora	217	493.96	1	Leaf litter	Cuba
Longispora	200	100154	1	Dead leaves from rain forest	Trinidad, Cuba
Oenanthes	216	252.76	1	*Coccus viridis* on Coffea arabica	La Habana, Cuba
Cylindrospora	174	7122	3	Whitecourt	Ontario, Canada
Cylindrospora	175	7211	3	Paper towelling	New Yourk, USA
Cylindrospora	97	No. 1	2	Forest soil	Unknown
Cylindrospora	213	878.68	1	Wheat field soil	Kiel-Kitzeberg, Germany
Chartarum	182	6425	3	Wood, paper & tiles	Ontario, Canada
Chartarum	183	7900	3	Indoor air ex RCS strip	Alberta, Canada
Chartarum	94	No. 4	2	Forest soil	Unknown
Chartarum	95	No. 5	2	Forest soil	Unknown

Chartarum	96	No. 6	2	Forest soil	Unknown
Kampalensis	177	7746	3	Forest soil	New Guinea
Kampalensis	214	388.73	1	old stem of *Euphorbia tirukalli*	India
Microspora	178	7747	3	Ex *Arachis hypogaea*	Unknown
Teleomorph stage of Stachybotrys					
Memnoniella echinata	180	842	3	Unknown	Edmonton, Canada.
Echinata	181	3195	3	Tent canvas	Solomon Island
Echinata	185	9200	3	Human toe	New York, USA
Heterogeneous organisms					
Fusarium solani	205	No. 2	4	Soil	Ontario, Canada
Solani	206	No. 1	5	Soil	Quebec, Canada
Pythium ultimum	161	N1	5	Grassland soil	Colorado, USA
Botrytis cinerea	162	F-014	6	Unknown	Unknown
Alternaria alternata	165	F-005	6	Unknown	Unknown

Rhizoctonia solani AG-3	12		6	soil	Quebec, Canada
Verticillium albo atrum	208	Vaa AT3	4	Potato	Ontario, Canada
Streptomyces scabies	201	EF-35	7	Potato	Qubec, Canada
S. scabies	202	CG-1	7	Potato	Quebec , Canada

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Table 2. List of primers and fragments used for PCR amplification to detect *S. elegans* and *R. solani*

	Specific primers	Primer length (bp)	Sequence (5' to 3')	Size of amplicon (bp)
S. elegans	SE-13F	24	AAGCGACCTGAAAACCGAGGCTTC	880
	SE-13R	24	GCCATGTTCATCCTGGACACTTGG	
R. solani AG-3	SBU-177	20	TTTGGATGTGGGGTCTTTGC	179*
	SBU-336	20	AACATAGTGCCTTCTCTTCA	

* Bounou *et al.*, 1999.

Table 3. Effect of *S. elegans* on *Rhizoctonia* root rot disease of beet seedlings in greenhouse soil*

Treatment	Seedling survival (%)	Disease incidence (%)	Disease severity (score 1-4)	Tap root length (cm)***
Sterile soil				
Control	100 a **	0 a **	0.00 ± 0.00 a**	8.05 ± 2.11 a ***
+ *S. elegans*	100 a	0 a	0.00 ± 0.00 a	7.85 ± 1.62 a
+*S. elegans* and *R. solani*	100 a	22.5 b	0.25 ± 0.49 b	6.79 ± 1.70 b
+ *R. solani*	92.5 b	80 c	1.68 ± 1.36 c	5.59 ± 1.82 c
Non sterile soil				
Control	100 A#	17. 5 A	0.28 ± 0.64 A	7.86 ± 1.22 A
+ *S. elegans*	100 A	7.5 A	0.15 ± 0.53 A	9.11 ± 1.06 B
+ *S. elegans* and *R. solani*	100 A	30.5 B	0.35 ± 0.53 B	8.26 ± 1.44 A
+*R. solani*	95 B	86.9 C	1.90 ± 1.37 C	6.71 ± 2.24 C

*Experiment was conducted using green house soil inoculated with spores of *S. elegans* and sclerotia of *R. solani*. Three replicates per treatment and ten table beet seeds per replicate were sown. Data represent the mean of three replicates ± standard deviation of the mean.

** Mean values followed by the same letters within the same column are not significantly different according to the least significant difference test ($P \geq 0.05$).

*** Averages followed by the same letters within the same column are not significantly different according to Kruskal-Wallis non-parametric test.

#The lower and upper case letters in columns represent results of different tests.

Table 4. Relationship between the quantity of of *S. elegans* and *R. solani* in green house soil and the amplified quantity of DNA *

Treatment**	*S. elegans* (spores/g soil)	*S. elegans* amplified DNA (ng/g soil)	*R. solani* disease severity (score 1-4)	*R. solani* amplified DNA (ng/1g soil)
Sterile soil				
Control	0.00 ± 0.00 a **	0.00 ± 0.00 a	0.00 ± 0.00 a**	0.00 ± 0.00 a
+*S. elegans*	$1x10^5 \pm 0.29x10^5$ b	$7.33x10^4 \pm 0.33x10^4$ b	0.00 ± 0.00 a	0.00 ± 0.00 a
+*S. elegans* and *R. solani*	$1.16x10^5 \pm 0.44x10^5$ b	$8.00x10^4 \pm 1.15x10^4$ b	0.25 ± 0.49 a	$1.13x10^4 \pm 0.47x10^4$ b
+*R. solani*	0.00 ± 0.00 a	0.00 ± 0.00 a	1.68 ± 1.36 b	$1.06 x10^5 \pm 0.07x10^5$ c
Non-sterile soil				
Control	0.00 ± 0.00 A#	0.00 ± 0.00 A	0.28 ± 0.64 A	0,00 ± 0.00 A
+*S. elegans*	$0.83 x10^5 \pm 0.17x10^5$ B	$7.00x10^4 \pm 0.57x10^4$ B	0.15 ± 0.53 A	0.00 ± 0.00 A
+ *S. elegans* and *R. solani*	$0.83 x10^5 \pm 0.17x10^5$ B	$7.33x10^4 \pm 0.33x10^4$ B	0.35 ± 0.53 A	$1.67x10^4 \pm 0.33x10^4$ B
+*R. solani*	0.00 ± 0.00 A	0.00 ± 0.00 A	1.90 ± 1.37 B	$1.03 \pm 0.09 x 10^5$ C

*Experiment was conducted using green house soil inoculated with spores of *S. elegans* and sclerotia of *R. soalni*. Three replicates per treatment and ten table beet seeds per replicate were sown.

** Data represent the average of three replicates ± standard deviation. The data was log transformed, and mean values followed by the same letter within the same column are not significantly different according to the least significant difference test ($P \geq 0.05$).

#The lower and upper case letters in columns represent results of different tests.

