Characterization of Phialophora spp. isolates from a Montana take-all suppressive soil and their use in suppression of wheat take-all caused by Gaeumannomyces graminis var. tritici (Ggt)
by Narjess Zriba

A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Plant Pathology
Montana State University

Abstract:
Sterile fungi isolated from a Montana take-all suppressive soil were identified as Phialophora spp. and were characterized morphologically. These Phialophora spp. isolates were nonpathogenic on wheat or barley in glasshouse experiments. They, however, did not confer a substantial protection of wheat and barley seedlings against Gaeumannomvces graminis var. tritici (Ggt) in glasshouse tests.

Ribosomal DNA (rDNA) fragments from four Phialonhora and two Gaeumannomvces isolates were amplified with Polymerase Chain Reaction (PCR) using universal primers, cloned, and sequenced. Sequence comparison to known sequences of Phialonhora spp. and Gaeumannomvces spp. revealed that the Phialonhora isolates included in this study were very closely related to Gaeumannomvces and less so to other Phialonhora spp. Sequence alignment allowed the design of primers to be used in detecting Phialonhora sp. I-52 in the soil and on cereal roots. Phialonhora sp. I-52 was tentatively identified as P. graminicola based on morphological and molecular analyses.

In vitro tests of antagonism involving Phialonhora spp. I-52, I-58, as well as a Bacillus sp. strain L that originated from the same soil as I-52 and I-58, resulted in significant inhibition of Ggt growth.

Phialonhora sp. I-52 was combined with Phialonhora sp. I-58 and tested for Ggt suppression in the field and with Bacillus sp. L under glasshouse conditions. Neither the combination of the two Phialonhora spp. nor that of I-52 and Bacillus sp. L conferred any advantage in controlling take-all over I-52 alone. Phialonhora sp. I-52 and Bacillus sp.

L were shown to successfully colonize wheat roots and were frequently and readily isolated over a two-month period.

Phialophora sp. I-52, when introduced on canola seed, proved to be an efficient biological control agent against wheat take-all in its original suppressive soil as well as in a highly conducive soil. In field experiments, Phialonhora sp. I-52 reduced take-all infection and increased shoot weight and grain yield.

The antagonistic ability of Phialonhora spp. towards Ggt is most likely due to competition for infection sites on wheat roots, competition for nutrients, particularly iron through the production of siderophores, and/or the production of diffusible antibiotic metabolites. Some evidence pointed to a possible role of I-52 in promoting the growth of wheat plants.

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APPROVAL

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This thesis has been read by each member of the graduate committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style, and consistency, and is ready for submission to the College of Graduate Studies.

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ABSTRACT

Sterile fungi isolated from a Montana take-all suppressive soil were identified as Phialophora spp. and were characterized morphologically. These Phialophora spp. isolates were nonpathogenic on wheat or barley in glasshouse experiments. They, however, did not confer a substantial protection of wheat and barley seedlings against Gaeumannomyces graminis var. tritici (Ggt) in glasshouse tests.

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Phialophora sp. I-52 was combined with Phialophora sp. I-58 and tested for Ggt suppression in the field and with Bacillus sp. L under glasshouse conditions. Neither the combination of the two Phialophora spp. nor that of I-52 and Bacillus sp. L conferred any advantage in controlling take-all over I-52 alone. Phialophora sp. I-52 and Bacillus sp. L were shown to successfully colonize wheat roots and were frequently and readily isolated over a two-month period.

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INTRODUCTION

In the last 20 years, much emphasis has been placed on biocontrol of plant pathogens. Biological control can be defined as the reduction in the amount of inoculum or disease producing activity of a pathogen accomplished by one or more organisms other than man (Cook & Baker, 1983).

Take-all of wheat and barley, caused by the soil-borne pyrenomycete fungus Gaeumannomyces graminis var. tritici (Sacc.) (Arx. and Olivier) Walker (Ggt), is a widespread root and crown disease of intensively grown cereals.

Due to the worldwide distribution of the disease and its economic importance, a great deal of research has been and is still being conducted since the causal agent was first described 150 years ago. There are no resistant commercial cultivars of wheat and barley. In fact, little or no resistance to take-all has been found in thousands of wheat lines and cultivars screened due to the complexity of the plant-pathogen interaction. Despite all the efforts, take-all remains one of the most difficult cereal diseases to control by genetic resistance.

In general, seed treatment chemicals have only a limited value because of their lack of efficacy or persistence (Prew & McIntosh, 1975). The inconsistency of chemical treatments has been explained by the

discontinuous distribution of the inoculum throughout the soil profile and at various depths in the soil, by the variation in the biology of the fungus in different areas, and by the response of the fungicides to differences in climate, soil physical and chemical characteristics, and soil microflora.

So far, the only recommended method for control of take-all is crop rotation due to the low competitive ability of the fungus and its low survival in absence of any host. However, crop rotation is neither economically nor environmentally feasible in many parts of the world.

An interesting alternative method for the control of take-all is the application of fungal and bacterial biocontrol agents based on the notion of specific suppression, also termed take-all decline (TAD), in addition to the general suppression which is a property of all natural soils. Suppressivity (or low receptivity) to take-all was first observed about 70 years ago. Gaeumannomyces graminis is susceptible to antagonism by microorganisms present in most natural soils and competes poorly with the general soil microflora for colonized or buried substrates (Garrett, 1970). Therefore, it has been proposed that the sensitivity of G. graminis to activities of the general microflora is important in limiting its activity and also as a background against which specific control agents must operate. These specific microorganisms

are present in only some soils or cropping sequences and include parasites like Didymella exitialis (Siegle, 1961), Pythium oligandrum (Deacon & Henry, 1978), and other antagonists and perhaps competitors. Phialophora spp. may offer good potential for biocontrol of take-all. However, they cannot be implicated in take-all decline as their population in cereal monoculture remains low except after grass (Deacon, 1973). Deacon (1976) reviewed evidence for a widespread natural biocontrol of the take-all fungus, Gaeumannomyces graminis, by Phialophora graminicola and prospects for application of biocontrol by this and similar mycelial fungi of cereals and grass roots.

With respect to take-all and other diseases, soils may be classified from suppressive to conducive. Although many soil characteristics have been shown to influence the level of soil suppressivity, it is often an acquired property of the soil due to changes in the microbial population (Alabouvette, 1990; Scher & Baker, 1980). It seems reasonable to assume that since soils suppressive to take-all have been reported in different environments, different mechanisms and, most probably, various microorganisms may be involved in this natural suppression of the pathogen and/or the disease. Understanding the mechanisms by which the biocontrol of take-all occurs is critical to the eventual improvement of the control methods. These mechanisms are generally classified as

competition, parasitism/predation, and antibiosis (Baker, 1968). In the case of take-all, competition (Wong, 1994) and antibiosis (Thomashow & Weller, 1988) play an important role in suppressing Ggt.

Organisms that colonize the roots of plants later become major constituents of the soil microflora on organic matter. These organisms can then act as an inoculum source for the following seasons (Bowen, 1979). In a cropping system, the build-up of certain microflora could be significant if pathogens are present in the population. In the presence of roots the interaction between microorganisms will exist particularly in the form of competition for nutrients (Fokkema & Van Der Meulen, 1976). Shaw and Peters (1994) suggested that a pathogen may in fact increase nutrient availability by damaging host tissue and so release host nutrients. This in turn may increase the population of non-pathogenic root parasites. An abundance of such a population may help rapid colonization of new roots and so decrease infection sites for pathogens.

There may be many reasons for soil suppressivity. For example, levels of fluorescent Pseudomonas spp. antagonistic to Ggt are higher in the rhizosphere of wheat from suppressive than non-suppressive soil (Weller & Cook, 1983). The infection of cereal roots by Ggt was reduced in pot experiments by prior colonization of non- or

weakly-pathogenic root-inhabiting fungi such as hypovirulent Ggt and Phialophora spp. from wheat or other grasses (Sivasithamparam, 1975; Wong, 1980), Phialophora graminicola (Balis, 1970; Deacon, 1974, 1976; Scott, 1970), Phialophora radicicola (Deacon, 1976), and G. graminis var. graminis (Wong, 1975). These fungi colonize the cortex of the root and may, therefore, induce resistance mechanisms in the host causing greater lignification and suberization of the endodermis and xylem vessels. In addition, they may compete with virulent Ggt for the same substrate and sites on the roots. They may also increase leakage of root exudates that contribute to proliferation of populations of other antagonistic rhizosphere microorganisms.

Competition between ectotrophic cereal root parasites is influenced by the natural progressive senescence of cereal root cortices. The importance of root cortical death (RCD) in relation to fungal colonization was realized by Holden (1975, 1976). In three-week-old wheat and barley plants, 61% and 41%, respectively, of cortical cells were anucleate in the oldest regions of the seminal root axes despite the healthy white outward appearance. Henry and Deacon (1981) found that behind the growing tip, cortical cells become progressively anucleate from the outer layers inward. The innermost cortical cells next to the endodermis remained alive. In soil, the rate of

senescence was not enhanced following inoculation with the weak parasites Idriella bolleyi and P. graminicola.

Weak parasites have been shown to successfully compete with Ggt in field conditions and this is particularly notable when the inoculum level of the pathogen is low (Wong, 1981). A weak parasite such as Phialophora graminicola is considered to be a successful parasite of grass roots because it is nonpathogenic and thus exists in a harmonious relationship with its host. In contrast, a pathogen such as Ggt is regarded as a less efficient parasite due to its destructive infection (Deacon, 1974; Garrett, 1970). Deacon (1976) reported that high populations of P. graminicola could be built up rapidly in grasslands and this population could be carried onto successive wheat crops resulting in restricted colonization by Ggt.

Despite several years of research, few biocontrol agents have been developed to a commercial level. Thus, it is important for the selection and development of new biocontrol agents to learn from those that have reached a commercial level. The addition of any microorganism to the soil will have little impact if the microorganism is not ecologically suited to that environment that will favor its activities, establishment, and proliferation. If a pathogen is to be controlled with reasonable success, the biocontrol agent must have an infection habit similar to

the pathogen and so colonize a similar niche or micro-site to that of the pathogen. Therefore, it should be obvious that the ecological attributes relating to the micro-environment of the pathogen are the bases of screening for the biocontrol agents. Biological control of take-all appears to be economically feasible, viable, and environmentally sound.

Inconsistent performance is a major limitation to the widespread use of biocontrol agents in commercial agriculture (Weller, 1988). Combining various biocontrol agents with different, ideally complementary, suppression mechanisms that are well adapted to the disease environment is likely to increase the amount and consistency of disease control. For instance, Duffy and Weller (1995) reported that combination treatments consisting of G. graminis var. graminis applied to the soil and fluorescent pseudomonads applied to the seed were significantly more suppressive to take-all than either treatment alone. Wong (1981) emphasized that control may only be effective in the early years of monoculture or following a break crop when take-all levels are reduced. Control is unlikely at the onset of severe take-all. In Australia, Wong et al. (1996) reported that cold tolerant isolates of Ggg and Phialophora sp. (lobed hyphopodia) were efficacious in controlling take-all in the field.

Previous work reported the presence of soils suppressive to take-all in Montana (Andrade, 1992). Out of eight soils collected from different wheat growing areas of Montana, three soils were selected as being either suppressive or conducive to take-all. Bozeman PostFarm soil, where wheat was grown in a four year rotation with green manure, exhibited properties conducive to take-all. In contrast, Larslan and Toston soils, where wheat had been grown as a monoculture for over 10 years, both exhibited suppressive properties toward Ggt. In addition, Andrade's results suggested that antagonism may also be associated with abiotic factors that activate the expression or repression of an antagonistic microflora under different conditions. These soils were characterized microbiologically and the organisms most probably involved in the suppression of the disease were further investigated. Two different mechanisms appeared to be involved in the suppression exhibited by both soils suppressive to take-all. In Larslan soil mycoparasitism appeared to be responsible for take-all suppression, whereas in Toston soil antagonism by actinomycetes and perhaps the involvement of Pseudomonas spp. in antagonism and/or iron depletion appeared to be the most likely mechanisms involved in the suppression of take-all (Andrade, 1992).

The present study on biological control of take-all had the following objectives:

- To identify and characterize, using morphological and molecular characteristics, two sterile fungi previously isolated from a Montana soil suppressive to take-all and other antagonistic fungi associated with wheat roots grown in the same soil,
- To test these fungi under glasshouse and field conditions to determine their potential in suppressing Ggt, improving the health of wheat roots and increasing yield in presence of take-all, and to try to elucidate the mechanisms involved in pathogen suppression, and
- To test whether a combination of one of these sterile fungi and a Bacillus sp. would increase the efficiency of control and to determine their survival and colonization of the roots.

CHARACTERIZATION OF *PHIALOPHORA* SPP. ISOLATED FROM A MONTANA TAKE-ALL SUPPRESSIVE SOIL

Introduction

The pathogens that cause take-all belong to the *Gaeumannomyces-Phialophora* complex of fungi that parasitize the roots and crown of graminaceous plants. These fungi are ecologically obligate parasites that grow over and on the roots of cereals and grasses. They all produce similar-looking dark runner hyphae. Four varieties of *G. graminis* have been described: *G. graminis* var. *tritici* (Ggt) is the etiologic agent of wheat (*Triticum aestivum* L.) and barley (*Hordeum vulgare* L.) take-all disease; *G. graminis* var. *avenae* (Gga) causes take-all in oats (*Avena sativa* L.) and take-all patch in turf grasses; *G. graminis* var. *graminis* (Ggg) is generally a benign parasite, infecting a number of grasses, including Bermudagrass (*Cynodon dactylon* L.) but is usually non-pathogenic to wheat (Smiley *et al.*, 1992; Walker, 1981); and *G. graminis* var. *maydis*, which has been recently characterized in China, attacks corn (*Zea mays* L.) (Yao, 1993).

Gga and Ggt have *Phialophora* anamorphs with simple hyphopodia while the Ggg anamorph has lobed hyphopodia.

Avirulent and weakly pathogenic Phialophora species also occur on cereal and grass roots. Phialophora sp. (lobed hyphopodia), which has some isolates that produce perithecia of Ggg (Walker, 1981), and Phialophora graminicola (Deacon) Walker, which has simple to slightly lobed hyphopodia and is the anamorph of G. cylindrosporus, are of particular interest in epidemiological and biological control research because of their ability to protect the roots against infection by the virulent take-all fungi.

Species in the deuteromycete genus Phialophora are ubiquitous and cosmopolitan and are important saprobes as well as human and plant pathogens. Accurate species determination is difficult because of the limited number of morphological characters and their pleomorphism (Yan et al., 1995).

Diagnosis of take-all may be difficult in the presence of other fungi, e.g., Pythium and Fusarium spp. that also cause root discoloration, and is often controversial. It is difficult to distinguish among pathogenic and non-pathogenic members of the Gaeumannomyces-Phialophora complex whose taxonomy is confused because it is based on complex properties, such as colony morphology, the ability to form phialides, hyphopodia, or sexual fruiting bodies in culture, and pathogenicity to cereals and grasses. Confirming diagnoses

of fungi isolated from diseased plants using selective media is useful but not accurate in most cases, and conventional pathogenicity testing plus attempts to produce perithecia are laborious, time consuming, and possibly inconclusive because some isolates fail to produce perithecia (Holden & Hornby, 1981).

Even though the genus Phialophora has received several monographic treatments (Cole & Kendrick, 1973; Moreau, 1963; Schol-Schwarz, 1970; Sivasithamparam, 1975), there still remains much to be done to establish the genus as homogeneous and stable. Phialophora radicicola Cain (1952) was isolated from infected maize roots in Canada by McKeen (1952) who noted its similarity to G. graminis in infection habit but complete absence of pathogenicity. Scott (1970) repeatedly isolated from old grassland a fungus with a similar infection habit to that of G. graminis, but which was non-pathogenic to wheat. This fungus is hereafter called P. radicicola even though it differs slightly from Cain's description, particularly in length of phialides and conidia. Two varieties of P. radicicola were recognized by Deacon (1974), P. radicicola var. radicicola and P. radicicola var. graminicola. However, Walker (1980) raised Deacon's variety (P.r. var. graminicola) to specific rank (P. graminicola).

Because of the problems identifying and classifying these fungi, molecular techniques are being investigated to establish a reliable means of confirming the morphological identification of Gaeumannomyces spp. and related fungi. A DNA hybridization probe and subsequently a polymerase chain reaction (PCR)-based assay were developed from a mitochondrial DNA fragment of Ggt (Henson, 1989; Henson et al., 1993; Schesser et al., 1991). Although these assays were relatively specific to G. graminis, the hybridization probe showed homology to other Phialophora spp. as well as Neurospora crassa, and the PCR-based assay resulted in amplification of DNA from Phialophora, Magnaporthe, and other species of Gaeumannomyces (Henson, 1992). The use of variable regions from internal transcribed spacers (ITS) of the nuclear rDNA is appealing because the nucleotide sequence of some regions of rDNA is highly conserved due to selection pressures to retain functionality. However, the transcribed spacer sequences between RNA genes do not encode gene products and thus appear to be relatively poorly conserved between species (Goodwin et al., 1995). Ward and Akrofi (1994) used PCR primers to amplify ribosomal ITS and 5.8S rDNA genes from nuclear DNA and then used restriction enzymes to generate RFLP patterns by digestion of the PCR fragments. This technique was relatively useful for discriminating between G. graminis

and P. graminicola and between varieties of G. graminis. Bryan et al. (1995) were able to distinguish between Ggt, Ggg and Gga by nucleotide sequence differences in the ITS regions. Furthermore, G. graminis variety-specific oligonucleotide primers were developed and used in PCRs to amplify DNA from cereal seedlings infected with Ggt or Gga (Bryan et al., 1995).

This study reports the identification of two previously unknown fungi isolated from a take-all suppressive soil (Andrade, 1992) as Phialophora spp. The results of a comparative study of morphology and behavior of these two and other Phialophora spp. isolated from this soil are also included in this study. Some of these isolates were further characterized molecularly by sequencing the ITS regions and 5.8S rDNA and comparing them to published ITS and rDNA sequences of Gaeumannomyces graminis, Phialophora spp. and related fungi.

Materials and Methods

Fungal Isolates

Known fungal isolates were obtained from laboratory collections (D. E. Mathre, J. Henson, Montana State University). Purified cultures included Phialophora-like fungi isolated from Larslan soil (Montana): I-52, I-58, 254-1, 254-2, 159-1, 17-1, 395, 336, 357-2; Gaeumannomyces graminis isolates Ggt 528, Ggt 532, Ggt 698, Ggt 554, Ggg

723, Ggg 724; P. graminicola strain 1802 (J. Henson); G. cylindrosporus 1850 (J. Henson); and G. incrustans. The Phialophora-like fungi were isolated from wheat roots grown in Larslan soil which is suppressive to take-all. The isolation technique consisted of submersing root tissue (3-5 mm) in 0.5% (v/v) NaOCl solution in distilled water for 2 min with stirring. Following surface sterilization, air-dried root tissues were placed on potato dextrose agar (PDA) plus streptomycin (100 mg/l). Colonies were then transferred to half-strength PDA (½ PDA) for subsequent DNA isolation and to compare colony characteristics and growth rates among isolates.

Colony Morphology and Growth Rates

Colony morphology was assessed for 10 days. Growth rates were obtained by incubating three culture plates of each isolate in the dark for 8 to 12 days at 2°C, 5°C, 15°C, 20°C, and 25°C. Two measurements of the diameter were taken at right angles using a ruler every 24 h and averaged. The data were analyzed by the analysis of variance using MSUSTAT (Lund, 1991), and significantly different means were separated by LSD at $p = 0.05$.

To compare cultural characteristics of some Gaeumannomyces and Phialophora isolates, the fungi were grown on PDA in the dark at 20°C on Lilly and Barnett's (1951) glucose-asparagine agar (GAA) (Weste, 1970) and on PDA in alternating light and darkness on the laboratory

bench for production of phialidic conidia. Cultures were examined microscopically after 6 and 28 days. To induce perithecia production, plates of Lilly and Barnett's GAA, corn meal agar (CMA), and PDA were incubated at room temperature (22°C) in diffuse light or were subjected to UV light at 20°C. Three plates of each isolate on each medium were observed after 21 days using a light microscope. In addition, for perithecial production, infected wheat roots were placed in test tubes with a cotton ball in the bottom to keep them moist and incubated indoors against a north facing window (Hornby, 1969) for 4-5 weeks. Perithecia that formed on the roots were examined microscopically.

To study the shape of hyphopodia on infected wheat coleoptiles (Walker, 1972), seeds were germinated on agar-plate colonies of Gaeumannomyces and Phialophora isolates and grown for up to 3 weeks in closed dishes in diffuse light, so that even non-pathogenic isolates would colonize the shoots of the senescing seedlings.