Table 5. Effect of *S. elegans* on *Rhizoctonia* root rot disease of beet seedlings in natural soil *

Treatment	Seedling survival (%)	Disease incidence (%)	Disease severity (score 1-4)	Tap root length (cm)**
Soil sample 3*				
Control	93.33 ± 3.33 a **	77 ± 7.66 a **	1.83 ± 1.62 a**	8.05 ± 2.11 a ***
+ *S. elegans*	96.77 ± 5.77 a	40 ± 4.03 b	0.50 ± 0.73 a	7.85 ± 1.62 a
+*S. elegans* and *R. solani*	96.77 ± 5.77 a	48 ± 0.30 b	0.76 ± 0.77 b	6.79 ± 1.70 b
+ *R. solani*	96.77 ± 5.77 a	90 ± 0.00 c	2.17 ± 1.21 c	5.59 ± 1.82 c
Soil sample 55				
Control	100 ± 0.00 a#	86.67 ± 1.15 a	1.80 ± 1.06 a	4.55 ± 1.25 a
+ *S. elegans*	96.77 ± 5.77 a	50.00 ± 1.00 a	0.53 ± 0.63 b	7.06 ± 0.97 b
+ *S. elegans* and *R. solani*	96.77 ± 5.77 a	54.67 ± 2.21 b	0.77 ± 0.85 b	7.31 ± 1.13 b
+*R. solani*	96.77 ± 5.77 a	93.33 ± 1.15 b	2.67 ± 1.09 c	4.68 ± 1.45 a
Soil sample 59				
Control	93.33 ± 3.33 a#	76.00 ± 1.44 a	2.00 ± 1.64 a	6.01 ± 3.05 a
+ *S. elegans*	93.33 ± 3.33 a	43.33 ± 1.15 b	0.60 ± 0.93 b	7.14 ± 0.98 a
+ *S. elegans* and *R.solani*	96.67 ± 5.77 a	50.00 ± 1.00 b	0.733 ± 1.01 b	6.63 ± 1.01 a
+ *R. solani*	96.67 ± 5.77 a	100 ± 0.00 c	2.53 ± 1.19 c	4.57 ± 1.17 a

*Experiment was conducted using different types of natural soil. Soil samples were obtained from three different places in Quebec where incidence of *Rhizoctonia* disease and potato scab has been reported. Soil 3 is fine sandy soil (Saint-Romuald), soil 55 is silty sandy soil (Saint-Germain), and soil 59 is coarse sandy soil (Isle-Verte). The soils were inoculated with spores of *S. elegans* and sclerotia of *R. solani*. Three replicates per treatment and ten table beet seeds per replicate were sown. Data represent the mean of three replicates ± standard deviation of the mean.

** Mean value s followed by the same letters within the same column are not vary significantly different according to the least significant difference test ($P \geq 0.05$).

*** Averages followed by same letters within same column are not significantly different according to Kruskal-Wallis non-parametric test.

#The tests were performed separately in different soil samples.

Table 6. Relationship between the quantity of of *S. elegans* and *R. solani* in natural soil and the amplified quantity of DNA *

Treatment**	*S. elegans* (spores/g soil)	*S. elegans* amplified DNA (ng/g soil)	*R. solani* disease severity (score 1-4)	*R. solani* amplified DNA (ng/1g soil)
Soil sample 3*				
Control	0.00 ± 0.00 a **	0.00 ± 0.00 a	1.83 ± 1.62 a	0.00 ± 0.00 a
+ *S. elegans*)	$1.66x10^5 \pm 0.33x10^5$ b	$9.67x10^4 \pm 0.33x10^4$ b	0.50 ± 0.73 b	0.00 ± 0.00 a
+ *S. elegans* and *R. solani*	$2.00x10^5 \pm 0.57x10^5$ b	$1.23x10^5 \pm 0.03x10^5$ b	0.76 ± 0.77 b	$1.64x10^4 \pm 0.33x10^4$ b
+ *R. solani*	0.00 ± 0.00 a	0.00 ± 0.00 a	2.17 ± 1.21 c	$1.33 x10^5 \pm 0.06x10^5$ c
Soil sample 55				
Control	0.00 ± 0.00 a#	0.00 ± 0.00 a	1.80 ± 1.06 a	0.00 ± 0.00 a
+ *S. elegans*	$0.83 x10^5 \pm 0.67x10^5$ b	$3.67x10^4 \pm 1.20x10^4$ b	0.53 ± 0.63 b	0.00 ± 0.00 a
+ *S. elegans* and *R. solani*	$1.00 x10^5 \pm 0.00x10^5$ b	$5.67x10^4 \pm 1.33x10^4$ b	0.77 ± 0.85 b	$0.67x10^4 \pm 0.13x10^4$ b
+ *R. solani*	0.00 ± 0.00 a	0.00 ± 0.00 a	2.67 ± 1.09 c	$1.40x10^5 \pm 0.00 x10^5$ c
Soil sample 59				
Control	0.00 ± 0.00 a#	0.00 ± 0.00 a	2.00 ± 1.64 a	0.00 ± 0.00 a
+ *S. elegans*	$1.83 x10^5 \pm 0.17x10^5$ b	$8.67x10^4 \pm 0.66x10^4$ b	0.60 ± 0.93 b	0.00 ± 0.00 a
+ *S. elegans* and *R. solani*	$2.00 x10^5 \pm 0.00x10^5$ b	$1.33x10^5 \pm 0.06x10^5$ b	0.733 ± 1.01 b	$6.68x10^4 \pm 0.66x10^4$ b
+ *R. solani*	0.00 ± 0.00 a	0.00 ± 0.00 a	2.53 ± 1.19 c	$1.13 x10^5 \pm 0.07x10^5$ c

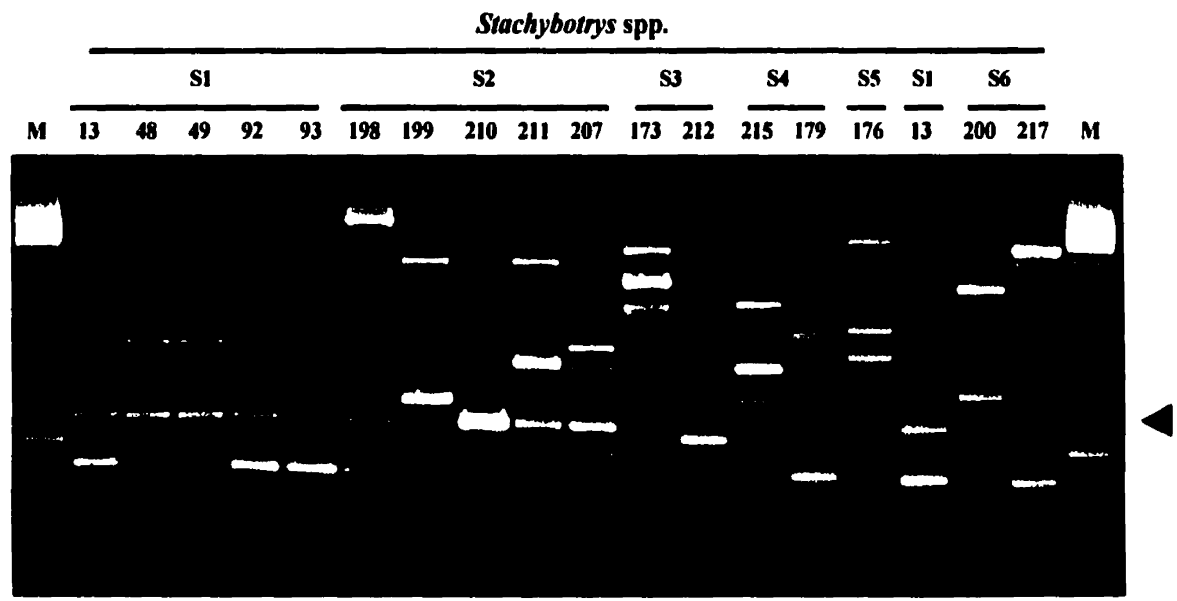
* Experiment was conducted using different types of natural soil. The natural soil samples are obtained from three different places in Quebec, Canada, where incidence of *Rhizoctonia* disease and potaot scab is high. Natural soil 3 is fine sandy soil (Saint-Romuald), soil 55 is silty sandy soil (Saint-Germain), and soil 59 is coarse sandy soil (Isle-Verte). The soils were inoculated with spores of *S. elegans* and sclerotia of *R. solani*. Three replicates per treatment and ten table beet seeds per replicate were sown. Data represent the mean of three replicates ± standard deviation of the mean.