Identification of Phialophora (I-52)

Based on some morphological characteristics I-52 was suspected to be either Phialophora graminicola or P. lignicola. Phialophora I-52 was thus compared to these two species for growth rate and cultural morphology, particularly phialide, phialospore, and collarette

morphology in an attempt to confirm its identification to the species level.

In vitro Growth Inhibition of *Gaeumannomyces graminis* var. *tritici* by *Phialophora* Isolates

To test the ability of the Phialophora isolates to inhibit the growth of Ggt, 7-mm agar plugs of the antagonist were placed on 2% malt extract agar (MEA) and on half-strength PDA (½ PDA) plates at equal distance from a central 7 mm disc of Ggt. Ggt growth and the percentage of inhibition were determined after 7, 14, and 21 days of incubation at room temperature. The Phialophora isolates were added to the plate at the same time as Ggt or 3 days earlier. The experiment was repeated using 2% MEA and ½ PDA containing 20 mM 2-[N-morpholino]ethansulfonic acid (MES) to see whether inhibition in vitro occurs on buffered media. The pH of the media was adjusted to pH 6 using 1 M NaOH before autoclaving. In a separate experiment, each Phialophora or Gaeumannomyces isolate was paired with Ggt 698 on PDA, two plates per combination, to test for inhibition zone and/or hyphal interaction.

A technique using wells made in ½ PDA and filled with sterile soil was used to test antagonism in vitro in an attempt to bring soil nutritional factors into play that may affect the expression of antibiosis (Andrade, 1992). Larslan soil, from which the Phialophora isolates originated, was utilized in addition to the conducive

PostFarm soil, Toston soil (somewhat suppressive), and a take-all suppressive soil from Oregon cultivated to wheat for 60 years (R. Smiley, Pendleton, OR). Four 10-mm wells per plate were made equidistant from each other and 15 mm from the edge of the plates. A 7-mm plug of Ggt grown for one week on ½ PDA was then placed in the center of each plate. Sterile soil-containing wells with only the addition of agar plugs to the soil surface were used as checks. One fungal isolate was tested per plate and two plates were prepared per isolate. Plates were kept at room temperature and the zone of inhibition and Ggt linear growth were measured 7 days later.

Use of Cell-Free Culture Medium

Four 7-mm plugs of each fungus grown for 5 days on ½ PDA were placed in 250 ml Erlenmeyer flasks containing 50 ml of sterile one-fifth-strength potato dextrose broth (1/5 PDB). Cultures were grown on a rotary shaker for 5 days at room temperature. The broth was filter-sterilized through a 0.2 µm filter (Nalgene), added to a 15 mm diameter sterile filter disc, and placed on ½ PDA, 15 mm from the edge of the plate. A 7-mm plug of Ggt grown on ½ PDA was immediately placed in the center of the plate. Four discs containing four different fungal extracts were placed on each plate, equidistant from one another. Each plate was replicated three times. Controls consisted of sterile filter discs dipped into

sterile-filtered 1/5 PDB. Plates were maintained at room temperature and Ggt growth was measured after 7 days.

Hypholytic Activity

To test for hypholytic ability, Phialophora isolates were placed on the living mycelium of Ggt which had been growing for one week on ½ PDA or 2% MEA (Zogg & Jaggi, 1974). After 5 days the plates were examined microscopically for Ggt hyphal lysis.

Assay for Siderophore Production

Detection of siderophore production by Phialophora and Gaeumannomyces isolates was based on the universal chrome azurol S (CAS) assay (Schwyn & Neilands, 1987). The presence of siderophores is detected readily on CAS blue agar plates by the appearance of orange halos around colony growth.

Pathogenicity Assay

The pathogenicity of Phialophora isolates was tested on wheat and barley. One 2-day-old seedling per 16 cm x 3 cm plastic tube was grown in sand. The inoculum, consisting of a 10-mm mycelial disc taken from the edge of a 7-day-old culture, was placed right below the seedling and covered with sand. The experiment was conducted in a completely randomized design with five replicates per isolate. Controls consisted of agar plugs without any fungus (healthy control) and Ggt plugs (diseased control)

respectively placed beneath the seedlings. After 4 weeks growth under glasshouse conditions, the roots of the test plants were washed free of sand and examined for disease symptoms. The roots were inspected for runner hyphae. Shoots were dried at 70°C for 48 h and weighed.

Assay of Suppression of Take-All by *Phialophora* Isolates

This assay was similar to the pathogenicity assay described above except that the Ggt inoculum on autoclaved oat kernels was mixed with the sand (1%, w/w) before the wheat seedlings were planted on top of an agar disc of the test antagonist and covered with sand.

DNA Extraction and Purification

DNA was prepared from the isolates I-58, 357-2, 336, 17-1, 395, 254-2, 159-1, I-52 by grinding them in liquid nitrogen using a mortar and a pestle. Two 7-mm mycelial plugs were taken from 5-day-old cultures grown on ½ PDA, then transferred to 250 ml Erlenmeyer flasks containing 100 ml of 1/5 PDB. Broth standing cultures were grown for 10 days at room temperature, then mycelium was filtered through cheesecloth and washed with sterile water. Air-dried mycelium on filter paper was ground in liquid nitrogen, resuspended in Tris-EDTA-RNase A and incubated for 5 min at room temperature. SDS (sodium dodecyl sulfate) was added to a final concentration of 2% and Protein K to a final concentration of 200 µg/ml. After

incubation at 55-60°C for 30 min, NaCl (5 M) and 10% CTAB (cetyltrimethyl ammonium bromide) were added and incubated at 65°C for 10 min. Then chloroform-isoamyl-alcohol (1 vol.) was added and incubated for at least 30 min on ice. After centrifugation (14,000 rpm) for 10 min, isopropanol (0.55 vol.) was added to the aqueous layer, mixed and kept on ice for 30 min (David Long, personal communication). After centrifugation, the pellet was resuspended in sterile double distilled water.

PCR Amplifications

Oligonucleotide primers used for amplification were identified from conserved sequences of the 17S, 5.8S and 26s rDNA of Neurospora crassa, Schizosaccharomyces pombe, and Saccharomyces cerevisiae (White et al., 1990). The primers were the following: psnDNA2p (5'-GTCCACACACCGCCCGT-3') (Chambers et al., 1986), pITS2 (5'-GCTGCGTTCTTCATCGATGC-3'), pITS3 (5'-GCATGCATGAAGAACGCAGC-3'), and pITS4 (5'-TTCTTCGCTTATTGATATGC-3') (White et al., 1990). All PCR reactions were performed essentially as described by Sambrook et al. (1989). Amplification reactions were carried out in a volume of 50 µl in the presence of 10 µM of each primer and 10 µM deoxynucleoside triphosphates (Invitrogen) in a buffer containing 100 mM Tris-HCl pH 8.3, 500 mM Kcl, 15 mM MgCl2, 0.01% gelatin, and 1.25 U of Taq DNA polymerase. Cycling conditions were 94°C for 5 min and then 94°C for 45 s, 50°C for 30 s, and

72°C for 1 min (35 cycles), followed by a 10-min extension at 72°C.

Sequence Analyses

The products amplified using the psnDNA2p and pITS4 primer pair were cloned into the plasmid vector PCR™2 (Invitrogen). The ligation mixture was used to transform competent cells of E. coli INVαF' by the calcium chloride method (Sambrook et al., 1989) or E. coli XL2 by the electroporation method (Chuang et al., 1985). Plasmid DNA was isolated by the alkaline lysis method (Sambrook et al., 1989) and purified using the Qiagen plasmid miniprep kit. The regions containing the rDNA ITSs and the 5.8S gene of Phialophora isolates I-52, 17-1, 159-1 and 357-2, and Gaeumannomyces Ggt 698, and Ggg 724 amplified by psnDNA2p and pITS4 were sequenced at the DNA Sequencing and Synthesis Facility (Iowa State University) with Universal (-21M13) and Reverse (M13) primers. DNA sequences were aligned using the University of Wisconsin Genetics Computer Group programs Fasta and Pileup (Pearson & Lipman, 1988). Sequence data were compared with listings in the GenBank database.

Results

Growth Rate and Mycelial Characteristics

The isolates were separated into three groups based on their growth rate at 20°C (Table 1). Slow-growing isolates had an average growth rate less than 4 mm per day at 20°C (range 3.1 to 3.9 mm per day) and included P. graminicola 1802, Phialophora sp. 395, and Ggt 554. The second group had an average growth rate between 5 and 8 mm per day and included I-52, 254-1, 159-1, 336, 138, G. incrustans, Ggg 723, Ggg 724, Ggt 698, Ggt 528, and G. cylindrosporus. Fast-growing isolates had an average growth rate higher than 8 mm per day (range 8.2 to 9.6 mm per day) and included 17-1, 254-2, I-58, 357-2 and Ggt 532. Phialophora graminicola 1802 showed a low growth rate and a mycelial morphology different from the rest of the Phialophora spp. isolates but was similar to the Gaeumannomyces isolates. When initially isolated from roots or first transferred on PDA, the colony color of most Gaeumannomyces isolates was grayish white turning dark gray or black with age. In contrast, the majority of Phialophora isolates were creamy- or greenish-white. After 7 to 10 days of growth, colony pigments of Phialophora isolates were either brownish yellow, orange yellow, or greenish-gray. The majority of isolates produced a white or a creamy-white band, respectively, for Gaeumannomyces

Table 1. Growth of Gaeumannomyces and Phialophora isolates at various temperatures.

Isolate	Growth rate (mm/d) on 1/2 PDA at various temperatures					
	2°C	5°C	10°C	15°C	20°C	25°C
P. graminicola 1802	0.5	0.9	1.1	2.2	3.9	4.3
Ggt 554	0.3	0.8	0.6	2.0	3.1	6.5
395	1.3	2.9	3.2	5.6	3.6	4.6
I-52	1.4	2.4	2.8	4.3	5.9	7.7
254-1	1.5	2.8	2.9	5.5	5.8	5.8
159-1	1.5	2.7	2.9	4.6	7.6	9.8
336	1.1	1.9	2.8	4.9	6.0	6.7
G. incrustans	-[a]	-	2.3	3.3	5.1	7.2
Ggt 698	0.6	1.2	1.8	5.5	5.1	6.2
Ggt 528	0.6	0.7	1.4	5.5	6.2	8.6
Ggg 723	-	0.7	1.2	7.7	5.8	8.1
Ggg 724	-	0.6	0.7	7.1	6.2	8.3
G. cylindrosporus 1850	0.4	-	1.6	3.4	5.4	6.8
138	0.8	2.8	2.6	3.8	6.3	7.1
254-2	1.8	3.0	3.6	6.5	9.6	10.5
I-58	1.1	1.7	3.3	5.4	8.5	9.8
17-1	1.5	6.8	3.8	7.1	8.5	10.0
357-2	1.4	2.9	4.0	7.7	8.2	8.8
Ggt 532	-	0.7	1.6	5.2	8.3	10.3
LSD (0.05)	0.3	0.2	0.1	0.2	0.1	0.1

a: No growth.

and Phialophora, in the new growth zone at the perimeter of the colony. The leading mycelium of all Gaeumannomyces isolates and P. graminicola 1802 distinctively curled back to the center of the colony. However, none of the Phialophora isolates showed any mycelial curling.

Only Gaeumannomyces isolates produced perithecia on PDA, CMA, and Lilly and Barnett's GAA. Most of the remaining isolates produced phialospores and a few produced sclerotia (isolates 17-1 and 249). Neither of the methods employed induced perithecia in Phialophora isolates. However, Ggt 698 consistently produced perithecia using both methods.

Most Phialophora and Gaeumannomyces isolates produced simple or slightly lobed hyphopodia (Table 2).

Characterization of *Phialophora* (I-52)

Initially, the mycelium of Phialophora sp. I-52 was creamy white (Figure 1), becoming brown-yellow after 1 week (Figure 2), and finally turning orange yellow to red after two weeks of growth. On PDA, a diffusible yellow pigment was produced. Moniliform hyphae were common. Sporulation was abundant on WA, 1/10 PDA, 1/4 PDA and ½ PDA, and less abundant on full-strength PDA or WA amended with 100 ppm of $FeCl_3$ (Figure 3). After 10 days growth, colonies reached 6.8 cm in diameter on PDA, 8.0 cm on 2% MEA, and 4.5 cm on WA. Hyphae sometimes aggregated in strands. Hyphal width varied from 1.5 to 5-6 µm. Phialides

Table 2. Hyphopodia production and pathogenicity on wheat coleoptiles by some Phialophora and Gaeumannomyces isolates.

Isolate #	Presence(+) or absence(-) of hyphopodia	Pathogenicity
20-3*	+ simple to slightly lobed	brown lesions
254-1	+ simple to slightly lobed	light brown lesions
395*	+ simple	dark brown lesions
273-1	+ simple	dark brown to black lesions
249*	+ simple to slightly lobed	dark brown lesions
I-52**	+ simple	no lesions on coleptile[a]
17-1*	+ lobed	brown[b] lesions
357-2**	-	no lesions to very light lesions
336*	+ simple	dark brown to black lesions
159-1**	+ simple to slightly lobed	light brown lesions
Ggt 698	+ simple	dead seedlings[c]
G.c 1850	+ simple	dark brown to black lesions
138	+ simple	dark brown to black lesions

Production of root hairs was increased (*) or highly increased (**) by certain isolates.
a: Presence of brown lesions on seminal roots.
b: Host-cells packed with hyphopodia and phialospores.
c: Coleoptiles and roots were all black with mycelium growing profusely, host-cells packed with Ggt mycelium.

Figure 1. Phialophora sp. I-52. Mycelium is creamy white when first isolated from wheat roots on PDA plus streptomycin (100 ppm) at room temperature and diffuse light.

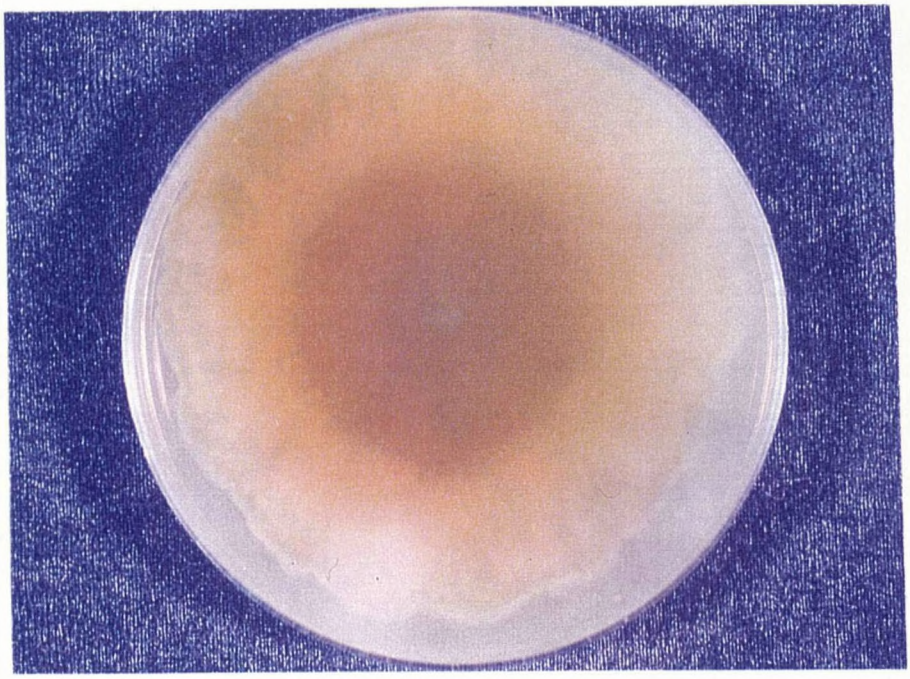

Figure 2. Phialophora sp. I-52. Mycelium is yellow-brown after 1 week growth on PDA plus streptomycin (100 ppm) at room temperature and diffuse light.

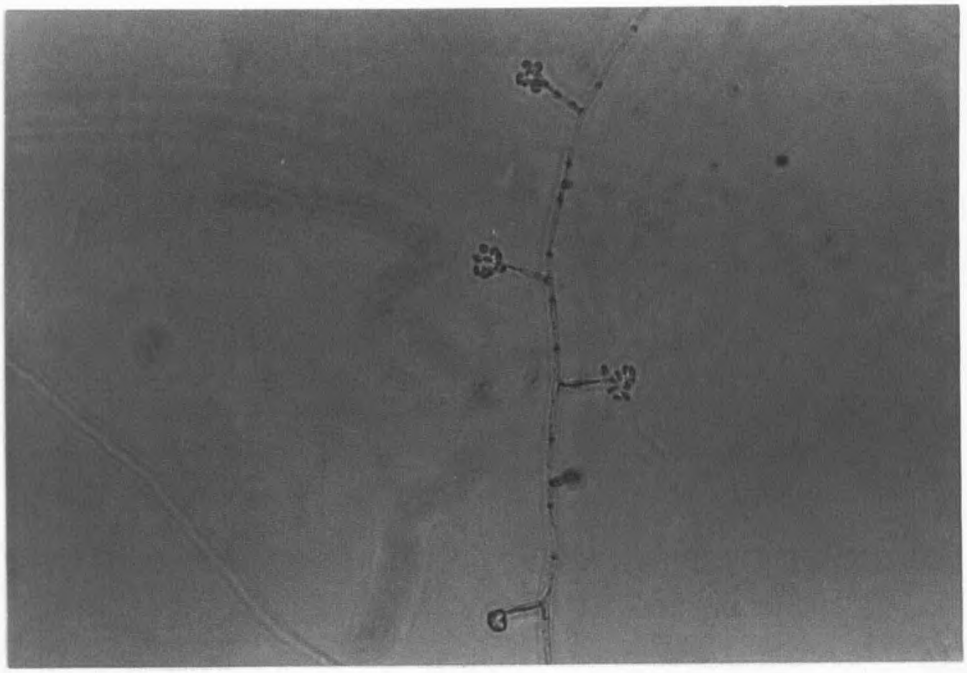

Figure 3. Phialophora sp. I-52. Phialides and phialospores (x 400) produced on WA-coated glass slides.

were usually lateral and sometimes branched. Their size was variable, being 9-15 x 1-2 x 0.5-1 µm in length, width at the base and at the tip, respectively. Phialospores were hyaline, becoming yellow brown with age, generally ovoid, 1-2.5 x 1.5-6 µm in size (Table 3). Phialospores were usually grouped in a slimy cluster at the tip of the phialide with some released in its vicinity.

In vitro Inhibition of Ggt Growth by *Phialophora* Isolates

Table 4 shows the effect on growth of Ggt 698 when paired with each of the other Gaeumannomyces and Phialophora isolates on PDA. Measurements of inhibition

Table 3. Morphological characters of some Phialophora sp. isolates.

Isolate #	Phialospore Size* (µm)	Phialide Size (µm)	Hypha[a] Width (µm)
17-1	2-3/2-2.5	12-16/2.5/1[b]	5
254-1	5-6/2.5-3	-	3-5
249	3.5-4/1-2	18/4.5/2	1.5-6
336	2-3/2-3	5-19/2-2.5/1-1.5	1-5
159-1	1.5-2.5/2-3	-	2-4
273-1	2-2.5/3.5-4	16/2.5/1	1-3
159-2	2-2.5/2.5-4	16/1.5/1	2-5
279	2-3/2.5-4	19/2/1	1-5
334	2.5/2.5-3	14-17/1.5/2.5	1-5
138	2-3/2-3	12-14/2-2.5/1	1-5
395	2.5-5/2-7[c]	18/2.5/1	2-4
I-52	1-2/1.5-6[c]	9-15/1-2/0.5-1	1.5-6

*: Means of 10-15 observations.
a: Width of macrohyphae and microhyphae.
b: Phialide width at the tip.
c: Germinating phialospores.

Table 4. Antagonism of Phialophora and Gaeumannomyces isolates towards Gaeumannomyces graminis var. tritici.

Isolate	Inhibition zone (mm)			Diameter of Ggt culture (mm)		
	5 d[a]	10 d	15 d	5 d	10 d	15 d
I-52	2.0[b]	2.0	2.0	39.0[b]	39.5	39.0
17-1	5.0	3.0	2.5	40.0	41.0	41.0
159-1	2.0	2.0	2.0	40.0	39.5	39.5
254-1	7.0	7.0	6.0	39.5	41.0	40.5
I-58	10.5	0.0	0.0	38.5	42.0	43.0
357-2	5.0	0.0	0.0	37.0	38.5	38.0
336	15.0	9.5	9.0	37.0	44.5	45.0
395	13.0	7.0	5.5	39.0	44.0	46.5
254-2	2.0	0.0	0.0	39.0	38.0	37.5
138	7.0	3.0	2.5	40.0	42.0	41.5
Ggt 554	19.5	0.0	0.0	38.5	47.0	47.0
Ggt 528	9.5	0.0	0.0	39.0	43.0	43.5
Ggt 532	9.5	0.0	0.0	39.5	42.0	42.0
Ggg 723	9.5	0.0	0.0	37.5	42.0	41.5
Ggg 724	8.5	0.0	0.0	38.5	41.5	41.5
G. c[c] 1850	15.0	0.0	0.0	39.5	47.5	47.5
P. g[d] 1802	16.5	0.0	0.0	40.0	50.0	50.0
Control Ggt698	-	-	-	41.0	57.0	73.0

a: Inhibition zone (mm) and Ggt diameter (mm) were measured after 5,10, and 15 days of incubation at 25°C.
b: Isolates were paired on PDA.
c: Gaeumannomyces cylindrosporus.
d: Phialophora graminicola.

zone and linear growth of Ggt 698 were taken after 5, 10, and 15 days of incubation at 25°C. In general, Phialophora isolates reduced Ggt growth regardless of whether an inhibition zone was present or not, whereas Gaeumannomyces isolates did not cause any inhibition.