** Data represent the average of three replicates ± standard deviation. The data was log transformed and mean value followed by the same letter within the same column are not significantly different to least significant difference test ($P \geq 0.05$).

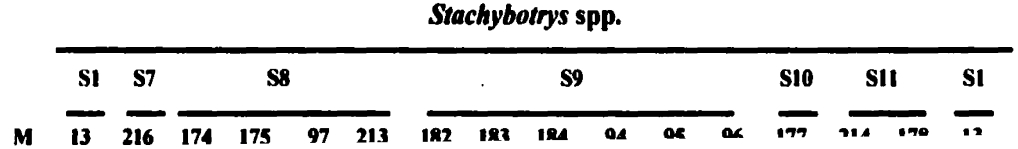
The tests were performed separately in different soil samples.

Figure 1. RAPD profiles of DNA from *S. elegans*, other *Stachybotrys* species, fungal and bacterial species and plant using primer OPN-16. Refer to Table 1 for isolate ID number used in electrophoresis. **A:** isolates belonging to *S. elegans* (S1), *S. bisbyi* (S2), *S. albipes* (S3). *S. parvispora* (S4), *S. dichora* (S5), *S. longispora* (S6). **B:** isolates belonging to *S. oenanthes* (S7), *S. cylindrospora* (S8), *S. chartarum* (S9), *S. kampalensis* (S10), and *S. microspora* (S11). **C:** Representative isolates of *M. echinata* (M), *P. ultimum* (P), *F. solani* (F), *Potato* (PL), *B. cinerea* (B), *A. alternaria* (A), *R. solani* (R), *S. scabies* (St) and *V. albo atrum* (V). L stands for molecular 1Kb ladder The black arrows indicates the position of 951 bp amplicon.

A


Stachybotrys spp.
S1
S2
S3
S4
S5
S1
S6
M 13 48 49 92 93 198 199 210 211 207 173 212 215 179 176 13 200 217 M
951 bp

B


Stachybotrys spp.
S1
S7
S8
S9
S10
S11
S1
M 13 216 174 175 97 213

C

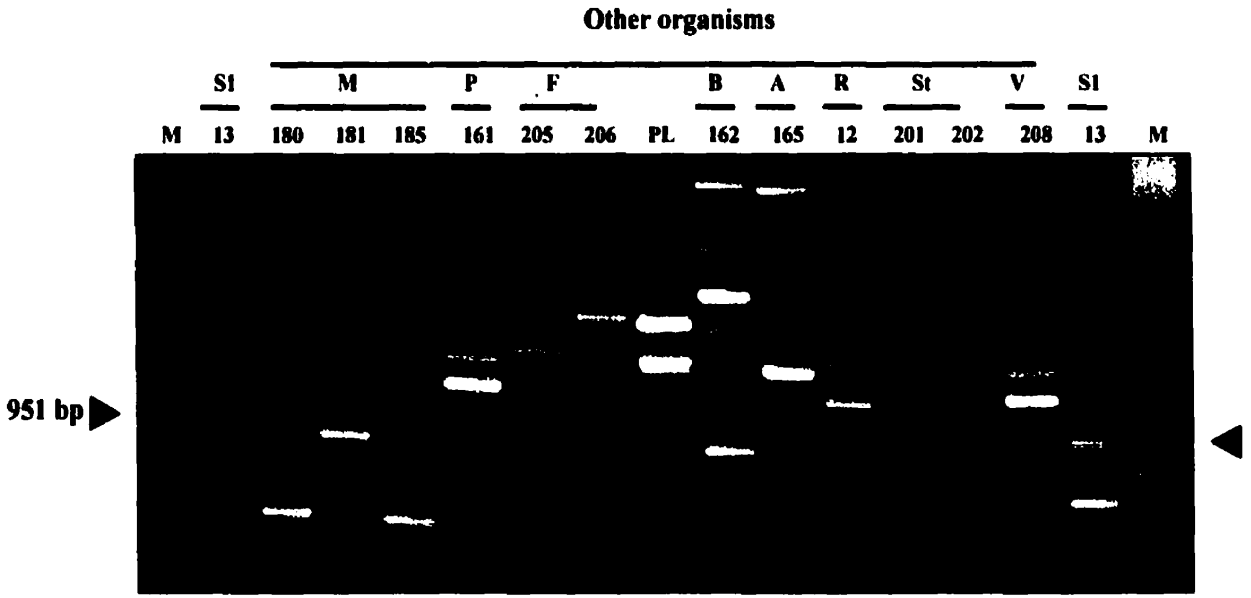

Other organisms
Sl
M
P
F
B
A
R
St
V
Sl
M
13
180
181
185
161
205
206
PL
162
165
12
201
202
208
13
M
951 bp

Figure 2. Slot blot of amplified DNA products from fungal, bacterial and plant DNA using OPN-16 primer. The blot was probed with radiolabelled 951bp fragment of *Stachybotrys elegans* isolate 13.