Table 5 provides data on the inhibition by Phialophora isolates of Ggt growth on ½ PDA and 2% MEA. In general, when the Phialophora isolates were added 3 days earlier than Ggt, a high percentage of inhibition was observed, such as 86.7% with 254-1 and 254-2 and 85.8% with I-52. However, when Phialophora and Ggt were added at the same time, the inhibition was only 67% on ½ PDA and 72% on 2% MEA. Phialophora graminicola 1802 showed the lowest inhibition of Ggt growth, e.g., 37.3% on ½ PDA and 44.6% on 2% MEA when both fungi were plated simultaneously. On buffered media (Table 6), a similar inhibition of Ggt growth was noted (the highest was 71.5% by 254-2 on ½ PDA and 75.5% by 254-1 on 2% MEA).

When wells were made in ½ PDA and filled with sterile soil to test whether the expression of the antibiosis of Phialophora isolates was influenced by the soil characteristics, no such effect was observed. Inhibition of Ggt growth by Phialophora isolates varied between 42.3% (by P. graminicola 1802) and 76.8% (by Phialophora 254-1). Phialophora and Gaeumannomyces isolates showed similar

Table 5. In vitro inhibition of Gaeumannomyces graminis var. tritici growth by Phialophora isolates from a suppressive Montana soil.

Phialophora Isolate	1/2 PDA				2% MEA			
	Ggt[a] mm	P.I[b] %	Ggt[c] mm	P.I[b] %	Ggt[a] mm	P.I[b] %	Ggt[c] mm	P.I[b] %
395	26.5	55.1	21.7	63.8	18.0	70.8	9.0	84.6
254-2	19.5	66.9	8.0	86.7	17.2	72.1	8.0	86.3
336	30.5	48.3	16.0	73.3	20.0	67.6	9.0	84.6
357-2	23.7	59.8	9.5	84.2	20.5	66.8	8.0	86.3
159-1	24.2	59.0	8.0	86.7	18.5	70.0	8.0	86.3
17-1	24.5	58.5	11.5	80.8	21.5	65.1	9.0	84.6
254-1	21.5	63.5	8.0	86.7	17.5	71.6	11.0	81.2
I-58	23.5	60.2	15.5	74.2	20.7	66.4	10.0	82.9
I-52	23.5	60.2	8.5	85.8	17.5	71.6	8.5	85.5
P.g 1802*	37.0	37.3	22.5	62.5	34.2	44.6	23.7	59.5
Control Ggt 698	59.0	-	60.0	-	61.7	-	58.5	-
LSD (5%)	6.5		1.7		1.5		3.2	

a: Ggt diameter in mm measured after 7 days of incubation at 25°C. Ggt and Phialophora spp. were plated simultaneously.
b: Percent inhibition (%) compared to the Ggt control.
c: Ggt diameter in mm measured after 7 days of incubation at 25°C. Ggt was added 3 days later than Phialophora.
*: Phialophora graminicola 1802 obtained from J. Henson (Dept. of Microbiology, M.S.U).

Table 6. *In vitro* inhibition of *Gaeumannomyces graminis* var. *tritici* growth by *Phialophora* isolates from a suppressive Montana soil on buffered media.

Phialophora Isolate	1/2 PDA (pH 6.0)		2% MEA (pH 6.0)	
	Ggt[a] (mm)	% Inhibition	Ggt[a] (mm)	% Inhibition
395	24.0	63.1	22.5	71.0
254-2	18.5	71.5	20.5	73.5
336	29.0	55.4	23.7	69.3
357-2	23.7	63.5	26.0	66.4
159-1	22.5	65.4	23.0	70.3
17-1	22.2	65.8	25.0	67.7
254-1	19.5	70.0	19.0	75.5
I-58	28.0	56.9	24.5	68.4
I-52	20.5	68.5	20.5	73.5
P.g 1802*	36.0	44.6	34.5	55.5
Control Ggt 698	65.0	-	77.5	-
LSD (5%)	1.5	-	1.6	-

a: Ggt diameter measured after 7 days of incubation at 25°C.
Ggt and *Phialophora* spp. were plated simultaneously.
*: *Phialophora graminicola* 1802 obtained from J. Henson (Dept. of Microbiology, M.S.U).

growth morphology and rate when grown on the various soils as well as on agar media.

After 7 days of incubation at 25°C, there was no significant difference in linear growth between the Ggt cultures growing in the presence of the Phialophora cell-free filtrates and the control cultures using filter sterilized 1/5 PDB.

When Phialophora isolates were placed at the edge of Ggt cultures to test their hypholytic ability, the following isolates induced growth cessation of the Ggt hyphae where the zone was completely cleared: 254-2, 17-1, 254-1, 395, I-52, 357-2, and 336. I-58 grew on the surface of Ggt mycelium but did not cause hyphal lysis. Some hyphal distortion and disintegration was also observed, most likely induced by diffusible antibiotic metabolites produced by the Phialophora isolates.

Siderophore Production

Table 7 shows that the majority of Gaeumannomyces isolates did not produce any siderophores on CAS blue agar except for G. cylindrosporus. Most Phialophora isolates, except 17-1 and I-58, showed a strong positive reaction.

Pathogenicity Assay on Wheat and Barley

Of all the Phialophora isolates tested for pathogenicity on wheat and barley, only P. graminicola

Table 7. Siderophore production by *Phialophora* and *Gaeumannomyces* isolates.

Fungal Isolates	Siderophore production
Ggg 723	–
Ggg 724	–
Ggt 554	–
Ggt 528	–
Ggt 532	–
Phialophora graminicola 1802	++
Gaeumannomyces cylindrosporus 1850	+
Ggt 698	–
138	++
17-1	–
357-2	–
254-2	+
159-1	++
I-52	++
I-58	–
254-1	++
336	++

* After 4 days of incubation on CAS blue agar at room temperature, (-) negative reaction, (+) positive reaction (diameter of orange halo 10 to 20 mm), (++) positive reaction (diameter of orange halo > 20 mm).

1802 caused a few dark brown lesions on some seminal roots of wheat and barley seedlings. Most of the remaining Phialophora isolates produced a little yellow to light brown discoloration (Table 8).

Ggt reduced shoot dry weight of wheat and barley seedlings by 60% and 53.7%, respectively. However, the majority of Phialophora isolates did not cause any substantial reduction in shoot weight (Table 8).

Suppression of Take-All by *Phialophora* Isolates

When wheat and barley seedlings were planted on top of agar discs of the Phialophora isolates tested, the control (agar disc without any fungus) showed the highest take-all infection, which was higher on wheat than on barley (Table 9). Although Phialophora isolates conferred some protection, take-all infection ratings were still high, ranging from 3.2 to 4.4 on wheat and from 1.2 to 2.6 on barley roots. Even though take-all infection was quite high compared to the healthy plants, shoot dry weight was little affected. Overall, shoot dry weight associated with Phialophora was higher than the Ggt control and this result was more consistent for wheat than barley (Table 9).

Table 8. Pathogenicity of *Phialophora* isolates on wheat and barley under glasshouse conditions.

Host/Isolate	Disease Rating on the roots (0-5)*	Shoot Dry Weight (mg)
Wheat		
Control	0.0	150.3 ef**
Ggt	4.2	60.3 a
Phialophora I-52	0.0	150.7 ef
Phialophora I-58	0.0	144.3 def
Phialophora 395	0.0	131.3 cbdef
P. graminicola 1802	0.6	133.7 cdef
Phialophora 138	0.0	115.0 cb
Phialophora 336	0.0	138.3 cdef
Phialophora 254-1	0.0	120.3 cbd
Phialophora 17-1	0.0	114.3 cb
Phialophora 254-2	0.0	105.3 b
Barley		
Control	0.0	154.0 f
Ggt	2.6	71.3 a
Phialophora I-52	0.0	129.3 cbdef
Phialophora I-58	0.0	106.3 b
Phialophora 395	0.0	105.0 b
P. graminicola 1802	0.4	137.3 cdef
Phialophora 138	0.2	149.7 ef
Phialophora 336	0.0	137.7 cdef
Phialophora 254-1	0.0	127.0 cbde
Phialophora 17-1	0.2	119.3 cbd
Phialophora 254-2	0.0	153.0 ef

* Take-all rating (0-5 scale), 0: no disease, 1: less than 25% of the roots are black, 2: 25-100% of the roots are black, 3: black lesions at tiller base, 4: black lesions moving up the tiller, and 5: plant severely stunted or dead.

**Values followed by the same letters are not significantly different ($p = 0.05$) according to LSD test.

Table 9. Take-all suppression by Phialophora isolates on wheat and barley under glasshouse conditions.

Host/Isolate	Disease Rating on the roots (0-5)*	Shoot Dry Weight (mg)
Wheat		
Healthy control	0.0 a	64.0 efd
Ggt alone	4.2 jk	27.3 a
Ggt/Phialophora I-52	3.2 ghi	53.0 ecd
Ggt/Phialophora I-58	4.4 k	38.3 abc
Ggt/Phialophora 395	3.4 hi	54.7 ecd
Ggt/P. graminicola 1802	3.8 jki	53.3 ecd
Ggt/Phialophora 138	3.6 ji	53.7 ecd
Ggt/Phialophora 336	3.4 hi	37.7 abc
Ggt/Phialophora 254-1	3.2 ghi	53.0 ecd
Ggt/Phialophora 17-1	4.2 jk	34.3 ab
Ggt/Phialophora 254-2	3.2 ghi	48.7 bcd
Barley		
Healthy control	0.0 a	140.7 g
Ggt alone	2.8 gfh	60.7 efd
Ggt/Phialophora I-52	1.8 cbd	68.0 ef
Ggt/Phialophora I-58	2.4 fde	59.0 efd
Ggt/Phialophora 395	2.2 fde	67.7 ef
Ggt/P. graminicola 1802	1.8 cbd	69.3 ef
Ggt/Phialophora 138	2.4 fde	54.3 ecd
Ggt/Phialophora 336	2.6 gfe	49.0 bcd
Ggt/Phialophora 254-1	1.2 b	63.0 efd
Ggt/Phialophora 17-1	2.0 cde	48.3 bcd
Ggt/Phialophora 254-2	1.4 cb	73.7 f

* Take-all rating (0-5 scale), 0: no disease, 1: less than 25% of the roots are black, 2: 25-100% of the roots are black, 3: black lesions at tiller base, 4: black lesions moving up the tiller, and 5: plant severely stunted or dead.

**Values followed by the same letters are not significantly different (p = 0.05) according to LSD test.

PCR Amplification and Sequencing of rDNA

An rDNA PCR fragment was amplified consisting of the 3′ 126 nucleotides of the 18S gene, ITS 1, the 5.8S gene and ITS 2 using the primers psnDNA2p (Bryan et al., 1995) and pITS4 (White et al., 1990). These fragments were both amplified from purified fungal DNA and directly from boiled fungal tissue. This fragment was cloned and the nucleotide sequence of both strands was obtained. For purified DNA preparations, negative controls consisted of the standard PCR reaction mixture but with DNA template replaced by an equal volume of water. After amplification, the PCR products were checked by electrophoresis in 1% agarose gels and the DNA stained with ethidium bromide. The primers psnDNA2p and pITS4 amplified DNA efficiently from only six Gaeumannomyces graminis and Phialophora sp. isolates. These primers amplified a region stretching from the 3′ end of the small ribosomal subunit to the 5′ end of the large ribosomal subunit and included the 5.8S gene and the two internal ITS regions. The sizes of the fragments amplified by these two primers were approximately 650-750 bp. The nucleotide sequences of this region were aligned for comparison with Gaeumannomyces and Phialophora isolates from GenBank. The sequence comparison shows the close relationship between the Gaeumannomyces and Phialophora isolates included in this study as well as those from the GenBank (Figure 4).

Figure 4. Sequence comparison of ITS 1 and ITS 2. Sequences from Gaeumannomyces graminis var. tritici (isolate 698), Ggg 724, and Phialophora spp. I-52, 17-1, 159-1 and 357-2 are aligned with the corresponding regions from Ggt 747 (U17221), Ggg (U17213), Phialophora sp. (PSP) (U17216), P. graminicola (PG) (U17218), and P. parasitica (PP) (ATCC26366).

```
          1                                                        50
    Ggg   .AGGGATCAT T.ACAGAGTT GAAAAACTCC AACCCCTGTG AACCTACC.T
 GGT747   .AGGGATCAT T.ACAGAGTT GAAAAACTCC AACCCCTGTG AACATACC.T
 GGT698   .AGGGATCAT TAACAGAATT GAAAAACTCC AACCCCTGTG AACATACCTT
    PSP   GAGGGATCAT T.ACAGAGTT GAAAAACTCC AACCCCTGTG AACCTACC.T
 GGG724   .AGGGATCAT TAA.AGAGTT GAAAAACTCC AACCCCTGTG AACCTACC.T
     PG   .AGGGATCAT T.ACAGAGTT GAAAAACTCC AACCCCTGTG AACCTTACCT
   17-1   .......... .......... .......... .......... ..........
   I-52   .......... .......... .......... .......... ..........
  357-2   .......... .......... .......... .......... ..........
     PP   .......... .......... .......... .......... ..........
  159-1   .......... .......... .......... .......... ..........
          51                                                      100
    GGG   TTACTGTTGC TTCGGCGGAA GATGGCCCCC CC...GGGCC GGACGCCGCC
 GGT747   TTACTGTTGC TTCGGCGGAC GATGGCCCCC CCCGGGGGCC GGACGCCGCC
 GGT698   TTACTGTTGC TTCGGCGGAC GATGGCCCCC CCCGGGGGCC GGACGCCGCC
    PSP   TTACTGTTGC TTCGGCGGAC GATGGCCCCC .....GGGCC GGACGCCGCC
 GGG724   TTACTGTTGC TTCGGCGGAA TATGGCCCCC C....GGGCC GGACGCCGCC
     PG   CT.TTGTTGC TTCGGCGTGC CCTCTGGAGG CCGCAGGTCC GCCCCTCACC
   17-1   .......... .......... .......... .......... .......AGG
   I-52   .......... .......... .......... .......... .......AGG
  357-2   .......... .......... .......... .......... ..........
     PP   .......... .......... .......... .......... .......AGG
  159-1   .......... .......... .......... .......... ..........
          101                                                     150
    GGG   GGAGGTTACA AACCCTGAAT TTTAGTGTAT CTCTGAGTAC AAAACCAAAT
 GGT747   GGAGGTTACA AACCCTGAAT TTTAGTGTAT CTCTGAGTAT AAAACCAAAT
 GGT698   GGAGGTTACA AACCCTGAAT TTTAGTGTAT CTCTGAGTAT AAAACCAAAT
    PSP   GGAGGTTACA AACCCTGAAT TTTAGTGTAT CTCTGAGTAC AAAACCAAAT
 GGG724   GGAGGTTACA AACCCTGAAT TTTAGTGTAT CTCTGAGCAC AAAACCAAAT
     PG   GGGTGGCCGC GGCGCCCGGC GGTCGGCACG CCGGAGGTTC CAAACTCTGA
   17-1   GATCATTACA GAGTTGCAAA ANTCCNTAAA CCATTGTGAA CGTTACTTAA
   I-52   GATCATTACA GAGTTGCAAA ACTCCCTAAA CCATTGTGAA CGTTACCTAC
  357-2   .......... .......... .......... .......... ..........
     PP   GATCATTAAC GAGTTTCGTA CT..CCAAAC CCTTTGTGAA CATACCTGTT
  159-1   .......... .......... .......... .......... ..........
          151                                                     200
    GGG   AATT.A.... .......... .......... .......... ..........
 GGT747   AATTAA.... .......... .......... .......... ..........
 GGT698   AATTAAAACT TTCAACAACG GATCTCTTGG TTCTGGCATC GATGAAGAAC
    PSP   AATTAAAACT TTCAACAACG GATCTCTTGG TTCTGGCATC GATGAAGAAC
 GGG724   AATTA..... .......... .......... .......... ..........
     PG   ATTTA..... .......... .......... .......... ..........
   17-1   ACCGTTGNTT CGGCGGGCGG CCCC..GGGG TTNACCCCCC GGGCGCCCNT
   I-52   ACCGTTGCTT CGGCGGGCGG CCCCCGGGGT TTACCCCCCG GGGCGCCCCT
  357-2   .......... .......... .......... .......... ...GGCCCNT
     PP   TTCGTTGCTT CGGCAGGTGA AGGCGGACGC TCCGGGCCTG AAGCCGCCGC
  159-1   .......... .......... .......... .......... .......CCG
          201                                                     250
    GGG   .......... .......... .......... .......... ..........
 GGT747   .......... .......... .......... .......... ..........
 GGT698   GCAGCGAAAT GCGATAAGTA ATGTGAATTG CAGAATTCAG TGAATCATCG
    PSP   GCAGCGAAAT GCGATAAGTA ATGTGAATTG CAGAATTCAG TGAATCATCG
 GGG724   .....GAAAT GCAATAAGTA ATGTGAATTG CAGAATTCAG TGAATCATCG
     PG   .......... .......... .......... .......TAG TGTATCTCTG
   17-1   GGGCC..... ........CC ACNGCGGGCG CCCGCCGGAG GTCACCAAAC
   I-52   GGGCC..... ........CC ACCGCGGGCG CCCGCCGGAG GTCACCAAAC
  357-2   GGGCC..... ........CC ACCG.GGGGG CCCGCCGGAG TCACCAAAAT
     PP   CGGCCGCCTC TCGCGGGGCG GCCGGGTGGG CCTGCCGGAG GGCACAGACT
  159-1   TCGCTACTAC CGATTGAATG GCTCAGTGAG GCTTCCGGAC TGGCCCANAG
```