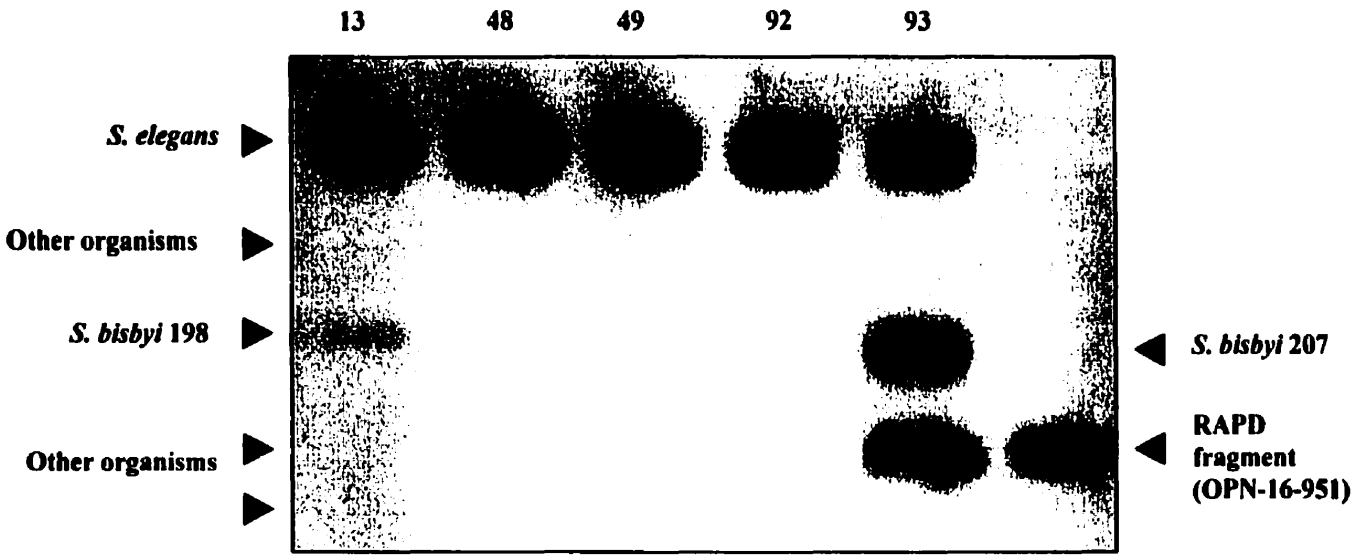
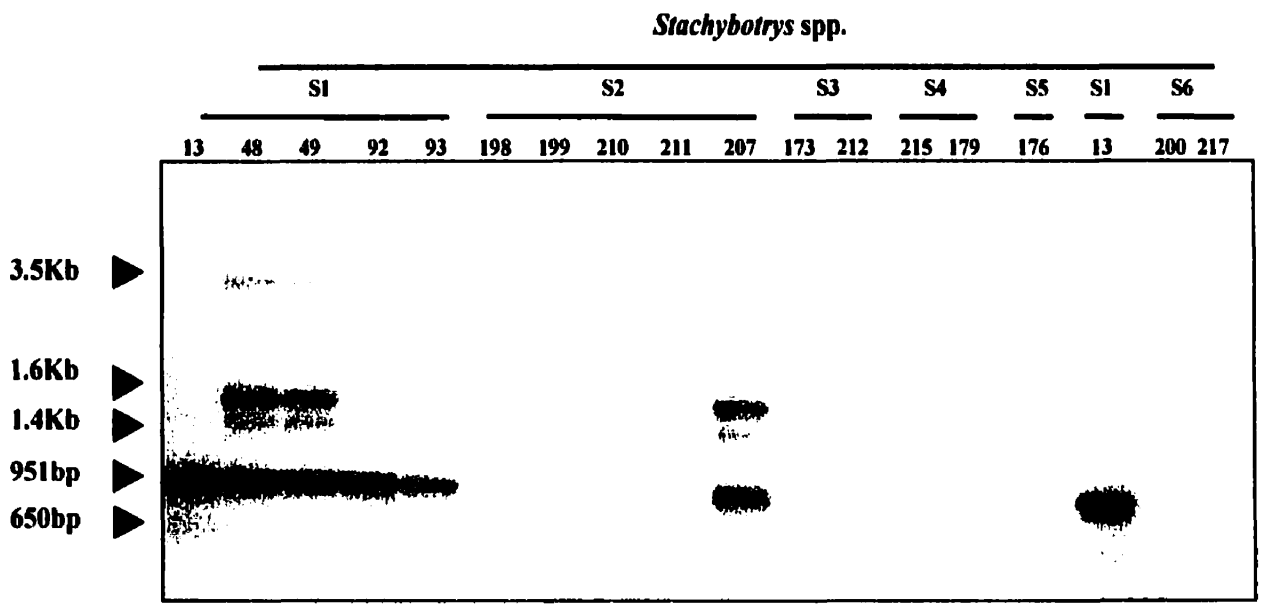
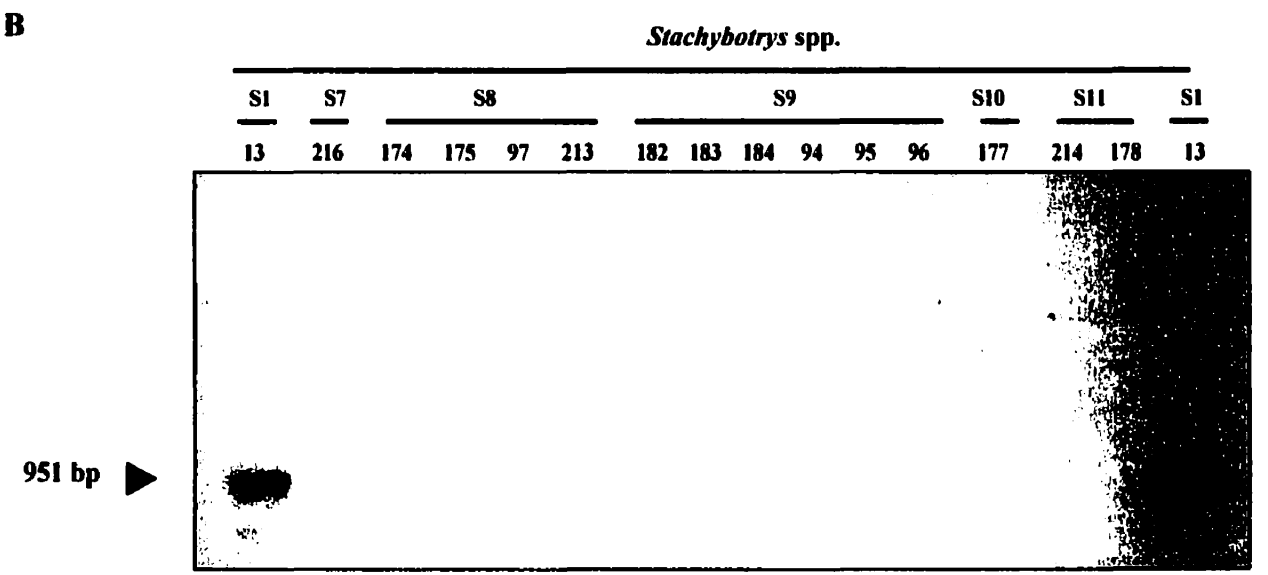

13
48
49
92
93
S. elegans
Other organisms
S. bisbyi 198
Other organisms
S. bisbyi 207
RAPD
fragment
(OPN-16-951)

Figure 3. Southern blot of amplified DNA fragments from *Stachybotrys* species, other fungal, bacterial and plant species. Refer to Table 1 for the isolate ID number. **A:** isolates belonging to *S. elegans* (S1), *S. bisbyi* (S2), *S. albipes* (S3). *S. parvispora* (S4), *S. dichora* (S5), *S. longispora* (S6). **B:** isolates belonging to *S. oenanthes* (S7), *S. cylindrospora* (S8), *S. chartarum* (S9), *S. kampalensis* (S10), and *S. microspora* (S11). **C:** Representative isolates of *M. echinata* (M), *P. ultimum* (P), *F. solani* (F), *Potato* (PL), *B. cinerea* (B), *A. alternaria* (A), *R. solani* (R), *S. scabies* (St) and *V. albo atrum* (V). L stands for molecular 1Kb ladder The black arrow indicates the position of 951 bp amplicon. The probe used in the blot was the radiolabeled RAPD fragment OPN-16-951 produced by *S. elegans* isolate 13.

A


Stachybotrys spp.
S1
S2
S3
S4
S5
S1
S6
13 48 49 92 93 198 199 210 211 207 173 212 215 179 176 13 200 217
3.5Kb
1.6Kb
1.4Kb
951bp
650bp

B


Stachybotrys spp.
S1
S7
S8
S9
S10
S11
S1
13 216 174 175 97 213 182 183 184 94 95 96 177 214 178 13
951 bp

C

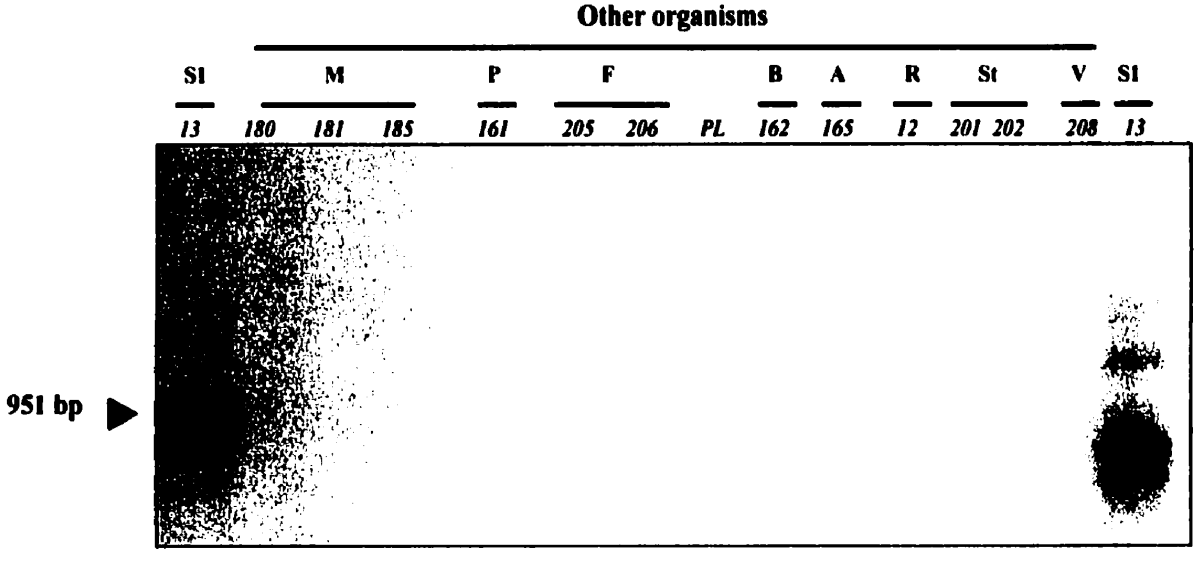
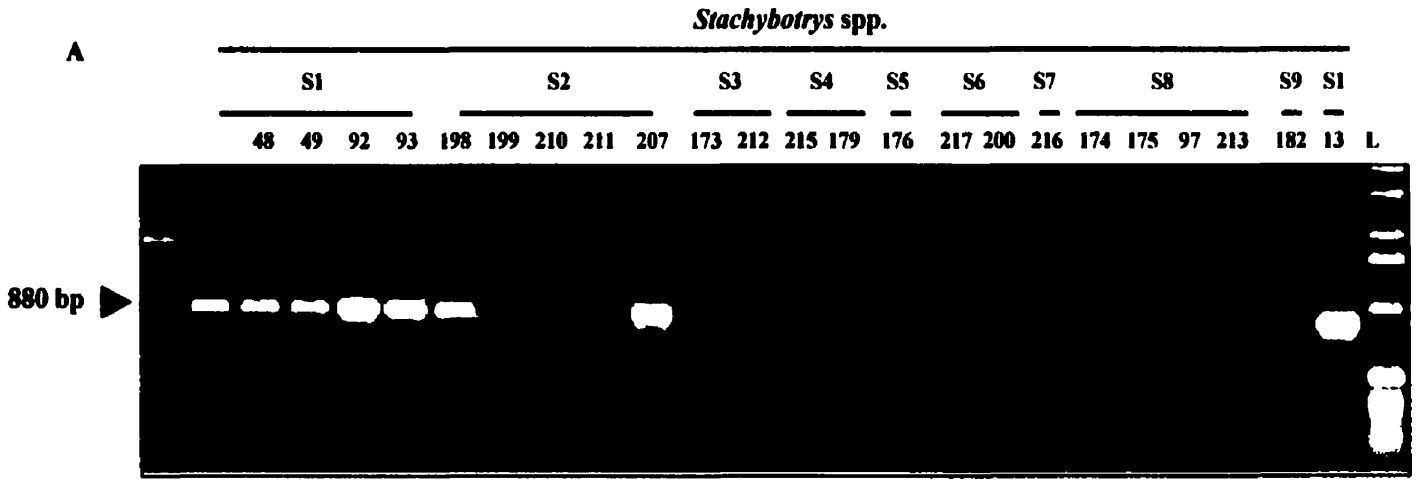

Other organisms
Sl
M
P
F
B
A
R
St
V
Sl
13
180
181
185
161
205
206
PL
162
165
12
201
202
208
13
951 bp

Figure 4. Specificity of the designed primers (SE-13R/F). Refer to Table 1 for the isolate ID number. **A:** isolates belonging to *S. elegans* (S1), *S. bisbyi* (S2), *S. albipes* (S3). *S. parvispora* (S4), *S. dichora* (S5), *S. longispora* (S6). *S. oenanthes* (S7), *S. cylindrospora* (S8), *S. chartarum* (S9), **B:** *S. kampalensis* (S10) and *S. microspora* (S11). Representitive isolates of *M. echinata* (M), *P. ultimum* (P), *F. solani* (F), *V. albo atrum* (V), *B. cinerea* (B), *A. alternaria* (A), *R. solani* (R), *S. scabies* (St) and potato tissue (PL). The black arrow indicates the position of 951bp amplicon. L stands for 1Kb molecular ladder.


A
Stachybotrys spp.
S1
S2
S3
S4
S5
S6
S7
S8
S9
S1
48 49 92 93 198 199 210 211 207 173 212 215 179 176 217 200 216 174 175 97 213 182 13 L
880 bp

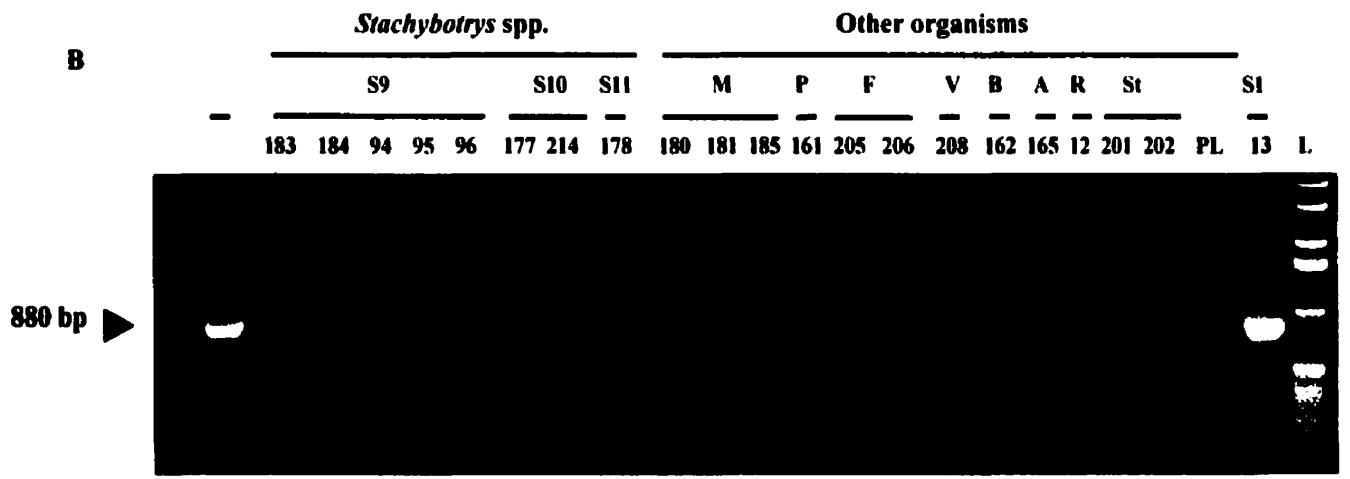

B
Stachybotrys spp.
Other organisms
S9
S10
S11
M
P
F
V
B
A
R
St
S1
183 184 94 95 96 177 214 178 180 181 185 161 205 206 208 162 165 12 201 202 PL 13 L
880 bp

Figure 5. PCR detection of *S. elegans* and *R. solani in vitro* using primers SE-13R/F and SBU-177/336. Zone 1: DNA of *S. elegans* was extracted from areas away from the interaction zone, Zone 2: DNA was extracted from the interaction zone of *S. elegans* and *R. solani*, Zone 3: DNA of *R. solani* extracted from areas away from the interaction zone. The black arrow indicates the size of 880 bp and 179 bp. L stands for 1Kb molecular ladder.