Figure 4. Continued.

```
          251                                                        300
   GGG    .......... .......... .......... .......... ..........
GGT747    .......... .......... .......... .......... ..........
GGT698    AATCTTTGAA CGCACATTGC GCCCGCCGGT ATTCCGGCGG GCATGCCTGT
   PSP    AATCTTTGAA CGCACATTGC GCCCGCCGGT ATTCCGGCGG GCATGCCTGT
GGG724    AATCTTTGAA CGCACATTGC GCCCGCCGGT ATTCCGGCGG GCATGCCTGT
    PG    AGATTAAAAA CAAATA.... .......... .......... ......ATCA
  17-1    TCTTGATAAT TTAT.GGCCT CTCTGAGTCT TCTGTACTGA ATAAGTCAAT
  I-52    TCTTGATAAT TTATGGGCCT CTCTGAGTCT TCTGTACTGA ATAAGTCAAC
 357-2    TCTTGATAAT TTATGGCCTN TTCTGGTCTT TCTGTACTGA ATAAGTCAA.
    PP    CTGTATTACA TAACGTACCT CTCTGAGTTA TATTTTACAA ACAAGT..AA
 159-1    AGGTCGGCAA CGACCACTCA GGGCCGGAAA GCTATCCAAA CTCGGTCATT
          301                                                        350
   GGG    CCGAGCGTCA TTTCACCACT CAAGCCCAGC TTG...GTGT TGGGGCACCC
GGT747    CCGAGCGTCA TTTCACCACT CAAGCCCAGC TTG...GTGT TGGGGCACCC
GGT698    CCGAGCGTCA TTTCACCACT CAAGCCCAGC TTG...GTGT TGGGGCACCC
   PSP    CCGAGCGTCA TTTCACCACT CAAGCCCAGC TTG...GCGT TGGGGCACCC
GGG724    CCGAGCGTCA TTTCACCACT CAAGCCCAGC TTG...GTGT TGGGGCACCC
    PG    ACGAGCGTCA TTTCACCACT CAAGCCCAGC TTG...GTGT TGGGGCACCC
  17-1    TCGAGCGTCA TTTCAACCAT CAAG.CCCCC GGGCTTGTGT TGGGG.....
  I-52    GAGCG.TTCA TTTCAACCAT CAAGCCCCCC GGGCTTGTGT TGGGG.....
 357-2    ACGAGCGTCA TTTCAACCAT CAAG.CCCCC GGGCTTGTGT TGGGG.....
    PP    CCGAGCGTCA TTTCAACCCT CAGGCCCTGG TTGCCTGGTG TTGGGGCGCC
 159-1    TAGAGGGTCA .CCAAACTCT TGATAATTTA TGGCCTCTCT GAGTCTTCTG
          351                                                        400
   GGG    GGCCGCCCGG CGG..TCGG. .GGCCCCCAA GAACATCGGC GGTCTCGCCA
GGT747    GGCCGCCCGG CGG..TCGG. .GGCCCCCAA GAACATCGGC GGTCTCGCCA
GGT698    GGCCGCCCCG CGG..TCGG. .GGCCCCCAA GAACATCGGC GGTCTCGCCA
   PSP    GGCCGCCCGG CGG..TCGG. .GGCCCCCAA GAACATCGGC GGTCTCGCTA
GGG724    GGCCGCCCGG CGG..TCGG. .GGCCCCCAA GAACATCGGC GGTCTCGCCA
    PG    GGCCGCCCAG CGGCCCCGG. .GGCCCCCAA GTCCATCGGC GGGCTCGTCG
  17-1    .....ACCTG CGGCTGCCGC AGGCCCTGAA AAGCAGTGGC GGGCTCGCTG
  I-52    .....ACCTG CGGCTGCCGC AGGCCCTGAA AAGCAGTGGC GGGCTCGCTG
 357-2    .....ACCTG CGGCTGCCGC AGGCCCTGAA AAGCAGTGGC GGGCTCGCTG
    PP    GCGCACCCTC AGCGGGCCGC GGGCCCCGAA AGTCAGTGGC GGGCTCGCCA
 159-1    TACTGAAATA ANTCAAAACT TTCCAACAAC GGATCTCTTG GTTCTGGCAT
          401                                                        450
   GGG    GGACCCTGAA CGCAGTAACT CGCGGTAAAA CGCGCTTCGT TCGGAGGC.T
GGT747    GGACCCTGAA CGCAGTAACT CGCGGTAAAA CGCGCTTCGT TCGGAGGC.T
GGT698    AGAACCTGAA CGCAGTAACT CGCGGTAAAA CGCGCTTCGT TCGGAGGC.T
   PSP    GGACCCTGAG CGCAGTAACT CGCGGTAAAA CGCGC..... ..........
GGG724    GGACCCTGAA CGCAGTAACT CGCGGTAAAA CGCGCTTCGT TCGGAGGCTT
    PG    GGACCCTGAG CGCAGTAACA CGCGGTAAAA CGCGC.CCTC GCTCGGCGGT
  17-1    TCACACCGAG CGTAGTAGCA TAC..... ..ATCTCGCT CTGGGCGTGC
  I-52    TCACACCGAG CGTAGTAGCA TAC..... ..ATCTCGCT CCGGTCGTGC
 357-2    TCACACCGAG CGTAGTAGCA TAC..... ..ATCTCGCT CTGGGCGTGC
    PP    GGACTCCGAG CGCAGTA.AT TCT..... ..CTCTCGCT GTGGAGCGCC
 159-1    CAATAAAAAA CNCACGAATG CAATAATTAA TGTGAATGCN NAAATTC...
          451                                                        500
   GGG    TCCCGGCGGG CTCCAGCCGC TAAACCCCCT AAAC..... ..........
GGT747    TCCCGGCGGG CTCCAGCCGC TAAACCCCCT AAACTTCTTA GGTT......
GGT698    TCCCGGCGGG CTCCAGCCGC TAAACCCCCT AAAAT..... ..........
   PSP    .......... .......... .......... .......... ..........
GGG724    TCCCGGCGGG CTCCAGCCGC TAAACCCCCT AAAC..... ..........
    PG    TCCAGTCGGG TTCCAGCCGC TAAACCCCCC TAAAC..... ..........
  17-1    TGCGGG.... TTCCGGCCGT TAAACCACCT TTTAACCCAA GGTTGACCTC
 I-52     TGCGGG.... TTCCGGCCGT TAAACCACCT TTTAACCCAA GGTTGACCTC
 357-2    TGCGGG.... TTCCGGCCGT TAAACCACCT TTTAACCCAA GGTTGACCTC
    PP    TGGTGGGTTT TACCAGCCGT AAAACACCCC AACTTCCAAA GGTTGACCTC
 159-1    .......... .......... .......... .......... ..........
          501                                 531
GGT747    .......... .......... .......... .
GGT698    .......... .......... .......... .
   PSP    .......... .......... .......... .
GGG724    .......... .......... .......... .
    PG    .......... .......... .......... .
  17-1    GGATCAGGTA GGAAGACCCG CTGAACTTAA G
  I-52    GGATCAGGTA GGAAGACCCG CT........ .
 357-2    GGATCAGGTA GGAAGACCCG CTGAACTTAA G
    PP    GGATCAGGTA GGAATACCCG CTGAACTTAA G
 159-1    .......... .......... .......... .
```

When the sequences of entire rDNA frament were compared, Phialophora I-52 showed a 83.3% identity with Ggt 698, 82.6% with Ggg 724, 83.9% with Ggt 747 (U17221), 83.6% with Ggg (U17213), 83.6% with Phialophora sp. (U17216), 82.9% with P. graminicola (U17218), 79.4% and with P. parasitica (ATCC26366) (Bryan et al., 1995). On the other hand, I-52 showed a 90.1% identity with Phialophora 159-1, 97.3% with Phialophora 17-1 and 92.4% with Phialophora 357-2. Phialophora isolates included in this study (I-52, 159-1, 17-1 and 357-2) showed a high percent identity in rDNA sequences even though they were morphologically distinct.

When only ITS 1 and ITS 2 sequences were compared, I-52 showed a 79% identity with Ggt 698, 73% with Ggg 724, 74.6% with Ggg (U17213), 74.9% with Ggt 747 (U17221). Percent identity between I-52 and Phialophora spp. was surprisingly lower than those between I-52 and Gaeumannomyces isolates. However, comparison of ITS regions of the four Phialophora isolates from Larslan soil showed a strong relatedness among them. Percent identity between I-52 and 159-1 was 98.3%, 97.4% between I-52 and 17-1, and 93.7% between I-52 and 357-2. ITS 1 and ITS 2 contained higher levels of nucleotide sequence divergence than either the 3′ 126 nucleotides of the 18S subunit or the 5.8S subunit. The sequences of ITS 1 and ITS 2 were highly similar within the Phialophora isolated from

Larslan soil which appeared to be closer to Gaeumannomyces than to any of Phialophora sp., P. graminicola or P. parasitica.

The primers selected to be used in Phialophora sp. I-52 detection were pI-52/1 (forward primer): 5' CTTGATAATTTATGGGCCTC 3' and pI-52/2 (reverse primer): 5' TATGCTACTACGCTCGGTG 3', with respective melting temperatures of 54°C and 55°C, the optimal annealing temperature being around 56°C and the estimated product size about 310 bp.

Discussion

The primary objective of this study was to characterize several Phialophora-like fungi isolated from a take-all suppressive soil in Montana employing cultural and molecular methods.

The majority of these Phialophora isolates exhibited a strong in vitro inhibition of Ggt growth. They were nonpathogenic on wheat and barley. They, however, did not confer substantial protection of wheat and barley seedlings against take-all under greenhouse conditions. This result might be due to a limitation in the technique adopted which favored Ggt growth at the expense of Phialophora and resulted in a high take-all infection. Ggt inoculum produced on autoclaved oat kernels was mixed with sand at a rate of 1% (w/w), while the fungal antagonists

were only supplied as 10-mm discs placed in contact with the germinating seed. Better protection against take-all might have occurred if the Phialophora spp. were added in the same way and rate as Ggt, i.e., thoroughly mixed with the sand as inoculum produced on a food substrate. In addition, sand was utilized, which eliminates the occurrence of any general suppression found in natural soils, and the plants were watered copiously every day, which favored Ggt growth and infection of wheat and barley roots.

Fungi belonging to the genus Phialophora have been encountered in the root region of wheat in Western Australia. Phialophora verrucosa, P. cyclaminis, P. fastigiata, P. malorum, P. hoffmannii, P. mutabilis, P. lignicola, the Phialophora state of Gaeumannomyces graminis, and some isolates that could not be matched with any described species of the genus Phialophora were described by Sivasithamparam (1975). Phialophora fastigiata, P. malorum and P. hoffmannii were consistently effective in reducing take-all disease. However, P. hoffmannii did not produce any diffusible antibiotics against the pathogen in agar media (Sivasithamparam, 1975). Walker (1972) suggested that some of the Phialophora-like fungi associated with cereals and grass roots may have Gaeumannomyces as their perfect stage. This possibility was tested in recent studies that confirmed

that P. graminicola is the anamorph of G. cylindrosporus (Bryan et al., 1995; Wetzel et al., 1996).

The Phialophora isolates examined here exhibited a diverse array of cultural characteristics. Their growth rate ranged between 5 and 9.6 mm/day, and the mycelium color varied between isolates and even within isolates depending on media, age of the colony, and subculturing. The majority of the Phialophora isolates studied produced simple to slightly lobed hyphopodia on wheat coleoptiles. Sizes of phialospores and phialides were variable and overlapping among the different isolates. Six isolates out of ten produced siderophores on CAS blue agar (P. graminicola 1802, Phialophora spp. 138, 357-2, 254-2, 159-1, I-52, 254-1, and 336). Inhibition of Ggt on agar media was not due to a pH effect but most likely due to competition for nutrients and/or production of diffusible compounds with toxic or fungistatic effect on Ggt. However, more tests need to be performed to determine if there is a correlation between siderophore and/or fungistatic metabolite production and the ability of these Phialophora to inhibit Ggt and protect wheat and barley roots against take-all infection.

One beneficial trait favoring Phialophora as efficient biocontrol agents is their ability to grow at low temperatures (5 and 10°C) as compared to Gaeumannomyces isolates.

Phialophora isolates were not pathogenic on wheat or barley. However, they were successful in colonizing the roots as was shown by the yellow to light brown discolorations on wheat and barley roots and by the presence of runner hyphae.

Schol-Schwarz (1970) revised the genus Phialophora which was originally created by Medlar in 1915 for a previously unknown pathogenic fungus isolated from the first reported case of Dermatitis verrucosa, a human skin disease. Numerous species of Phialophora have since been described. The genus comprises a large number of dematioid and hyaline fungi, frequently of ill-defined species that represent conidial states of members of the Helotiales or Sphaeriales. These species occur commonly on decaying wood, in soil, and in water, as well as on food and as the causal agents of skin infections. The genus shows a strong variability in hyphal pigmentation and conidiophore structures. One-celled slimy conidia are formed on typically flask-shaped phialides with a collarette. However, this study (Schol-Schwarz, 1970) did not include the complex of Phialophora radicicola (Cain, 1952) and the conidial state of Gaeumannomyces graminis.

The main character by which this genus has been separated from the rest of the related hyphomycetous genera has been the presence of a prominent collarette at the mouth of the phialide, which in the type species

P. verrucosa Medlar flares out into a cup-like structure. The collarette has been defined as "that part of the phialide wall from the conidiogenous locus to the open tip" (Subramanian, 1971). In Phialophora sp. I-52, the collarettes were usually inconspicuous (short and tubular), resembling those in P. parasitica (Yan et al., 1995). However, these collarettes were not easy to observe due to the slime present at the phialide mouth and, moreover, not all phialides bore collarettes.

The two ITS regions in filamentous fungi have been found to show a higher level of nucleotide sequence variation than either the 18S, 5.8S or 26S rDNA sequences (White et al., 1990). Therefore, we compared the nucleotide sequences of the complete region containing the 3′ 126 nucleotides of 18S, ITS 1, 5.8S and ITS 2 and the ITS regions only of Gaeumannomyces and Phialophora isolates in an attempt to support the identification of the Phialophora isolates which originated from Larslan soil and to establish the relationships between them and Gaeumannomyces isolates used in this study as well as with other Gaeumannomyces and Phialophora spp. whose sequences were reported in the literature.

This analysis allowed the clear identification of the four isolates (I-52, 159-1, 17-1, and 357-2) as Phialophora and showed that they were very closely related to Ggt 698 and Ggg 724. On the other hand, this study

showed that these Phialophora isolates were closer in their rDNA sequences to Gaeumannomyces than to Phialophora spp. These findings support previous results (Deacon, 1974; Schol-Schwarz, 1970; Sivasithamparam, 1975) that Phialophora contains a wide range of species depending on their geographic habitat, host-parasite relationships and evolution. For instance, one might not expect an isolate from soil, living as a nonpathogenic parasite on grasses or cereals, to be very closely related to another isolate which causes human keratomycosis.

Furthermore, it is noteworthy that Phialophora sp. (P2 and P9), included in rDNA sequence comparison, were isolated from wheat in Czechoslovakia and the UK and were characterized by lobed hyphopodia whereas the Phialophora spp. isolates included in this study have simple to slightly lobed hyphopodia except for isolate 17-1 which produced lobed hyphopodia. Often, new isolates from different sources (crops or countries) do not exactly fit the Phialophora and Gaeumannomyces taxa (Deacon, 1974; Nilsson, 1972). Martyniuk (1987) suggested that strains of P. graminicola isolated from various hosts may differ markedly in many respects. P. graminicola (U17218) was isolated from wild grass in the UK and one cannot help but ask what should be the delimitation of a species whose morphology is highly variable and whether this variability would be reflected at the rDNA sequence level.

The ITS regions used in this study are known to be highly variable between fungi. Amplification of rDNA regions from fungi suspected to belong to the Gaeumannomyces-Phialophora complex and comparisons of their sequences constitute a useful identification method of members of this complex. However, as this study showed, there are some intermediate isolates that can cause problems in identification by traditional as well as molecular techniques. As pointed out by Ward and Akrofi (1994), there is increasing evidence that not all isolates of the Gaeumannomyces-Phialophora complex fall into known taxa and that the species boundaries may overlap.

The molecular data support the conclusion that all isolates studied were related since there were sufficient DNA sequence similarities within the ITS regions. The ITS evolves rapidly and distant taxa cannot be easily compared using this region (Bruns et al., 1991).

After comparing the molecular information from the rDNA regions with morphological and cultural characters, Phialophora sp. I-52 was tentatively identified as P. graminicola (Deacon, 1974). Regarding the remaining Phialophora spp. isolates, further tests need to be conducted and more isolates need to be included to be able to construct a model to help identify Phialophora spp. at the species level. Ribosomal DNA sequences showed that some of these Phialophora isolates were very closely

related to Phialophora sp. I-52 even though there was some morphological and cultural variability between them. Therefore, no final conclusion could be drawn from the data available regarding their identity. This close relationship yet distinct separation between various species of Phialophora is supported by other studies (Yan et al., 1995) and points to the necessity for extreme care when attempting to group isolates from different regions in individual taxonomic categories.

A practical application of the nucleotide sequence comparison in this study was the design of primers for use in Phialophora sp. I-52 detection in the soil and on cereal and grass roots. These primers should be tested on a wide range of soil and rhizoplane fungi in future work. If specific to I-52, these primers would be of valuable epidemiological importance for this potential biological control agent against take-all.

Phialophora spp. USED AS INTRODUCED AND RESIDENT ANTAGONISTS AGAINST THE TAKE-ALL PATHOGEN

Introduction

Take-all of wheat and barley, caused by the soilborne fungus *Gaeumannomyces graminis* var. *tritici* (Ggt), can serve as both a model system for biological control research and a practical disease problem of major significance waiting to be solved by use of biological control (Cook, 1994). In fact, biological control of take-all of wheat has been studied intensively because of the economic importance of the disease and the absence of other economically viable methods of control. Ryder *et al.* (1990) suggested that biological control agents for a root disease should be sought in those soils that show a natural ability to suppress the disease. Cook (1994) emphasized the benefits and applications of a combination of introduced and resident antagonists which supports the theory that biological control can be "achieved through the product and/or the practice."

The natural suppression of take-all, known as take-all decline, develops in fields where wheat is continuously grown for several successive years. Soils may be naturally suppressive of disease or suppression may be

induced by manipulation of the abiotic and biotic environments (Baker & Chet, 1982). Suppressive soils are defined by Baker and Cook (1974) as those soils in which "either the pathogens cannot establish, they establish but fail to produce disease, or they establish and cause disease at first but diminish with continued culture of the crop." Hornby (1983) pointed out that the terms "disease suppression" and "pathogen suppression" are often used interchangeably. However, pathogen suppression occurs when the pathogen is growing saprophytically or surviving in the soil, while disease suppression describes suppression of the pathogen growing parasitically in the host (Hornby, 1983). In the literature the term "general antagonism" (*sensu* Gerlagh, 1968) consistently describes that suppression resulting from the activity of the general soil microbiota which can be manipulated by practices such as crop rotation and cultivation. Gerlagh (1968) defined "specific antagonism" as suppression which develops in soil only in the presence of a virulent pathogen. However, other researchers have used the term "specific" to describe that suppression resulting from the antagonism of the pathogen within soil by specific groups of microorganisms or by populations of individual microorganisms. The term "transferable suppression" has been used synonymously with that of specific suppression (Shipton et al., 1973).

In France, Alabouvette (1986) considers that Fusarium-wilt suppressive soils "have both general and specific suppression, indicating that the two mechanisms are complementary rather than opposed." Simon (1989) reported that take-all suppression was induced by inoculation of natural soil with Trichoderma koningii but that disease-suppression was enhanced by incubating the pathogen and antagonist in soil before seeding, which indicated that pathogen-suppression played a significant role in the subsequent expression of disease-suppression.

The variability of the organisms associated with take-all suppression suggests that different mechanisms may be involved in different soils and in different wheat growing areas. Although antibiosis against Ggt in vitro has been the major criterion to test organisms for their antagonistic potential, lack of correlation between antibiosis in vitro and performance in the field indicates that other mechanisms such as competition, mycoparasitism, and/or induction of resistance may be responsible for suppression of Ggt.

Mycoparasites are fungi that parasitize other fungi. In practice, the term is used broadly to include presumptive parasites that coil around other fungal hyphae or overgrow other colonies on agar (Mulligan & Deacon, 1992). This may involve penetration of living host hyphae or antagonism by antibiotics (Dennis & Webster, 1971),

wall lytic enzymes (Chet, 1986), or toxic radicals (Kim et al., 1990). The destructive Didymella exitialis penetrates the hyphae of Ggt by producing penetrating hyphae, or by boring directly through the fungal host cell wall (Adams, 1990). Mycoparasites appear to have biocontrol potential. Some are responsible for natural suppressiveness of soils to plant pathogens, examples being Pythium nunn and P. oligandrum in Pythium-suppressive soils (Lifshitz et al., 1984) and Trichoderma hamatum in Rhizoctonia-suppressive soils (Liu & Baker, 1980).

It has been demonstrated that infection of wheat roots by the take-all fungus is reduced by prior colonization by non- or weakly-pathogenic root-infecting fungi in glasshouse and field experiments. Such fungi include a sterile black mycelial fungus in Germany (Speakman & Kruger, 1984), Idriella bolleyi in the UK (Kirk & Deacon, 1987a), Phialophora species in Canada and the UK (Balis, 1970; Deacon, 1976; Scott, 1970; Speakman & Lewis, 1978), G. graminis var. graminis in the USA (Wong & Southwell, 1980), a sterile dark mycelial fungus in Japan (Narita & Suzui, 1991), and a sterile red fungus in Australia (Dewan & Sivasithamparam, 1990).

In Montana, two sterile, previously unidentified, fungi isolated from a suppressive soil were shown to be antagonistic against Ggt in greenhouse experiments

(Andrade et al., 1994b). In the present study, to better understand the variables affecting the biocontrol potential of these fungi, they were added separately and in combination to their original soil and to two other Montana soils, one suppressive and the other conducive to take-all. To better understand the role of inoculum potential of the antagonistic fungi, two different substrates were utilized as a food base, and they were introduced at two inoculum levels.

Materials and Methods

Dual Culture Assays

Antagonism tests. The two Phialophora sp. isolates (I-52 and I-58) were tested for their ability to inhibit Ggt growth in vitro. These two fungi were plated at the edge of water agar (WA), potato dextrose agar (PDA), quarter-strength PDA (1/4 PDA), or one tenth-strength PDA (1/10 PDA) plates. Simultaneously a 7-mm-diameter plug of a fresh Ggt culture was placed in the center of each plate. Radial growth of Ggt was measured after 6 days and compared to a control plate without the antagonists.

To further test the ability of these Phialophora sp. isolates to inhibit the growth of Ggt, 7-mm agar plugs of the antagonist were placed on 2% malt extract agar (MEA) and on half-strength PDA (½ PDA) plates at equal distance from a central 7 mm disc of Ggt. Ggt growth and the

percentage of inhibition were determined after 7, 14, and 21 d of incubation. The Phialophora isolates were added to the plate at the same time as Ggt or 3 days earlier. The experiment was repeated using 2% MEA and ½ PDA containing 20 mM 2-[N-morpholino]ethansulfonic acid (MES) to see whether inhibition in vitro occurs on buffered media. The pH of the media was adjusted to pH 6 using 1M NaOH before autoclaving.