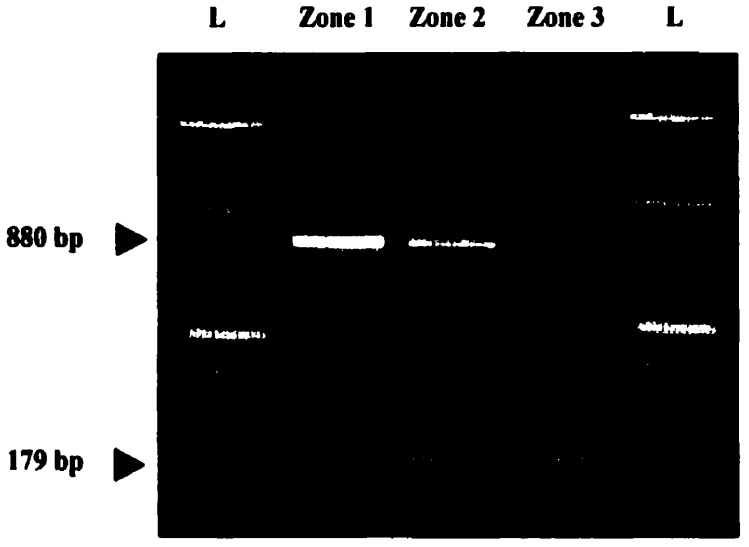

L
Zone 1
Zone 2
Zone 3
L
880 bp
179 bp

Figure 6. PCR detection of *S. elegans* and *R. solani* in green house soil and beet seedling root using primers SE-13R/F and SBU-177/336, respectively. **A:** PCR detection of *S. elegans* and *R. solani* in soil. DNA was extracted from both sterile and non-sterile soils. C: noninoculated soil, S: soil inoculated with *S. elegans* alone. R: soil inoculated with *Rhizoctonia solani* alone. S+R: soil inoculated with *S. elegans* and *R.solani*. The black arrow indicates the size of 880 bp and 179 bp. L stands for 1Kb molecular ladder.

B: PCR detection of *S. elegans* and *R. solani* on roots of beet seedling using primers SE-13F/R and SBU-177/336, respectively. B+C: beet seedlings grown in control soil. B+S: beet seedlings grown in soil inoculated with *S. elegans* alone. B+ R: beet seedligns grown in soil inoculated with *R. solani* alone. B+S+R: Beet seedlings grown in soil with *S. elegans* and *R.solani*. The black arrows indicate the size of 880 bp and 179 bp. L stands for 1Kb molecular ladder.