In an attempt to assess soil nutritional factors that may affect the expression of antibiosis, a technique involving wells made in ½ PDA and filled with sterile soil was used to test antagonism in vitro. Larslan soil, from which the Phialophora sp. isolates originated, was utilized in addition to the conducive PostFarm soil, Toston soil (somewhat suppressive), and a take-all suppressive soil from Oregon cultivated to wheat for 60 years (R. Smiley. Pendleton, OR). Four 10-mm wells per plate were made equidistant from each other and 15 mm from the edge of the plates. A 7-mm plug of Ggt grown for one week on ½ PDA was then placed in the center of each plate. Sterile soil-containing wells with only the addition to the soil surface of agar plugs were used as checks. One fungal isolate was tested per plate and two plates were prepared per isolate. Plates were kept at room temperature and the zone of inhibition and Ggt linear growth were measured 7 days later.

Use of cell-free culture medium. Four 7-mm plugs of each fungus grown for 5 days on ½ PDA were placed in 250 ml Erlenmeyer flasks containing 50 ml of sterile one-fifth-strength potato dextrose broth (1/5 PDB). Cultures were grown on a rotary shaker for 5 days at room temperature. Then the broth was filtered-sterilized through a 0.2 µm filter (Nalgene), added to a 15 mm diameter sterile paper filter disc, and placed on ½ PDA, 15 mm from the edge of the plate. Immediately, a 7-mm plug of Ggt grown on ½ PDA was placed in the center of the plate. Four discs containing four different fungal extracts were placed on each plate, equidistant from one another. Each plate was replicated three times. Controls consisted of sterile filter discs dipped into filter-sterilized 1/5 PDB. Plates were maintained at room temperature and Ggt growth was measured after 7 days.

Hyphal interaction. In an unconventional method (Zogg & Jaggi, 1974), Ggt was first grown in Petri dishes on ½ PDA or 2% MEA for one week. Thereafter, to test for the hypholytic ability of the fungal antagonists, they were placed on the living mycelium of Ggt. In another method to study hyphal interaction, sterilized glass slides were coated with PDA, 1/4 PDA, 1/10 PDA, or WA and placed in sterile plastic Petri dishes. The take-all fungus was placed 5 cm from I-52 or I-58 on the coated slides and incubated for 5 days at 20°C in the dark. The zone of

hyphal interaction was then stained with lactophenol cotton blue and observed under a light microscope. Hyphal lysis, protoplasm condensation, cessation of growth, or any other abnormality that was microscopically visible was recorded.

Detection of Presumptive Mycoparasites in Soil

Larslan and PostFarm soils were air-dried and sieved (2 mm). Half-PDA plates were inoculated centrally with Ggt and incubated at 25°C until the colony margin just reached the edge of the agar. Then, a sample of natural soil (0.5 g) was placed on the oldest part of the Ggt culture (Mulligan & Deacon, 1992). The plates were incubated at 25°C in the dark and examined (x 100 magnification) after 7, 14, and 21 days.

All tests mentioned above were conducted at least twice.

Glasshouse Tests

All experiments were carried out in a glasshouse in the MSU Plant Growth Center at Bozeman, Montana.

Effect of inoculum rate of I-52 on take-all disease and plant growth. PostFarm conducive soil mixed with sand (1:2, w/w) was utilized. Air-dried Ggt inoculum on autoclaved barley kernels was fractionated using a blender and sieved to a particle size ≤ or = 1 mm. Ggt inoculum

was added to the soil at two rates, 0.5% and 1% (w/w). I-52 was grown on autoclaved canola seed and added to the soil at various rates relative to Ggt inoculum (9:1, 20:1, 50:1, 100:1, 200:1, w/w). The treatments were Ggt alone, I-52 alone, Ggt + I-52, and a control where neither fungus was added. The experiment was conducted as a completely randomized design with five replications of each treatment combination. Plastic containers (3 cm diameter x 16 cm long) containing a cotton ball placed at the bottom were filled with an 11- to 12-cm-high column of infested soil. A single two-day-old pregerminated seedling of 'Pondera' spring wheat was planted in each container. A 1-cm layer of sterile sand was added over the soil to avoid contamination at irrigation. After 25 days, the soil was washed off the roots. The roots were rated for take-all infection (0-5 scale), and root and shoot dry weights were determined (dried at 70°C for 48 h).

Effect of I-52 broth filtrate on plant growth and root infection. Ten plugs of actively growing I-52 on ½ PDA were inoculated to an Erlenmeyer flask (1000 ml) containing 500 ml of 1/5 PDB. The flasks, with or without the fungus, were incubated at room temperature for 1, 2, or 3 weeks and the broth was filtered through sterile cheesecloth. The filtrates were used to water, every three days, Pondera wheat seedlings grown in PostFarm soil mixed with sand (1/1, w/w) with or without the addition of Ggt

(0%, 0.1% or 1% w/w). The control treatment consisted of watering with 1/5 PDB incubated for 1, 2, or 3 weeks without any fungus. Four weeks after planting, seedlings were removed and the soil washed off the roots. Root and shoot dry weights were determined and the roots were rated for length, presence of fine root hairs and take-all infection. This experiment was repeated using broth with only one week incubation and filtered or filter sterilized I-52 or 1/5 PDB broths, respectively. In experiment 2, seedlings were watered every other day.

Field Tests

Soils. Field tests were performed to determine whether the *in vitro* antagonistic activity of the *Phialophora* isolates correlated with their ability to suppress the take-all pathogen under field conditions. Three soils were utilized in this experiment in 1994. Larslan and Toston soils had previously been shown to have suppressive characteristics to take-all (Andrade *et al.*, 1994a). The PostFarm soil was shown in preliminary work to lack any level of suppressiveness and was conducive to take-all infection in the greenhouse, which confirmed earlier results (Andrade *et al.*, 1994a). In 1995, only Larslan and PostFarm soils were utilized. The soil characteristics are shown in Table 10.

Table 10. Location and soil analysis of three Montana soils used in glasshouse and field experiments.

Soils			
Location/ Analysis	Larslan	PostFarm	Toston
Location County Area	Valley Larslan, MT	Gallatin Bozeman, MT	Broadwater Toston, MT
Cropping system	spring wheat 18 years monoculture	wheat alfalfa alfalfa fallow	spring wheat 14 years monoculture
Soil analysis *			
Mg paste (mg/L)	73.0	28.2	22.1
Na paste (mg/L)	41.4	4.0	16.7
Ca paste (mg/L)	166	122	132
CEC (meq/100g)	16.1	24.9	15.4
EC mmhos/cm Sat Pst	1.88	1.0	0.96
Fe mg/Kg	23.4	16.9	3.1
Cu mg/Kg	1.02	3.37	5.33
Mn mg/Kg	62.7	44.5	13.0
Zn mg/Kg	3.62	1.18	2.18
Sand %	57	12	46
Silt %	26	56	38
Clay %	17	32	16
Org. Mat %	3.69	2.68	1.01
pH Sat Pst	8.0	8.1	8.2
SAR	0.7	< 0.1	0.4
Texture	sandy loam	silty clay loam	sandy loam

* Soil analysis carried out by the Soil Testing Lab, Dept. of Plant, Soil and Environmental Sciences, Montana State University, Bozeman, MT.

Inoculum preparation. A single pathogenic isolate of Ggt (Isolate #698) collected from artificially inoculated plants was utilized. Ggt inoculum was grown on autoclaved oat kernels for 2 to 3 weeks and then dried under a sterile air stream. Oat kernels and canola seed were used as inoculum substrate for the Phialophora antagonists. Actively growing cultures of I-52 and I-58 on PDA were transferred to autoclaved oat kernels and canola seed until the substrate was completely colonized. The inoculum was spread out to dry under a sterile air flow. These inocula of Ggt, I-52, or I-58 were mixed into each soil for about 10 minutes in a cement mixer.

Field experiments. The experiments were carried out at the Arthur H. Post Research Farm near Bozeman during two seasons. Microplots were set up using plastic pots that were buried 2/3 deep in the soil and arranged in a completely randomized design with four replications per treatment.

In the 1994 experiment (Experiment 1), I-52 and I-58 were added to the three soils separately and in combination on May 20 to test their ability to suppress Ggt. Small (1 L) and medium sized (3 L) pots of each treatment were placed next to each other. The small pots were used for an early evaluation of the disease 38 days after planting, and the medium pots were kept until plant maturity.

The factors considered in this experiment were the antagonists (I-52 and/or I-58), soils (Larslan, PostFarm, or Toston soils), substrates on which the antagonists were grown (oat kernels or canola seed), and, finally, the rate at which the antagonists were added to the soils relative to Ggt inoculum which was added at 1% w/w (low rate 3:1 w/w and high rate 9:1 w/w). Two types of checks were included. The positive check consisted of natural soil without addition of Ggt or any biological control agent. In the negative check, only Ggt inoculum was added to the soil at the same rate as the other treatments (1%, w/w). Ten kernels of the spring wheat cultivar Pondera were seeded per pot and later thinned to five plants per pot. The plants were irrigated when needed.

In 1995, this test was repeated with planting on May 31 (Experiment 2). Bottomless plastic pots were used to allow more natural root growth to occur in comparison to 1994 when pots with intact bottoms with four 3-cm drainage holes were used. Larslan suppressive soil and PostFarm conducive soil were tested and the antagonists, grown on canola or oats, were added only at the high rate 9:1 (w/w, antagonist/Ggt). Seedlings were evaluated for root infection, shoot and root dry weights. A chemical seed treatment (triadimenol 30% a.i, added at 0.11 ml + 9 ml H_2O/100 g of seed), as well as autoclaved oats or canola, were included to compare the antagonist's effect

to that of the fungicide, and to see whether the substrates have an effect on the disease and plant growth in the absence of the antagonists.

Plant and disease evaluation. The severity of take-all in the field was determined by several methods at different stages of wheat growth: (1) by determining the percentage of plants with chlorotic leaves, (2) by measuring plant height at 25 and 55 days after planting, (3) by counting total heads, and (4) by counting fertile and dead tillers (white heads). The severity of root symptoms was rated based on a 0 to 5 scale (0 = no disease, 1 = less than 25% of the roots are black, 2 = 25-100% of the roots are black, 3 = lesions at the base of the tillers, 4 = lesions moving up the tillers, 5 = plants severely stunted or dead) (Weller et al., 1985).

In addition, the severity of the disease on the shoots was evaluated at the seedling stage on a scale of 0 to 4 (0 = leaves green, 1 = one or two leaves with chlorosis, 2 = three, four or more than half of the leaves with chlorosis, 3 = most leaves with chlorosis, and 4 = plant dead). Plant evaluation was based on measuring the grain yield, dry weight of the shoots, and dry weight of the roots. The soil was washed off the roots which were rated for take-all infection. For seedling evaluation, the shoots were excised at the crown, placed into preweighed paper envelopes and dried at 70°C for 48 h. At maturity,

the plants were harvested and air-dried, and the dry weights of shoots and roots were measured.

Statistical Analyses

Statistical analyses were performed using the GLM procedure (SAS program) or ANOVA (MSUSTAT program). If a significant difference among treatments was noted, multiple comparisons were conducted by least significant difference (LSD) or Student-Newman-Keuls (SNK) tests.

Results

In vitro Antagonistic Activity

Figure 5 shows the percent inhibition of Ggt growth by I-52 and I-58. Inhibition associated with I-52 and I-58 ranged from 20 to 62% and from 30 to 58%, respectively. When Ggt radial growth (mm) data were analyzed, significant differences ($p < 0.01$) were found among antagonists, among periods of time after which the antagonists were added to the plates, and among media. Ggt growth was inhibited on agar media even when I-52 or I-58 were added 3 or 6 days later than Ggt. Inhibition was more pronounced on media richer than WA.

After 7-day incubation at 25°C, the Phialophora isolates I-52 and I-58 both inhibited Ggt growth by 60.2% on ½ PDA and by 71.6% and 66.4% on MEA. On buffered ½ PDA and MEA media (pH 6), the inhibition was respectively 68.5% (I-52), 56.9% (I-58), 73.5% (I-52), and 68.4%

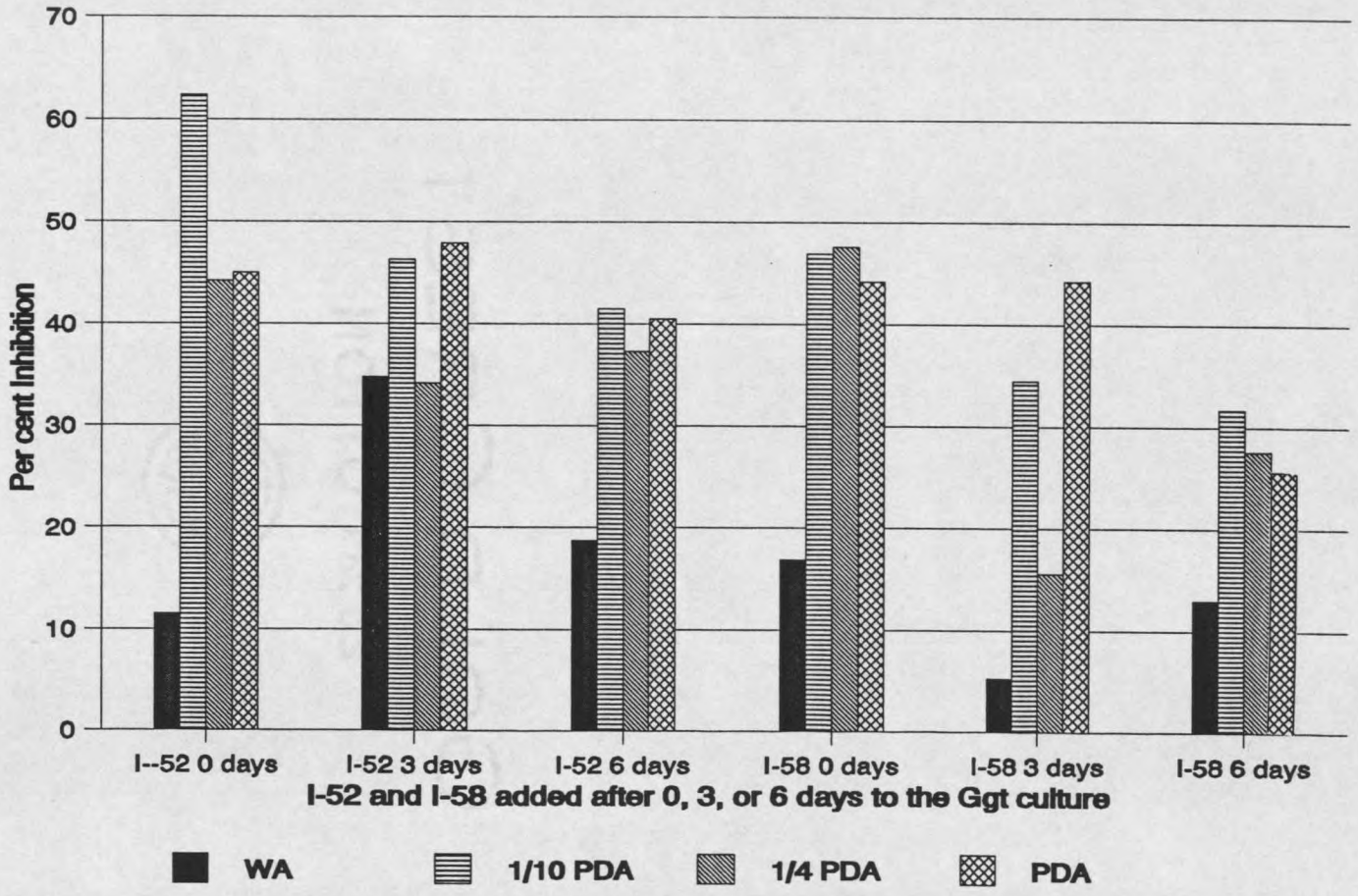

Figure 5. In vitro inhibition of Gaeumannomyces graminis var. tritici by the fungal antagonists I-52 and I-58.

(I-58). In general, inhibition of Ggt growth on both media and by either Phialophora isolate increased by 5 to 10% after 14 days and stabilized after 21 days (data not shown).

On the ½ PDA plates with the soil-containing wells, Ggt growth reduction between 55 and 65% was obtained with I-58 or I-52 regardless of which soil was in the well.

After 7-day incubation, the Ggt plates to which discs imbibed with the cell-free filtrate of I-52 or I-58 were added did not show any significant difference in radial growth compared to the control Ggt plates (data not shown).

Presumptive mycoparasites were detected by their sporulation or other structures on the Ggt culture. Identifications were confirmed with pure cultures obtained by subculturing the mycoparasites from Ggt plates onto plates of fresh ½ PDA. In Larslan soil Pythium spp., Trichoderma spp., and Penicillium spp. were observed. In PostFarm soil only Pythium spp. and Aspergillus spp. were detected.

No production of volatile inhibitory compounds by either antagonist was observed when culture plates were taped to the top of Ggt plates. Paired cultures on PDA or WA-coated glass slides did not manifest any hyperparasitic hyphal interaction between I-52 or I-58 and Ggt.

Glasshouse Tests

Effect of inoculum rate on plant growth and take-all infection. Almost 100% protection from take-all infection was obtained when Phialophora sp. (I-52) was added at ratios 9:1 or 20:1 (I-52/Ggt, w/w) and when Ggt inoculum was introduced into the soil at 0.5% (w/w). At 1% Ggt, 80% of the seedlings exhibited root infection scores lower than 2 (on a 0-5 scale) with I-52/Ggt ratios of 9:1 or 20:1. However, for higher I-52 rates (50:1, 100:1, 200:1, w/w I-52:Ggt), most of the seedlings died before the completion of the experiment. Shoot and root dry weights could not be determined. It is likely that when I-52 inoculum was added at high doses, the canola that was utilized as a food substrate for the fungus resulted in phytotoxicity.

Effect of I-52 broth filtrate on plant growth and root infection. Data analysis showed significant differences ($p < 0.05$) between wheat seedlings watered with 1/5 PDB and the ones watered with I-52 broth in root infection, root length, and root hairs. However, no differences were found for root and shoot dry weights. In general, the treatments with no Ggt inoculum added to the PostFarm soil, which is conducive to take-all, were associated with a lower root infection, longer roots, more root hairs, and higher root and shoot weights than with

0.1% or 1% Ggt levels. The results of the duration of incubation (1, 2, or 3 wks) were not clear, due to some bacterial contamination and perhaps production of secondary products by I-52 after an incubation longer than one week. Table 11 presents the results of this experiment averaged across the three incubation periods and the results of experiment 2 where only one week incubation period was included. Experiment 2 involved irrigation with PDB or I-52 broth, either filtered through a cheesecloth or filter sterilized through 0.22 µm cellulose acetate membrane (Corning).

Table 11. Effect of irrigation with I-52 filtrate on plant growth and root infection by Ggt.

	R.I[a]		R.L[b]		R.H[c]		R.W[d]		S.W[e]	
Ggt	PDB[f]	I-52[g]	PDB	I-52	PDB	I-52	PDB	I-52	PDB	I-52
Experiment 1										
0%*	0.5	0.3	13.5	14.8	0.7	1.4	31.8	37.3	67.9	68.4
0.1%	0.6	0.6	10.7	13.2	0.5	1.3	28.0	30.3	54.3	52.0
1%	2.0	1.5	9.3	8.2	0.7	0.8	32.6	27.8	51.7	41.1
LSD (5%)	0.3		1.0		0.3		3.8		7.1	
Experiment 2										
0%**	0.5	0.4	11.2	11.1	0.9	1.6	-[h]	-	83.3	77.9
0.1%	1.7	0.5	9.3	10.4	0.4	1.1	-	-	50.3	55.9
1%	2.1	1.1	8.4	9.6	0.5	1.1	-	-	53.3	61.8
LSD (5%)	0.9		1.9		0.5				10.8	

* Values are means across the three incubation periods: 1, 2 or 3 wks.
** Only an incubation period of 1 wk was included.
a: Root infection with Ggt (0: healthy roots, 5:plant dead).
b: Root length (cm).
c: Root hairs (0-3).
d: Root dry weight (mg).
e: Shoot dry weight (mg).
f: PDB filtrate (control).
g: I-52 culture filtrate grown in PDB.
h: Not determined in experiment 2.

As shown in Table 11, the use of I-52 broth to water wheat seedlings resulted in lower root infection by Ggt and a higher root hair production compared to the control. These data corroborate some preliminary results in suggesting the involvement of both antibiotic metabolites and plant growth-promoting substances in the suppression of take-all by I-52.