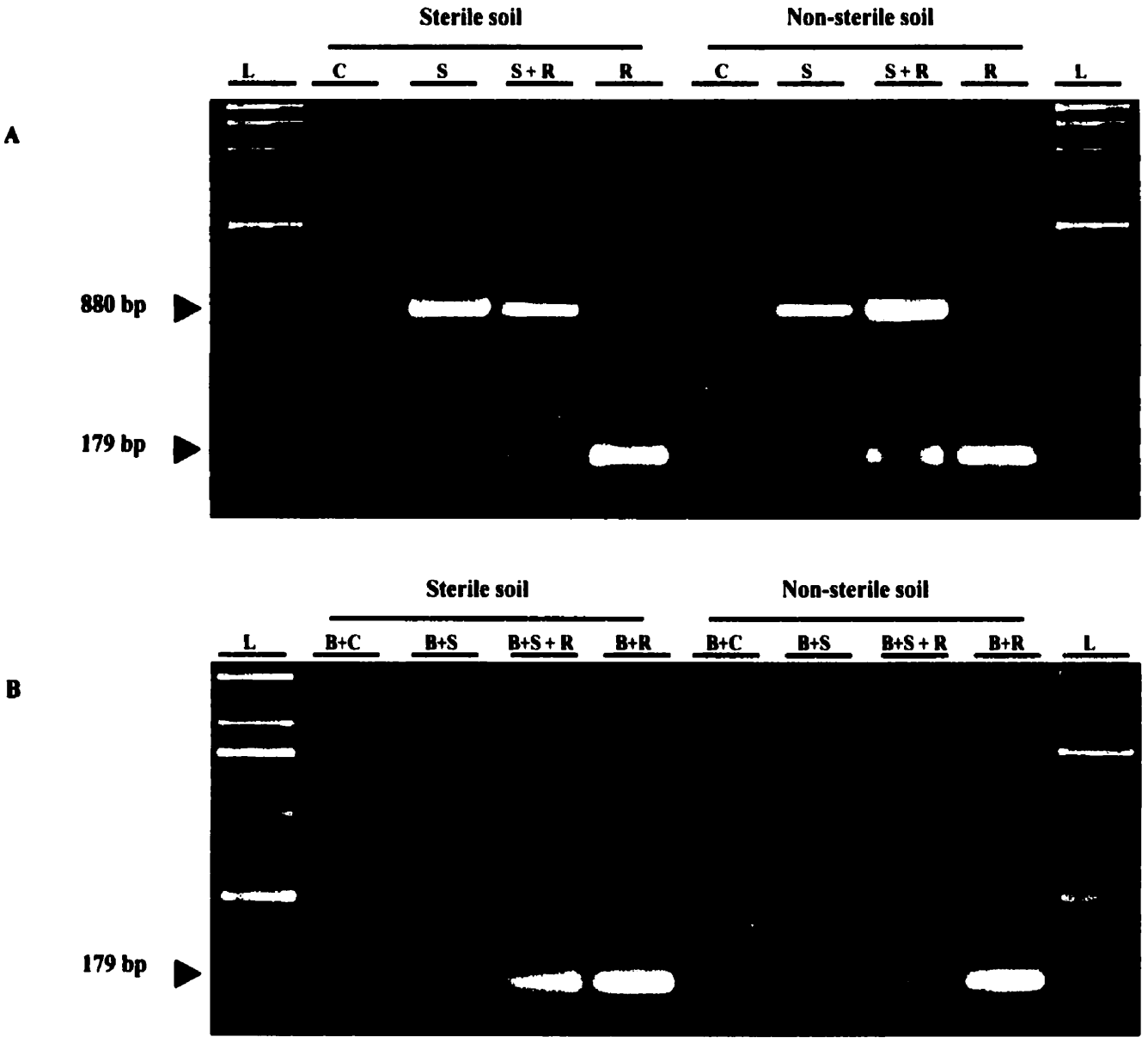
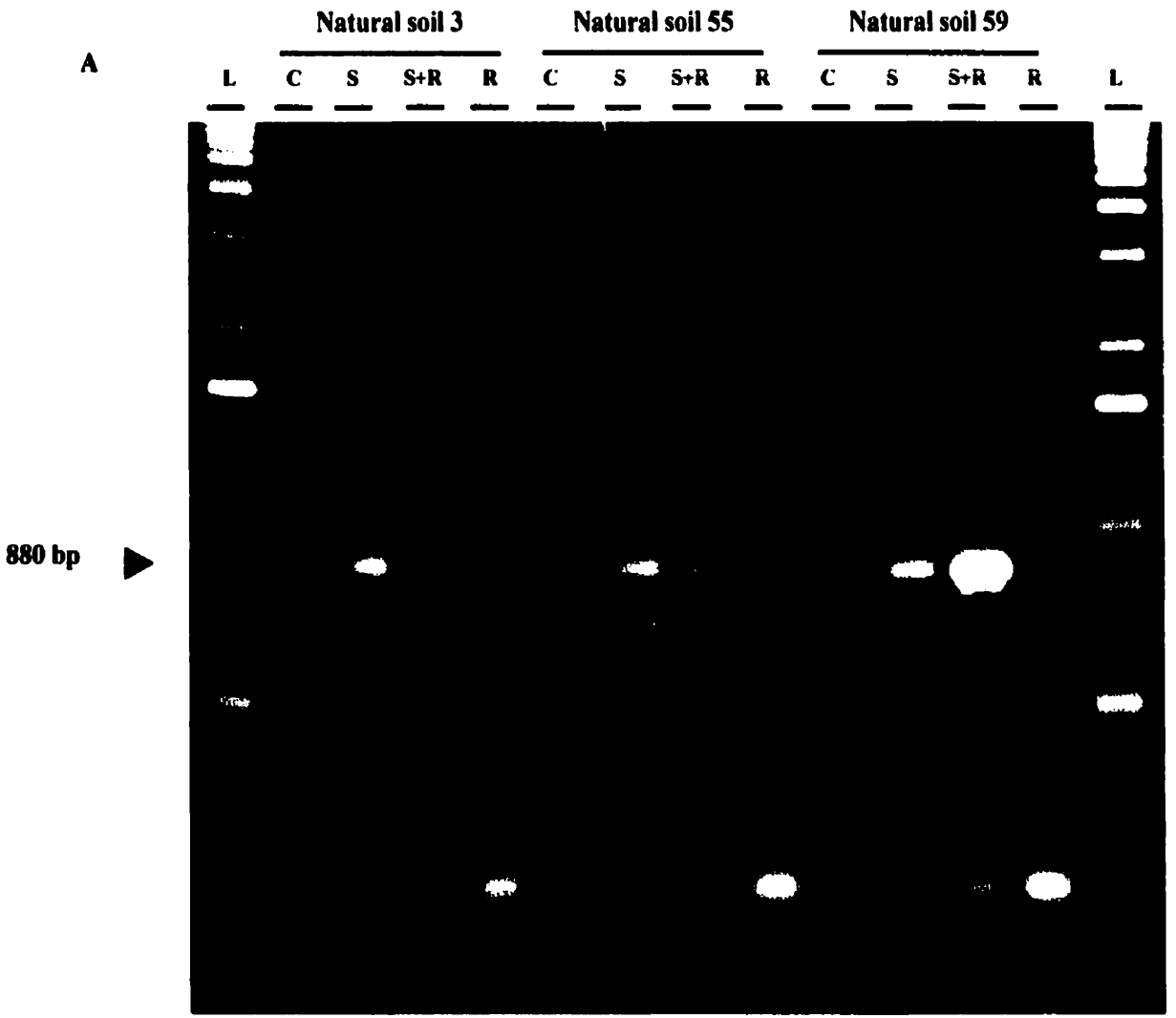
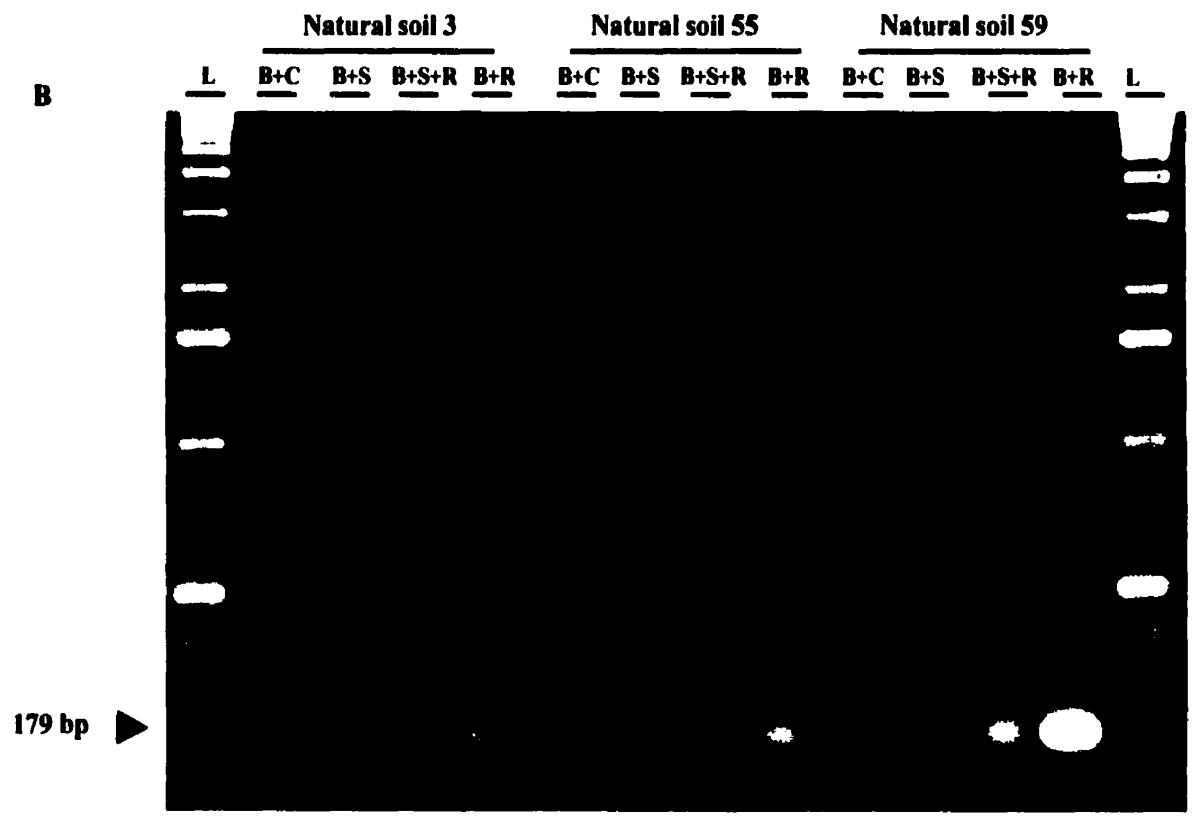

A
Sterile soil
Non-sterile soil
L
C
S
S+R
R
C
S
S+R
R
L
880 bp
179 bp
B
Sterile soil
Non-sterile soil
L
B+C
B+S
B+S+R
B+R
B+C
B+S
B+S+R
B+R
L
179 bp

Figure 7. PCR detection of *S. elegans* and *R. solani* in natural soil and beet seedling roots using primer SE-13F/R and SBU-177/336, respectively. Soil samples are obtained from Quebec where incidence of *Rhizoctonia* disease and potato scab is high. Natural soil 3 is fine sandy soil (Saint-Romuald, Quebec), Natural soil 55 is silty sandy soil (Saint-Germain), and Natural soil 59 is coarse sandy soil (Isle-Verte). **A**: PCR detection of *S. elegans* and *R. solani* in natural soil. C: noninoculated soil, S: soil inoculated with *S. elegans* alone. R: soil inoculated with *R. solani* alone. S+R: soil inoculated with *S. elegans* and *R.solani*. The black arrow indicates the size of 880 bp and 179 bp. L stands for 1Kb molecular ladder. **B:** PCR detection of *S. elegans* and *R. solani* on roots of beet seedling using primers SBU-177/336 and SE-13F/R, respectively. DNA of beet roots grown in three natural soil samples. B+C: beet seedlings grown in control soil. B+S: beet seedlings grown in soil inoculated with *S. elegans* alone. B+R: beet seedlings grown in soil inoculated with *R. solani* alone. B+S+R: beet seedlings grown in soil inoculated with *S. elegans* and *R. solani*. The black arrow indicates the size of 880 bp and 179 bp. L stands for 1Kb molecular ladder.