Field Experiments

Seedling evaluation. In experiment 1, wheat seedlings were removed from the small pots 38 days after planting and the soil washed from the roots. Disease rating on the roots and on the upper part of the seedlings as well as the shoot dry weight were determined (Table 12). The analysis of the leaf disease rating data showed highly significant differences among antagonists, substrates, and rates, a highly significant soil-antagonist interaction as well as significant soil-substrate and substrate-antagonist interactions. In addition, the shoot dry weight allowed detection of highly significant differences among the soils, antagonists, substrates and rates. However, root infection showed only highly significant differences ($p < 0.01$) among rates, substrates, soil-antagonist, rate-antagonist and significant ($p < 0.05$) substrate-antagonist interactions. Multiple comparisons using SNK test based on shoot dry

Table 12. Effect of Phialophora (I-52 and I-58) on plant growth and take-all infection on seedlings and mature plants under field conditions in 1994 (Experiment 1).

Substrate/ Antag.	Soil	Antag.*	Seedling Stage			Mature Plants					
			D.R[a]	D.R.L[b]	Shoot weight[c]	D.R	Shoot weight	# tillers	# fertile tillers	Root weight[c]	Yield (g/plant)
Canola											
	Larslan	I58	1.5	0.6	1.7	1.9	4.8	7.7	3.2	1.5	2.7
		I52+I58	0.2	0.1	2.2	1.5	8.5	11.0	5.9	2.3	4.2
		I52	0.6	0.2	2.5	1.5	8.6	8.9	4.6	2.4	4.4
		-	1.7	0.7	1.6	3.5	3.2	7.5	1.2	1.2	1.3
	PostFarm	I58	0.9	0.6	2.3	2.2	8.4	11.2	5.1	2.7	3.6
		I52+I58	0.6	0.4	2.5	2.1	7.6	11.9	4.2	2.8	3.0
		I52	1.0	0.2	2.1	1.7	8.5	12.0	4.9	2.6	3.7
		-	2.2	1.7	1.1	3.7	1.6	5.0	0.5	0.6	0.5
	Toston	I58	0.6	0.1	1.1	2.1	2.7	6.7	1.4	1.1	1.1
		I52+I58	0.6	0.4	1.3	2.1	4.3	8.6	2.4	1.6	2.1
		I52	0.6	0.1	1.4	1.9	5.2	8.4	2.9	1.4	2.4
		-	3.5	1.5	0.3	4.1	0.2	4.2	0.0	0.2	0.0
Oats	Larslan	I58	1.3	1.5	1.1	1.9	3.0	7.5	2.3	1.2	1.1
		I52+I58	1.1	0.0	2.1	1.8	4.7	7.7	3.1	1.4	2.2
		I52	0.9	0.1	1.9	1.6	5.0	7.4	3.2	1.3	2.4
		-	1.7	0.7	1.6	3.5	3.2	7.5	1.2	1.2	1.3
	PostFarm	I58	0.7	1.0	1.2	2.7	3.2	7.2	1.7	1.6	1.0
		I52+I58	0.9	0.2	1.8	2.9	3.6	7.9	1.6	1.5	1.4
		I52	1.4	0.5	1.8	1.9	3.6	7.5	1.7	1.5	1.4
		-	2.2	1.7	1.1	3.7	1.6	5.0	0.5	0.6	0.5
	Toston	I58	0.9	0.6	0.6	2.9	1.0	5.0	0.2	0.3	0.2
		I52+I58	1.2	1.0	0.8	2.4	2.1	6.0	1.2	0.6	0.8
		I52	0.9	1.0	0.8	2.4	2.9	6.5	1.9	0.9	1.2
		-	3.5	1.5	0.3	4.1	0.2	4.2	0.0	0.2	0.00
LSD (5%)			0.5	0.5	0.6	0.6	2.7	2.5	1.8	0.9	1.4

*: Antagonist (I52, I52 + I58, I58), average of 3:1 and 9:1 ratios (Antagonist/Ggt), added in the presence of Ggt (1% w/w).
a: D.R: root disease rating (0-5).
b: D.R.L : leaf disease rating (0-4).
c: Shoot and root dry weights in g.

weight grouped Larslan and PostFarm in one group (1.9 g) and Toston in another (1.0 g). Averaged across soils, substrates and rates, the antagonists I-52 and I-58, separated or combined, were associated with a lower leaf disease rating (0.4, 0.7, 0.3, respectively) (Figure 6). Concerning shoot weight, I-52 alone or combined with I-58, averaged across substrates, gave higher values (1.7 g and 1.8 g respectively) than I-58 alone (1.4 g).

In Larslan soil, I-52 significantly reduced take-all infection on the roots (Figure 7). The shoot dry weight significantly increased when I-52 was added at the high rate on canola and with I-58 on oats compared to the control infected by Ggt (Table 12). Figure 8 shows that I-52 alone or combined with I-58, resulted in the highest shoot dry weight, averaged across substrates, in the three soils. A more noticeable response was reported for the PostFarm soil where the addition of either I-52 and I-58 significantly reduced the disease on the roots and leaves, and increased the shoot dry weight (Table 12). Thus, the addition of antagonistic fungi appeared to effectively reduce the take-all infection in a soil with conducive properties. In Toston soil, the root and leaf disease ratings were reduced when I-52 or I-58 were added. The shoot dry weight increased significantly with the addition of I-52 or I-58 on canola (Table 12).

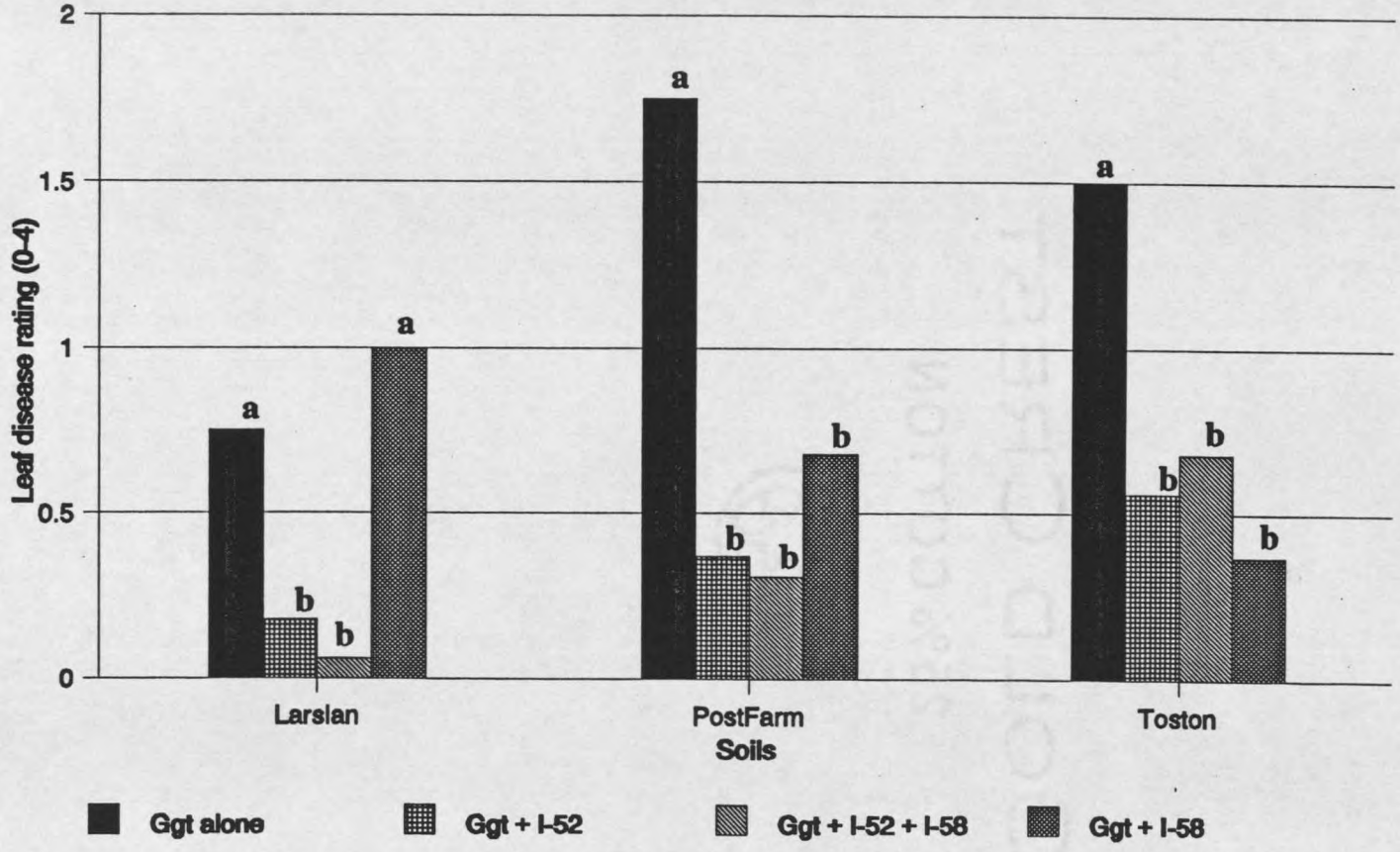

Figure 6. Effect of addition of Phialophora (I-52 and I-58) on leaf symptoms at the seedling stage (Experiment 1).

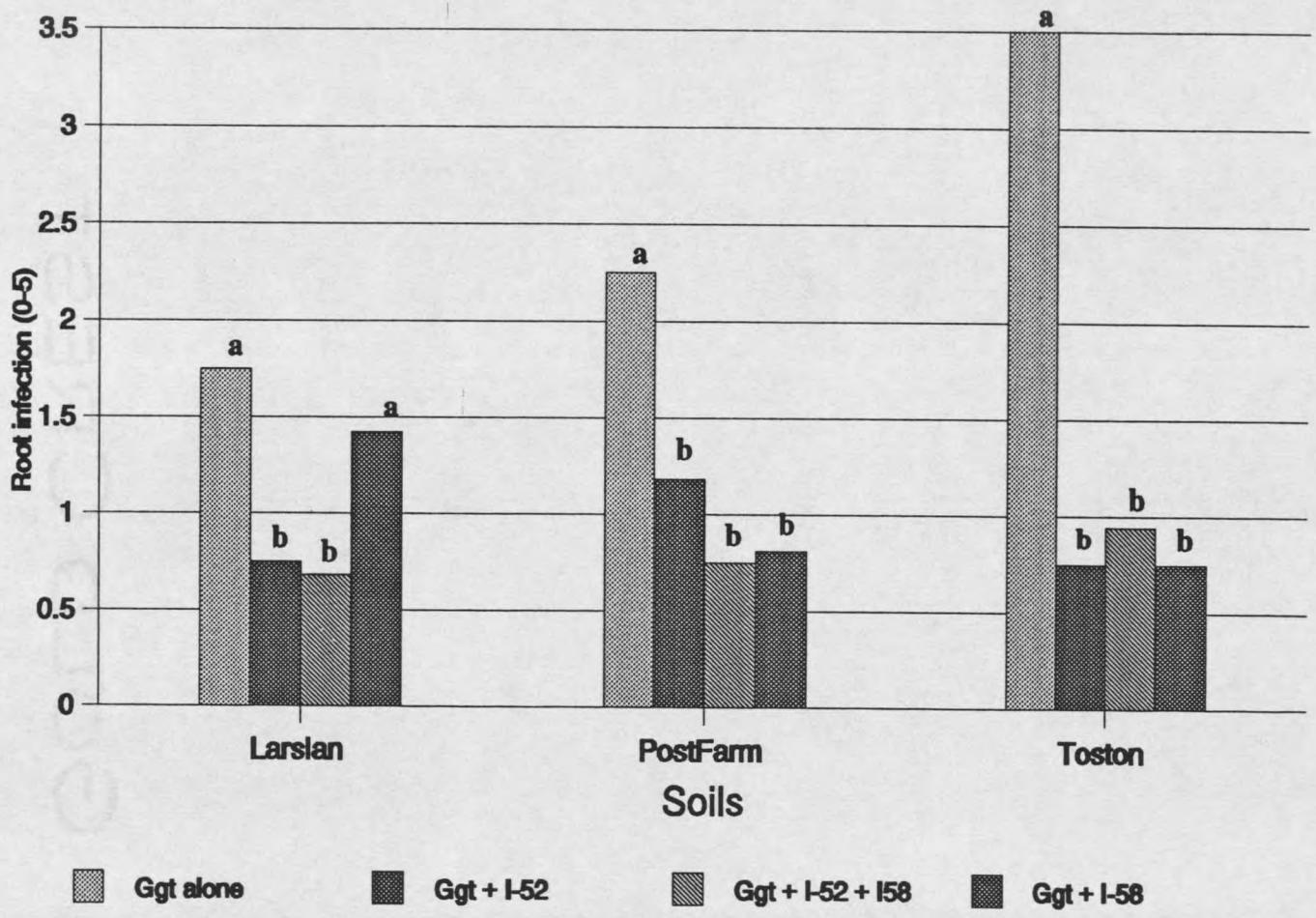

Figure 7. Effect of Phialophora (I-52 and I-58) on root infection at the seedling stage (Experiment 1).

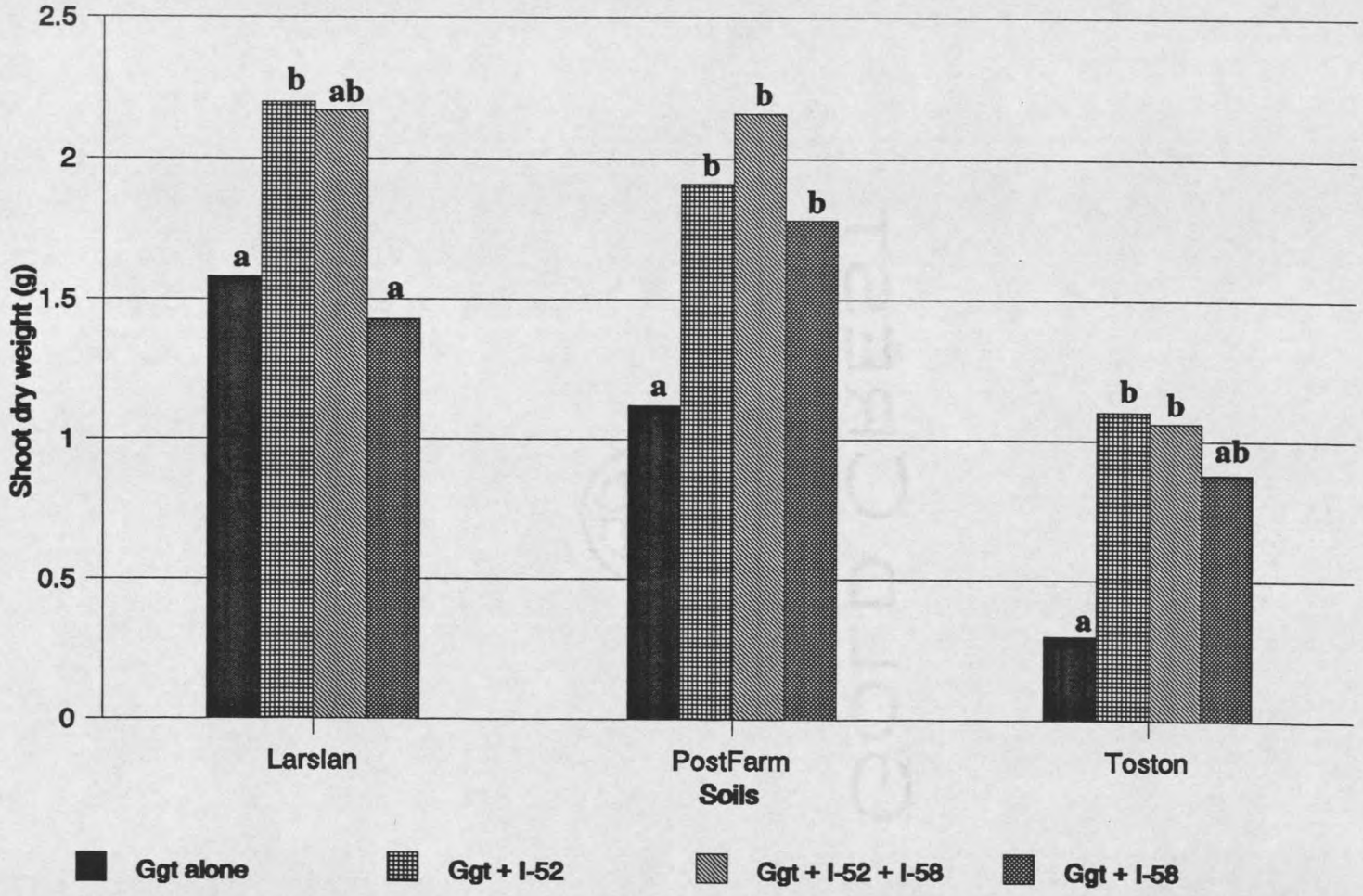

Figure 8. Effect of Phialophora (I-52 and I-58) on shoot weight of seedlings (Experiment 1).

In experiment 2, data analysis of root infection showed significant differences among soils and substrates ($p < 0.05$) and a highly significant difference among antagonists ($p < 0.01$). No significant difference ($p < 0.05$) was detected among plants treated with triadimenol (1.1), I-52 (0.6), I-58 (1.2), I-52+I-58 (1.0) for root infection averaged across soils, though much less infection was noted when I-52 was added alone to the Ggt-infested soil (Figure 9). In Ggt-infested soils amended with autoclaved oats or canola significantly less root infection was obtained (2.1) than in unamended Ggt-infested soils (2.9) (Table 13). The average root infection across all treatments was significantly lower ($p < 0.05$) in Larslan soil (1.2) than in PostFarm soil (1.5) which, once again, confirmed their different receptivity levels towards take-all. Analysis of root dry weight of seedlings showed no significant difference among antagonists, soils, nor substrates. Shoot dry weight data analysis, however, detected a highly significant difference ($p < 0.01$) among antagonists. Averaged over the three soils, I-52 was associated with the highest shoot weight (1.5 g). The treatments I-52+I-58 (1.4 g), I-58 (1.3 g), and triadimenol (1.3 g) did not differ significantly from each other in regard to shoot weight, and autoclaved substrate (1.1 g) and Ggt control (0.9 g) were not significantly different from each other.

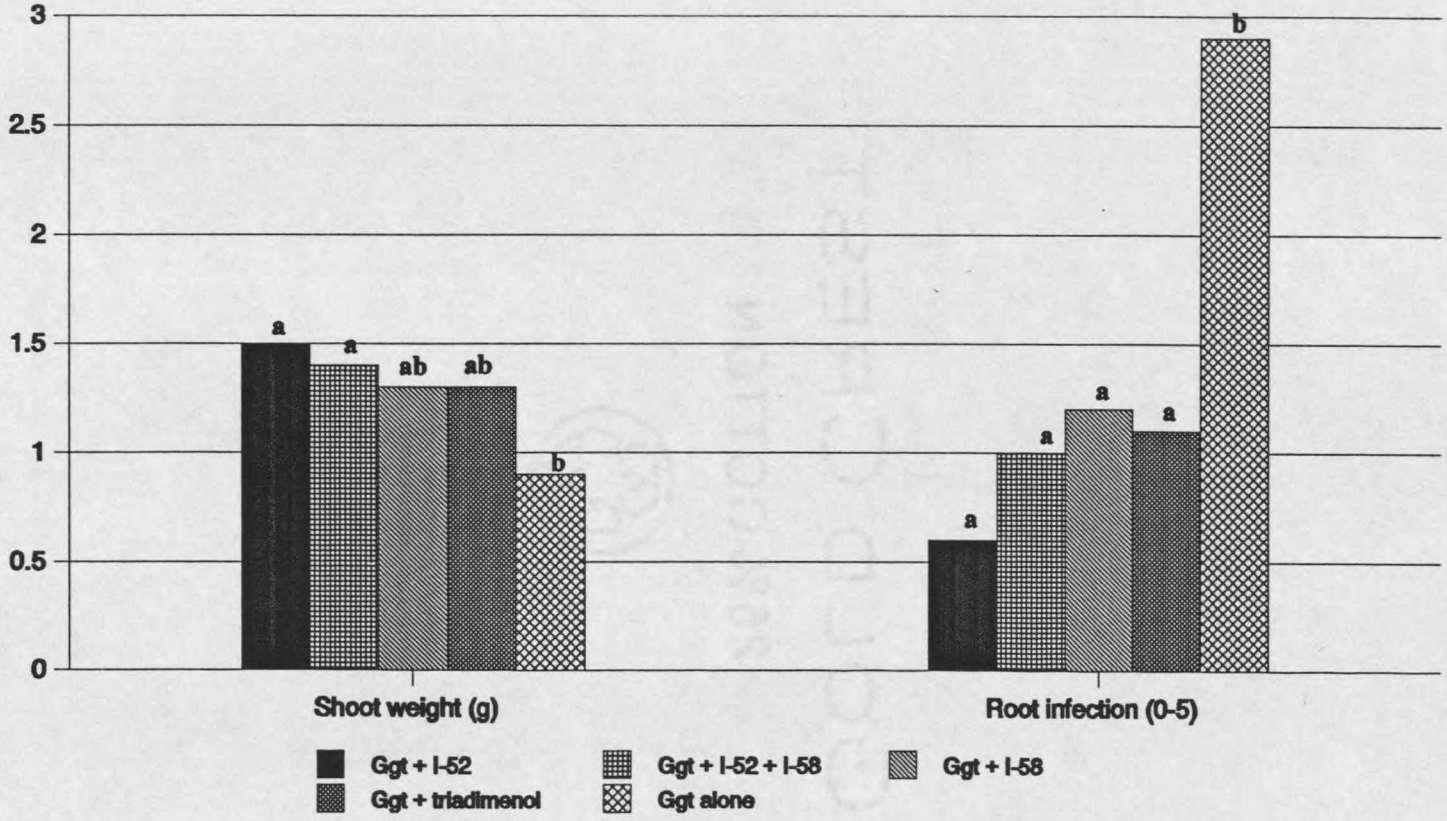

Figure 9. Effect of Phialophora (I-52 and I-58) on plant growth and root infection of seedlings in the field (Experiment 2).

Table 13. Effect of Phialophora (I-52 and I-58) on plant growth and take-all infection on seedlings and mature plants under field conditions in 1995 (Experiment 2).

Substrate/ antagonist	Soil	Antag.*	Seedling Stage			Mature Plants			
			D.R[a]	Root[b] weight	Shoot[b] weight	D.R	Root weight	Shoot weight	#[c] Heads
Canola	Larslan	I58	0.3	0.2	1.4	1.1	1.0	13.0	6.5
		I52+I58	0.2	0.3	1.6	1.4	2.1	21.1	9.0
		I52	0.2	0.3	1.6	1.4	3.2	44.4	22.0
		-	2.6	0.3	1.1	2.9	0.5	9.9	5.7
		triadim.[d]	0.4	0.1	1.2	2.2	0.9	12.2	6.7
		autoc.[e]	2.0	0.3	0.9	1.4	0.5	11.3	4.0
	PostFarm	I58	1.2	0.2	1.0	2.1	1.6	9.9	6.5
		I52+I58	1.1	0.3	1.3	2.0	1.5	12.4	9.0
		I52	0.7	0.2	1.3	1.9	1.3	18.7	5.7
		-	3.1	0.3	0.6	4.5	0.3	2.5	0.2
		triadim.	1.1	0.2	1.4	2.9	0.7	6.4	1.2
		autoc.	1.7	0.3	1.3	3.0	0.8	4.4	1.7
Oats	Larslan	I58	1.2	0.3	1.4	2.7	0.6	6.5	4.2
		I52+I58	1.1	0.3	1.3	2.4	1.6	16.1	8.5
		I52	0.7	0.2	1.5	1.4	1.9	25.9	9.2
		-	5.7	0.3	1.1	2.9	0.5	9.9	5.7
		triadim.	0.4	0.1	1.2	2.2	0.9	12.2	6.7
		autoc.	2.9	0.3	1.1	1.7	1.3	10.5	5.0
	PostFarm	I58	1.5	0.3	1.5	3.0	0.8	4.1	4.5
		I52+I58	1.6	0.3	1.2	1.9	1.3	7.1	4.7
		I52	0.6	0.3	1.6	2.6	0.8	13.9	5.7
		-	3.1	0.3	0.6	4.5	0.3	2.5	0.2
		triadim.	1.1	0.2	1.4	2.9	0.7	6.4	1.2
		autoc.	1.7	0.3	1.1	3.0	0.2	5.6	1.2
LSD (5%)			0.9	0.1	0.5	1.2	1.3	8.3	5.4

*: Antagonists (I52, I58, I52 + I58), autoclaved substrate, or chemical control added in the presence of Ggt.
a: D.R: disease rating on the roots (0-5).
b: Dry root and shoot weights in g.
c: Total number of heads.
d: Fungicide control (triadimenol).
e: Autoclaved substrate (canola or oats).

Mature plant evaluation. In experiment 1, in both Larslan and PostFarm soils, the number of heads increased significantly when I-52 was added alone or in combination with I-58 on canola and at a high rate. In Toston soil, I-52 and I-58, added either separately or together, on canola or oats, and at the high rate significantly increased the number of heads (Table 12, Figure 10). Root infection was significantly reduced, in all three soils, by the presence of the two antagonists, on canola or oats, and at low or high rate (Table 12, Figure 11). There was no significant difference between the antagonists, across the soils, for root dry weight (Figure 12), but root dry weight was significantly higher than the Ggt control. For shoot dry weight I-52, the presence of Ggt produced the highest value (5.6 g), averaged over the three soils (Figure 13). Addition of I-52 alone or with I-58 resulted in higher yield in the three soils compared to the control treatment where only Ggt was added (Figure 14). The increases in number of heads (harvestable tillers) and in grain yield obtained when these two antagonistic fungi were added to Ggt infested soils were over 100% that of the Ggt control. Larslan and PostFarm soils showed similar number of heads per plant (average of 3.6 and 3.0 respectively) while Toston soil was significantly lower (1.5). The use of canola as substrate for the antagonists, averaged over soils and treatments, was associated with

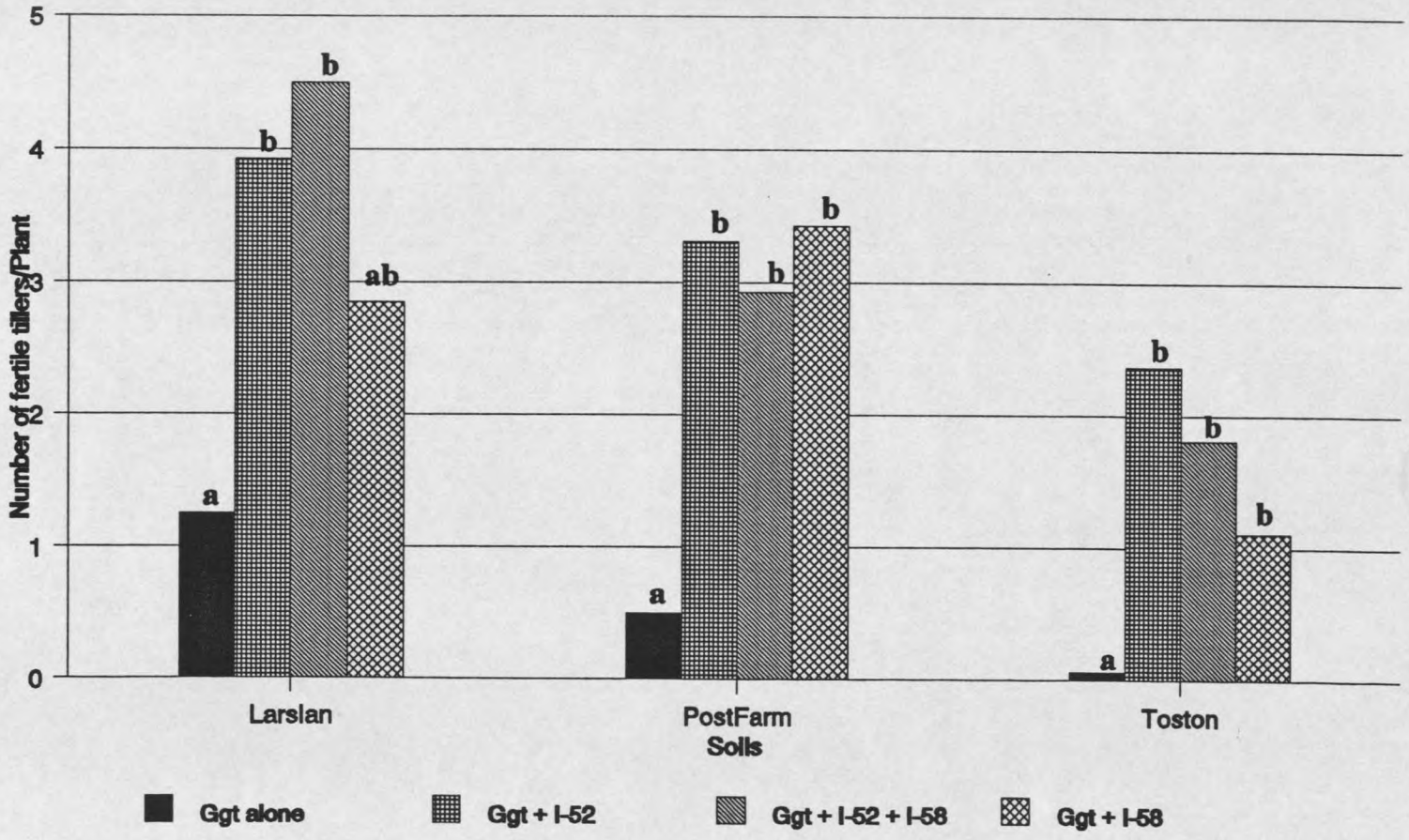

Figure 10. Effect of Phialophora (I-52 and I-58) on the number of fertile tillers (Experiment 1).

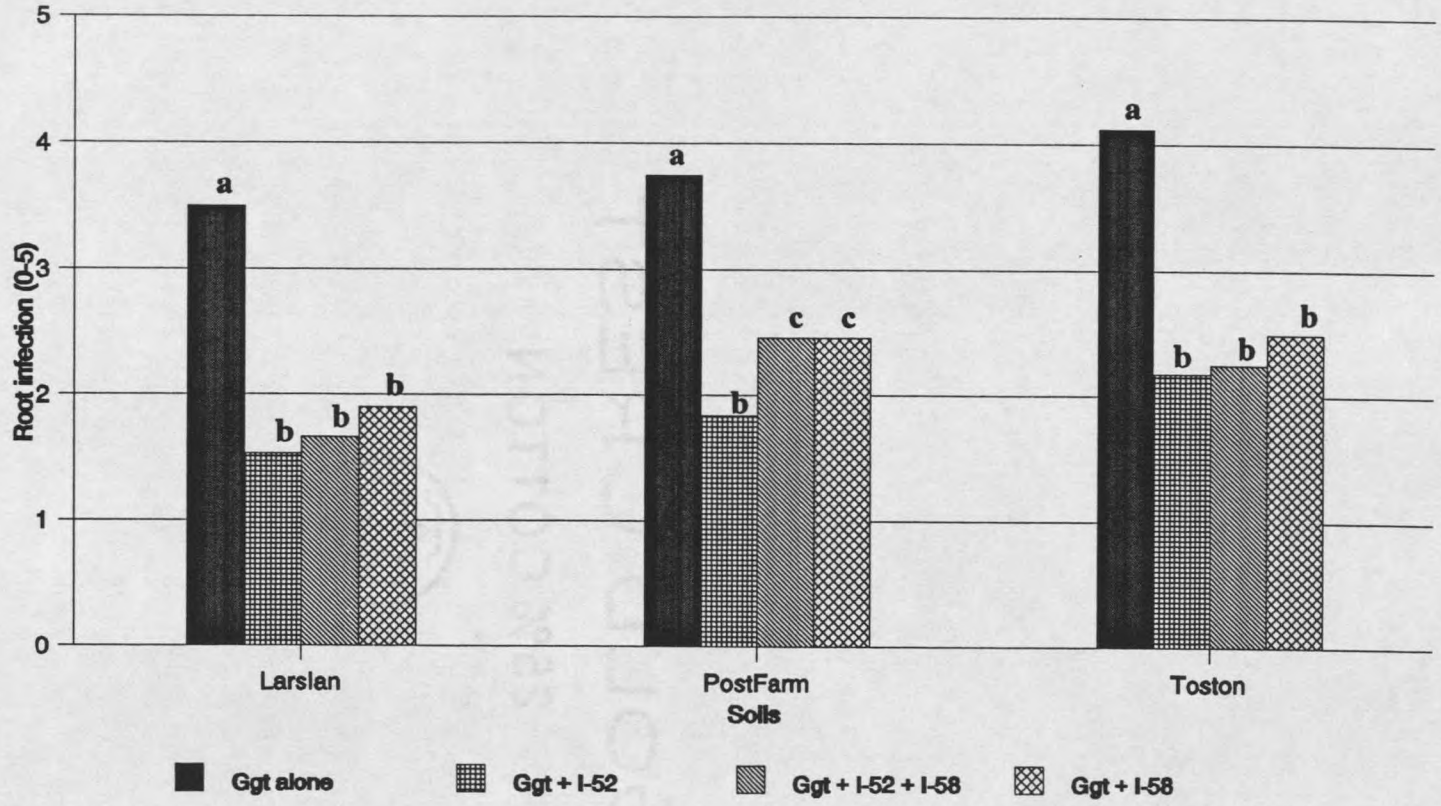

Figure 11. Effect of Phialophora (I-52 and I-58) on root infection on mature plants (Experiment 1).

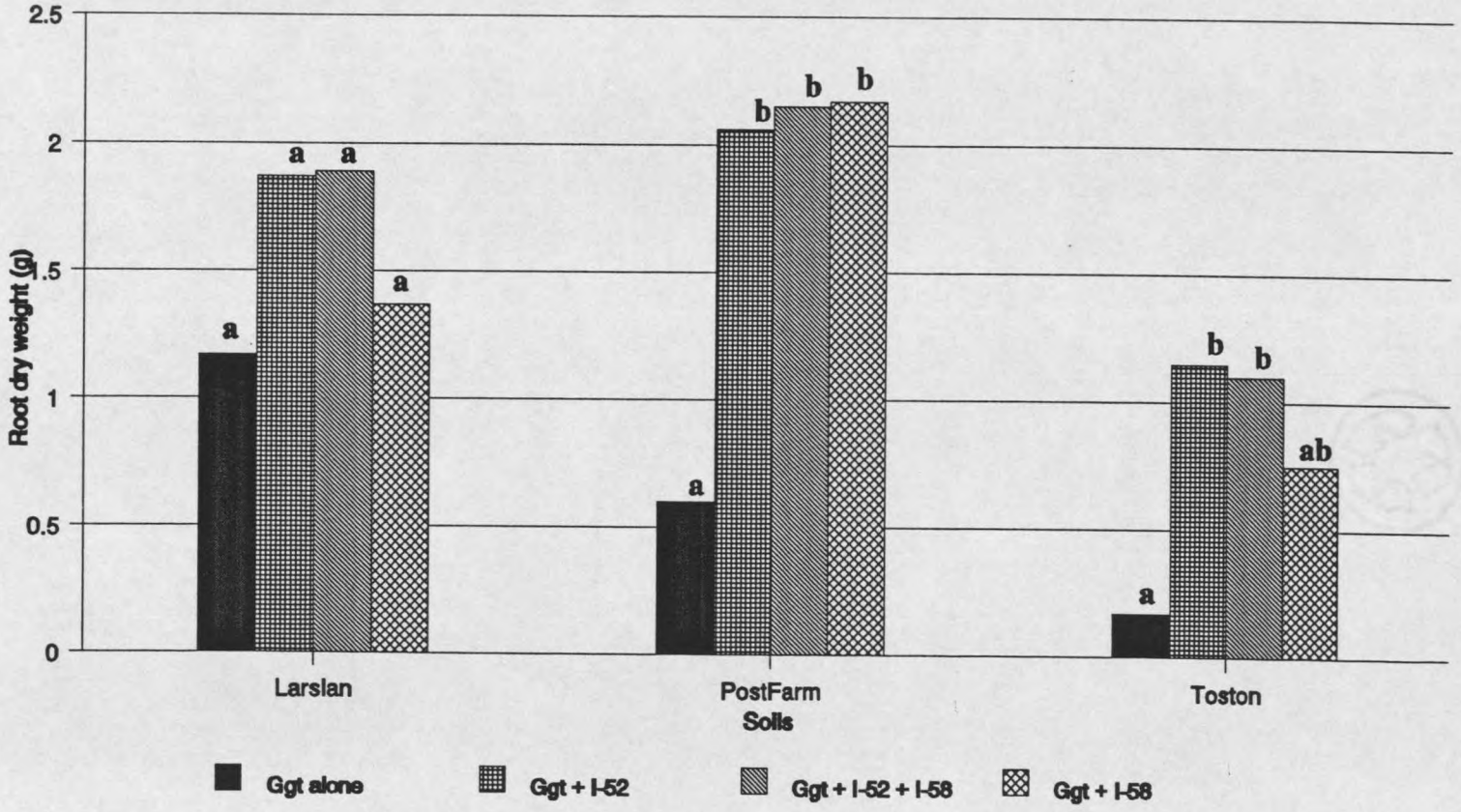

Figure 12. Effect of Phialophora (I-52 and I-58) on root weight of mature plants (Experiment 1).

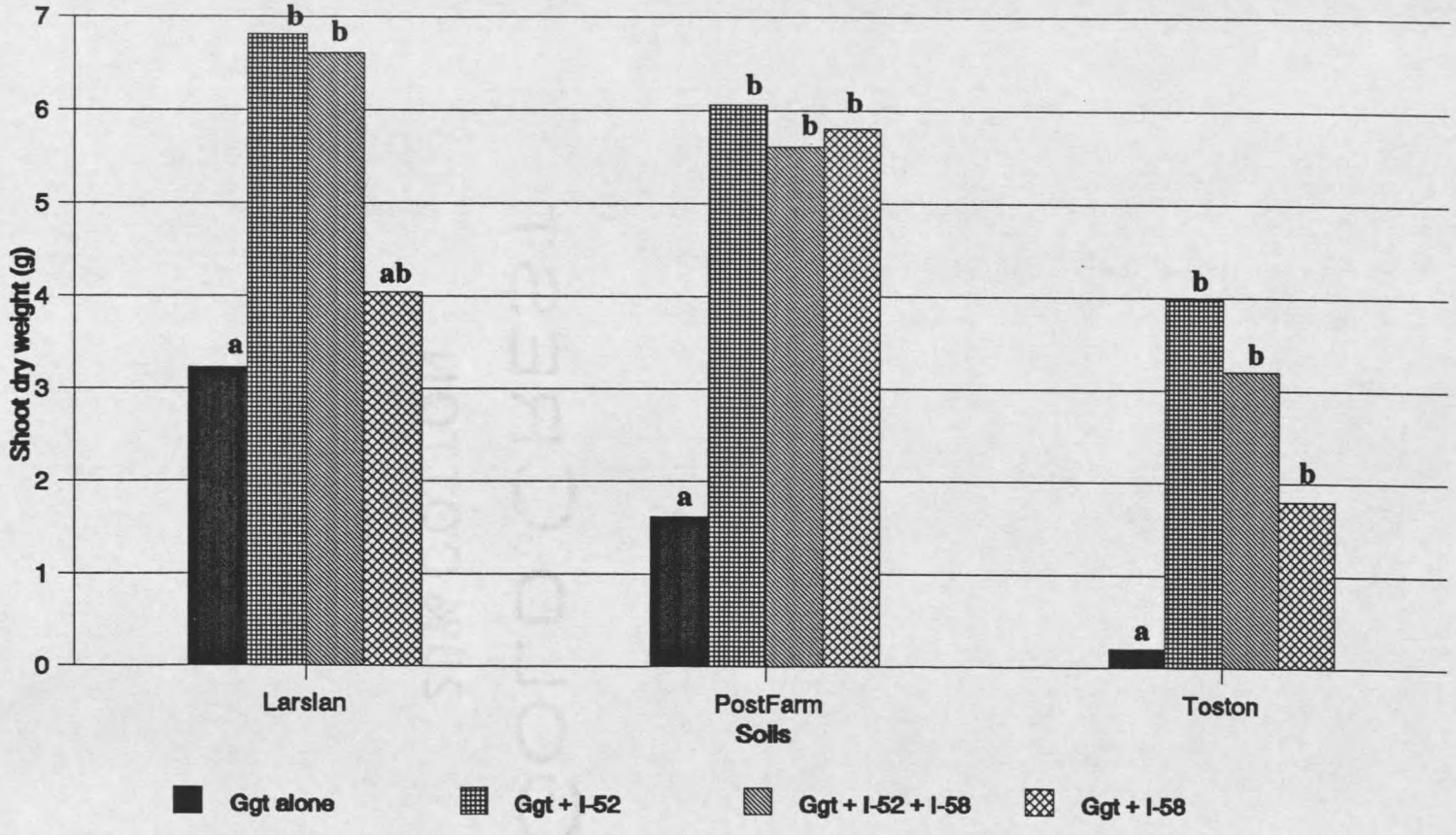

Figure 13. Effect of Phialophora (I-52 and I-58) on shoot weight of mature plants (Experiment 1).