A
Natural soil 3
Natural soil 55
Natural soil 59
L
C
S
S+R
R
C
S
S+R
R
C
S
S+R
R
L
880 bp


B
Natural soil 3
Natural soil 55
Natural soil 59
L
B+C
B+S
B+S+R
B+R
B+C
B+S
B+S+R
B+R
B+C
B+S
B+S+R
B+R
L
179 bp

Appendix 1. Results of Second Screening: (Operon Kit N, B)

Primer	Sequence	(G+C)%	Amplification (Reproducible)	Polymorphism			Potential Marker
				Within *S. elegans* (5 strains)	Within *S. bisbyi* (5 strains)	Between species (43 strains)	
OPB-07	5'-GGTGACGCAG-3'	70	Y	Yes, present in 5 *S. elegans*	Present in 3 *S. bisboy* 198, 199,207	Similar band in *S. parvispora, A. alternaria*	Strong band (1100 bp)
OPB-12	5'-CCTTGACGCA-3'	60	Y	Yes, present in 5 *S. elegans*	Present in 4 *S. bisbyi* 199,211,207,198	Similar band in *R. solani, S. albipes*	Normal band 1600 bp
OPB-18	5'-CCACAGCAGT-3'	60	Y	Yes, present in 5 *S. elegans*	Present in 1 *S. bisbyi* 207	Similar band in *Verticilium albo atrum*	Faint band 2300 bp
OPN-02	5'-ACCAGGGGCA-3'	70	Y	Yes, present in 5 *S. elegans*	Present in 3 *S. bisbyi* 199,211,207	Similar band in *S. chartarum* and *R. solani*	Strong band 2000 bp
OPN-05	5'-ACTGAACGCC-3'	60	Y	Yes, present in 5 *S. elegans*	Present in 1 *S. bisbyi* 207	No similar band	very faint band 400 bp
OPN-06			Y	Yes, present in 5 *S. elegans*	Present in 2 *S. bisbyi* 198, 207	Similar band in *Streptomyces*, potato tissue	Normal band 900
OPN-11	5'-TCGCCGCAAA-3'	60	Y	Yes, present in 5 *S. elegans*	Present in 3 *S. bisbyi* 199, 210,211	Similar band in *Fusarium solani*	Strong band 2200 bp
OPN-12	5'-CACAGACACC-3'	60	Y	Yes, present in 5. *S. elegans*	Present in 2 *S. bisbyi* 198, 207	Similar band in *S.cylindrospora*	Strong band 1600 bp
OPN-15	5'-CAGCGACTGT-3'	60	Y	Yes, present in 5 *S. elegans*	Present in 2 *S. bisbyi* 198, 207	Similar band in *F. solani* Potato tissue	Normal band 2200 bp
OPN-16	5'-AAGCGACCTG-3'	60	Y	Yes, present in 5 *S. elegans*	Present in 4 *S. bisbyi* 198,210,211,207	Similar band in *Verticillium albo atrum*	1 marker (strong) length=900 bp

Possibel primers

OPN-11: marker present in 5 *S. elegans* and 3 *S. bisbyi* (198, 207 absent) and in one stain of *Fusarium solani.*

OPN-16: marker present in 5 *S. elegans* and 4 *S. bisbyi* (199 absent) and in one stain of *Verticillium albo atrum.*

OPB-12: marker present in 5 *S. elegans* and 4 *S. bisbyi* (210 absent) and in one stain of *R. solani, S. albipes*

OPB-18: marker present in 5 *S. elegans* and 1 *S. bisbyi* (198, 199, 210, 211 absent) and in one stain of *Verticiliium albo atrum*

Appendix 2. Results of Third Screening: (Operon Kit N, B)

Third Primer screening			Amplification (Reproducible)	Polymorphism			Potential Marker
Primer	Sequence	(G+C)%		Within *S. elegans* (5 strains)	Within *S. bisbyi* (5 strains)	Between (43 strains)	
OPB-12	5'-CCTTGACGCA-3'	60	Y	Yes, present in 5 *S. elegans*	Present in 4 *S. bisbyi* 199,211,207,198	Similar band in lane 19, 47 (*S. albipes, R. solani*)	Normal band 1600 bp
OPB-18	5'-CCACAGCAGT-3'	60	Y	Yes,present in 5 *S. elegans*	Present in 1 *S. bisbyi* 207	Similar band in Lane 44 (*Verticillium albo atrum*)	Faint band (2300 bp)
OPN-16	5'-AAGCGACCTG-3'	60	Y	Yes, present in 5 *S. elegans*	Present in 4 *S. bisbyi* 198,210,211,207	No similar band	Strong band (951 bp)
OPN-11	5'-TCGCCGCAAA-3'	60	Y	Yes, present in 5 *S. elegans*	Present in 4 *S. bisbyi* 199, 210,211,207	Similar band in lane 43 (*Fusarium solani*)	Strong band (2200 bp)

Possible Primers: OPN-11 and OPN-16

OPN-16: marker present in 5 *S. elegans* and 4 *S.bisbyi* (199 absent). The amplification profile is similar to the profile showed in the second screening. It produces one strong band (950 bp). This band present in all five *S. elegans* isolate and no similar band was find in any other lanes.

OPN-11: marker present in 5 *S. elegans* and 4 *S. bisbyi* (198 absent). The amplification profile is reproducible. A strong band of 2200 bp present in all 5 *S. elegans* isolates and 4 *S. bisbyi* isolates. However one isolate of *F. solani* produced a band of similar size

Appendix 3. Result of Fourth Screening: (Operon Kit N, B)

Third Primer screening			Amplification (Reproducible)	Polymorphism			Potential Marker
Primer	Sequence	(G+C)%		Within *S. elegans* (5 strains)	Within *S. bisbyi* (5 S. strains)	Between (43 strains)	
OPN-16	5'-AAGCGACCTG-3	60	Y	Yes,present in 5 *S. elegans*	Present in 4 *S. bisbyi* 198,210,211,207	No Similar band in other Lanes	Strong band (951 bp)
OPN-11	5'-TCGCCGCAAA-3'	60	Y	Yes, present in 5 *S. elegans*	Present in 4 *S. bisbyi* 199, 210,211,207	Similar band in lane 44 (*Fusarium solani*)	Strong band (2200 bp)

Possible Primers: OPN-16

OPN-16: marker present in 5 *S. elegans* and 4 *S.bisbyi* (199 absent). This primer has been tested three times. The amplification profile is reproducible. Based on the intensity of the band, it should have multiple copies in the fungal genome. So when a set of primer is designed to amplify this region, there is a possibility that a moderate or strong amplification can be achieved in the conventional PCR. This will increase the sensitivity of the detection using PCR since signal is strong. The size of the band is 950 bp..