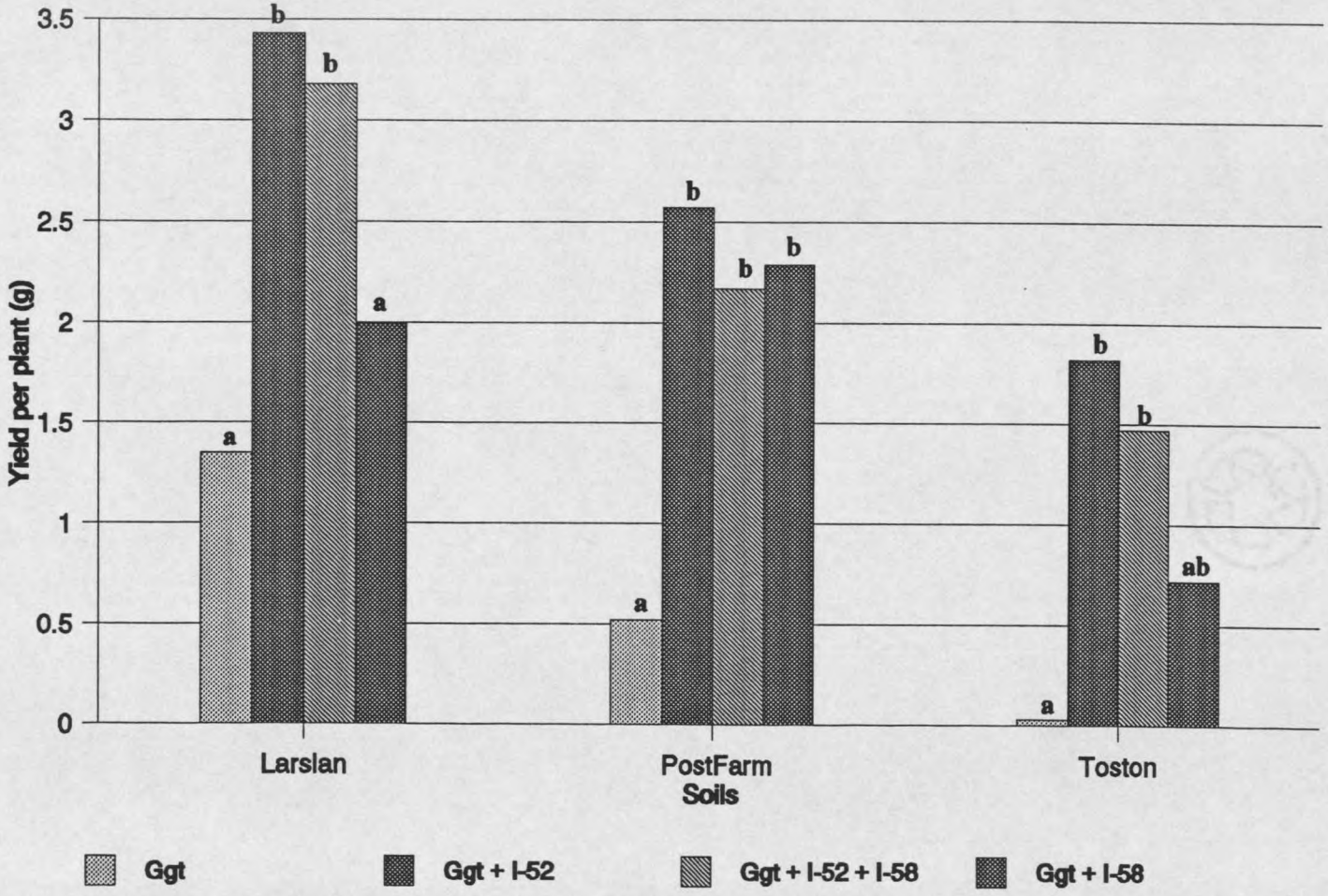

Figure 14. Effect of Phialophora (I-52 and I-58) on the grain yield of mature plants in Ggt-infested soils (Experiment 1).

significantly more heads per plant (3.8) than oats (1.9). I-52 alone produced greater, though not significantly, number of heads (3.2) than when combined with I-58 (3.1), or I-58 alone (2.3). Data analysis of the number of white heads per plant showed that Larslan and Toston soils were in the same group (0.1 and 0.2 white heads per plant respectively) and differed significantly from PostFarm soil (0.9), which could reflect the level of conduciveness present in PostFarm soil and a possible reduction in the effectiveness of the antagonists to suppress Ggt late in the season. The total number of tillers per plant was similar in PostFarm (9.3) and Larslan soils (8.3) which were higher than in Toston (6.7) whose compact structure could be responsible, in part, for the low tillering. The yield (kernel weight of five plants per pot) was considered the most important parameter to determine. Analysis of variance of yield data showed highly significant differences among soils, rates, substrates and antagonists. Over all treatments, plants grown in Larslan and PostFarm soils yielded significantly more (2.8 g and 2.2 g, respectively) than those in Toston soil (1.2 g). Canola as an antagonist carrier was associated with more yield (3.0 g) than oats (1.3 g). Finally, plants in pots where I-52 was added alone yielded more, though not significantly, (2.6 g) than those where I-52 was added in combination with I-58 (2.3 g) or I-58 alone (1.7 g).

Analysis of variance of the disease rating data on the roots at plant maturity detected highly significant differences among soils, rates, substrates and antagonists. The roots were more infected in PostFarm and Toston soils (2.4) than in Larslan soil (1.8), which confirms the suppressiveness of the latter against Ggt. Overall, the use of canola as substrate was associated with less infected roots (1.9) than oats (2.3). I-52+I-58 and I-58 were associated with more take-all symptoms on the roots (2.1 and 2.3, respectively) than I-52 alone (1.8), which might suggest that, in some cases, there may be a negative additive effect of I-58 on Ggt suppression by I-52. Analysis of shoot dry weight data at harvest showed highly significant differences among soils, rates, substrates, and antagonists. Larslan and PostFarm soils were similar and gave higher shoot dry weight (5.7 g and 5.5 g, respectively) than Toston soil (2.8 g). Shoot dry weight associated with canola (6.5 g), averaged over soils and treatments, was double that with oats (3.2 g). I-52 alone resulted in higher shoot dry weight (5.6 g) than when combined with I-58 (5.1 g) and than I-58 alone (3.9 g) (Table 12).

In experiment 2, data analysis of the number of total heads showed significant differences ($p < 0.01$) among soils, antagonists, and substrates, and a significant soil-antagonist interaction as well. Take-all infection

was lower in Larslan soil than in PostFarm soil, and shoot and root weights were significantly higher in the latter than in the former soil. The average total number of heads in Larslan soil was almost double that in PostFarm soil (Table 13). The antagonist I-52 was associated with the highest total number of heads (10.7) which was significantly higher ($p < 0.05$) than that associated with I-52+I-58 (7.8), I-58 (5.4), triadimenol (4.0), autoclaved substrate (3.0), and the Ggt control (3.0) (Figure 15). Overall, canola as a substrate resulted in more heads (8.1) than oats (5.4). Significant differences ($p < 0.01$) were found among soils, antagonists, and substrates, as well as a significant soil-antagonist interaction, for shoot dry weight. In Ggt-infested soils, I-52 gave the highest shoot dry weight (25.7 g), plants in Larslan soil had twice as much shoot dry weight as in PostFarm soil, and canola as substrate was associated with significantly ($p < 0.05$) higher shoot dry weight than oats. No significant shoot weight increases owing to amendment with autoclaved oats or canola inoculum were observed. Analysis of root infection data showed significant differences among soils, antagonists ($p < 0.05$) and substrates ($p < 0.05$). Plants in Larslan were less infected (1.9) than those in PostFarm (2.7). Canola (1.8) was associated with less root disease than oats (2.3) and the addition of I-52 showed the least, though not significantly, root

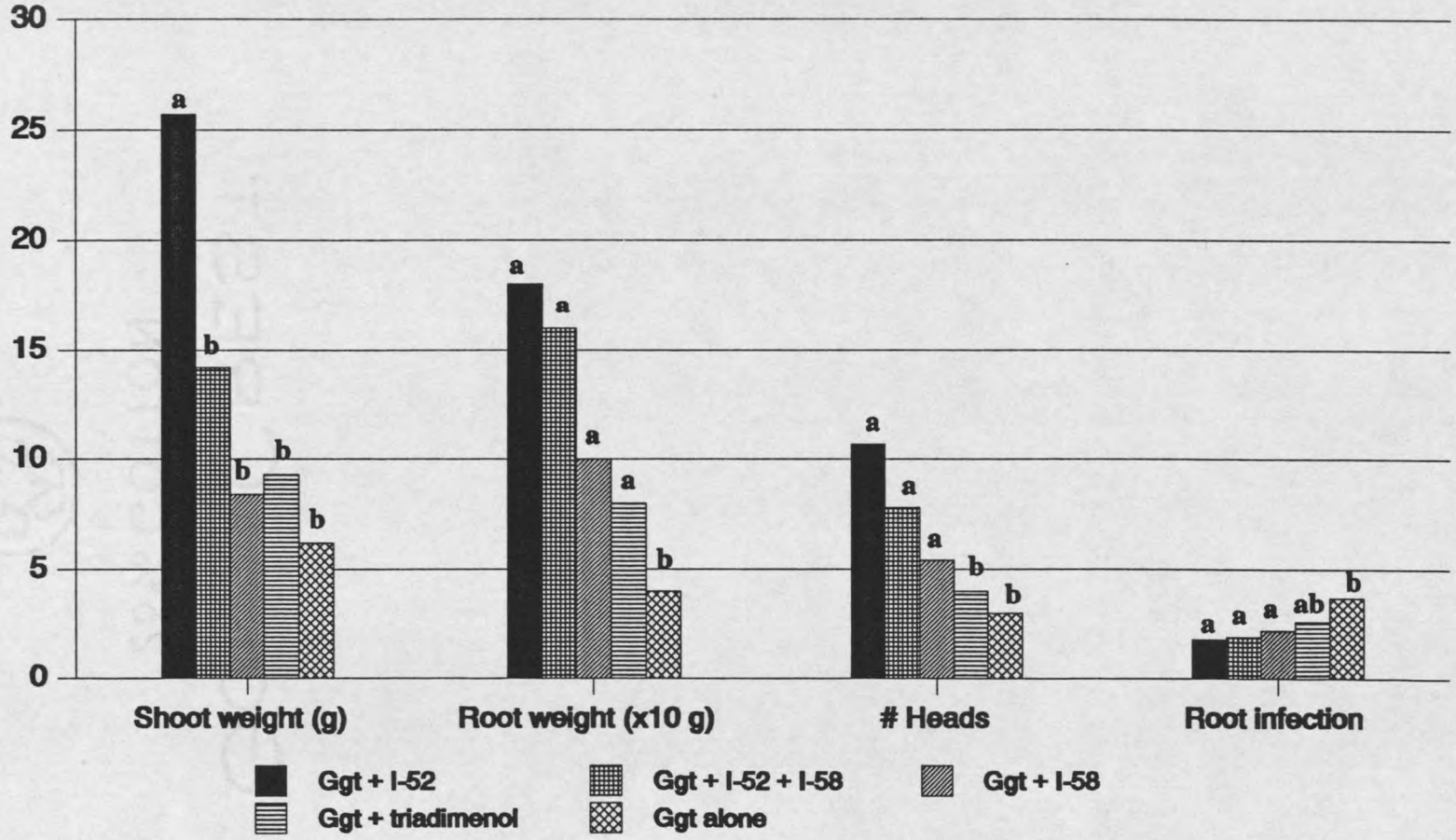

Figure 15. Effect of Phialophora (I-52 and I-58) on plant growth and root infection at harvest (Experiment 2).

infection (1.8) (Figure 15). Root dry weight was measured for mature plants and showed significant differences only among soils ($p < 0.05$) and antagonists ($p < 0.01$). Root dry weight was higher in Larslan (1.4 g) than in PostFarm soil (0.9 g) and the highest with addition of I-52 (1.8 g) (Table 13).

Discussion

This study showed that the biological activities of I-52 and I-58 may be affected by soil physical and microbial receptivity, substrate, and concentration of the antagonists in soil. These results indicate the importance of the disease rating on the roots and shoot dry weight in evaluating potential biological control agents in relation to other parameters such as the soil type, the substrate nature, and the ratio of antagonist to Ggt. It appears that the antagonistic activity of both I-52 and I-58 could be due, at least *in vitro* and particularly in the case of I-52, to the production of antibiotic compounds but not to volatile compounds nor to hyperparasitism. Furthermore, it seems likely that in the soil these two fungi control Ggt by various means including fast colonization of the roots, competition for nutrients in the rhizoplane, induction of plant resistance, and/or plant growth promotion.

In this study, effective biocontrol was demonstrated against take-all disease generated by artificial soil

infestation at the time of sowing. This was a more severe test on the biocontrol agents since the take-all inoculum was relatively high and close to the seed, which may not always be the case in naturally infested soils.

Dewan and Sivasithamparam (1990) reported that a sterile red fungus (SRF) was promising as a biological control agent of take-all and promoted growth of wheat by increasing the shoot and root weights as well as root length and the diameter of the roots. It has been suggested that take-all reduction by Phialophora graminicola may result from the lignification of cells within the roots especially those in the endodermis (Speakman & Lewis, 1978). However, Wong and Southwell (1980) found that, unexpectedly, P. graminicola did not confer any protection against Ggt although it colonized seminal roots to the same extent as the other Phialophora sp. They concluded that the degree of success in take-all control probably reflects the degree of effective competition between the antagonist and the pathogen.

Cowan (1979) discussed the possibility that P. graminicola and similar fungi might increase mineral nutrient uptake by plants in the same way as do mycorrhizal fungi. Deacon (1974) suggested competition as a mechanism by which P. graminicola controlled Ggt. Speakman and Lewis (1978) further showed that competition was important when they found that host-induced resistance

in the vascular system of the wheat root was proportional to the cylinder of cortex colonized by P. graminicola. Thus for the control to succeed, the population of the antagonist in the soil should far exceed that of the pathogen or the activity of the antagonist should have an advantage over the pathogen. In practice, this means a break or non-host crop should precede the inoculation of the fungal antagonists so that the population of Ggt in the soil would initially be small. Once the population of the pathogen has been reduced to a low level, it is conceivable to introduce the nonpathogenic fungi in the field and maintain them at high population level by applying particular cultural practices such as no-tillage.

The results of the two field experiments indicate that Phialophora sp. I-52 has the best potential to suppress Ggt in the field. In general, I-52 at a high rate on canola as a substrate was associated with a better control of take-all and a higher yield than I-58. From the data analysis of the treatments when the controls are excluded, we concluded that the parameters of plant height and number of plants with chlorotic symptoms recorded at two dates (25 and 55 days after planting) were not critical in determining clearly the performance of the antagonists. They might, however, be useful (especially after 25 days) in giving some early information about the

plant's general condition under various soil, substrate, rates, and antagonist environments.

In this study, take-all inoculum was added to the soil at a high level (1% w/w) compared to previous work by Andrade (1992) where 0.1% and 0.5 % (w/w) were applied. A significant protection of wheat plants by I-52 was still obtained both at the seedling and mature stages.

Biological control of take-all using bacteria such as Pseudomonas and Bacillus spp. has been investigated over many years (Capper & Campbell, 1986; Cook, 1974; Weller, 1988). The main limitations to the commercialization of these bacteria appear to be the generally modest levels of grain yield increases (10-20%) following inoculation and their inconsistent performance over time and space (Wong, 1994). Fungi such as G. graminis var. graminis (Ggg), Phialophora sp. (lobed hyphopodia), Phialophora graminicola and hypovirulent isolates of Ggt, have also been shown to protect wheat against take-all in glasshouse and field experiments in Australia (Wong, 1975, 1981; Wong & Southwell, 1980; Wong et al., 1996), Europe (Deacon, 1973; Speakman, 1984), and the USA (Duffy & Weller, 1995). In Germany, Speakman (1984) showed in a field experiment that a mixture of three isolates of Ggg and one of Phialophora sp. (lobed hyphopodia) reduced take-all disease in winter wheat and increased yield dramatically. However, yield increase was achieved at an inoculum rate

of Ggg-Phialophora sp. equivalent to 5.3 t/ha of oat inocula, which obviously is an uneconomic rate for commercially grown wheat.

Other studies in the USA (Cook, as cited in Wong et al., 1996) and in the United kingdom (Hornby, as cited in Wong et al., 1996) have been unsuccessful in achieving field control of take-all using Ggg.

Wong et al. (1996) used cold tolerant isolates of Ggg and Phialophora sp. (lobed hyphopodia) to control take-all of wheat in Australia. These isolates grew at 5°C at rates comparable to or greater than those of the Ggt tested. When added to the soil at a rate of 60 kg/ha of oat inoculum, these cold tolerant isolates consistently increased wheat yields by 33-45%.

It seems unlikely that a single organism or combination of organisms will afford effective protection against take-all of wheat in all soil types under all climatic conditions. However, until the determinants of root colonization and the factors affecting survival, ecological competence and mechanisms of suppression are understood, the manipulation of agronomic practices to favor the activity of biocontrol agents will not be efficacious and scientifically based.

The results of this study showed that addition of I-52 inoculum to the soil at rates equivalent to only 15 kg and 45 kg/ha increased yield, shoot and root weights

and induced a significant suppression of Ggt not only in a soil already suppressive to take-all but also in a conducive soil with a high Ggt infestation level. Therefore, it may be a candidate for commercial introduction into fields infested with Ggt or to prevent take-all establishment in fields newly converted to the monoculture of wheat.

APPLICATION OF *Phialophora* sp. IN COMBINATION WITH A *Bacillus* sp. TO WHEAT FOR THE BIOCONTROL OF TAKE-ALL

Introduction

Take-all of wheat (*Triticum aestivum* L.) and barley (*Hordeum vulgare* L.), caused by *Gaeumannomyces graminis* (Sacc.) Oliver and Von Arx var. *tritici* (Ggt), is important worldwide. Montana is a semi-arid region where much of the small grain cropland was originally a short grass prairie. However, take-all rapidly developed in Montana when wheat fields were first irrigated in the 1970s indicating that the inoculum had always been present (Andrade *et al.*, 1994a). Infection may cause premature senescence expressed as white heads.

Biological control of take-all has been studied intensively because of the economic importance of the disease and the absence of economically viable methods of control. There have been several reports of potentially useful microbial antagonists to Ggt. *Bacillus* spp. antagonistic to Ggt have been isolated from suppressive soils but lack of correlation between antibiosis *in vitro* and plant protection in the field has been reported (Capper & Campbell, 1986).

The actual mechanisms operating in the microbial control of take-all are complex. Some that have been described are antibiosis, enzymatic lysis, mycoparasitism, competition for space and nutrients, and the production of siderophores (Campbell & Faull, 1979; Hornby, 1979; Kloepper et al., 1980). Although it has been difficult to establish the role of antibiotics in the soil environment, some evidence suggesting that antibiotics and other diffusible compounds may have a role in biocontrol has been strengthened by the use of genetic techniques (Thomashow & Weller, 1988). They showed that a Pseudomonas fluorescens mutant deficient in phenazine production failed to inhibit Ggt on media supportive of antibiotic production and was significantly less suppressive than the parental strain to take-all on wheat seedlings (Thomashow & Weller, 1988). In order for a microorganism to produce antibiotics, it must be established on a nutrient resource and actively growing. Initial success of an organism in colonizing these resources cannot be attributed to antibiotic production (Deacon, 1991). Howell (1987) emphasized that although antibiosis may play a role in plant disease control, it is highly likely that it is not a mutually exclusive function, but probably occurs in conjunction with other mechanisms such as competition and parasitism.

It is most likely that disease suppression will be achieved predominantly through competition for nutritional microsites and that antibiosis is a secondary facilitating mechanism in defense of further intrusion (Baker, 1968; Deacon, 1991).

Competition is the endeavor of two or more organisms to gain a resource that each requires from the supply of the substrate when the substrate is not sufficient for both. Deacon (1991) suggested that microsites or "windows of opportunity" that are available for only a limited period are a major factor in determining the outcome of competition. Such ecological niches will be available to those best adapted to exploit them. The strategy for the use of competition in biocontrol is that one or more essential nutrients in the rhizosphere or rhizoplane must be limiting to inhibit growth. The principal limiting factors are carbon, nitrogen, vitamins, and iron (Baker, 1968; Lockwood, 1988). Iron is essential for the germination and growth of fungi. Bacteria and fungi can enter into direct competition by producing siderophores chelating any free available iron in the rhizosphere. Despite the fact that only a minority of the fungi have been studied so far, it appears that virtually all fungi depend on the production of iron-chelating compounds which are now collectively called siderophores.

Some weak parasites such as Phialophora graminicola and Idriella bolleyi have been shown to control take-all in grasslands and cereal monocultures by colonizing the naturally senescing root cortices and by suppressing the pathogen by competitive niche exclusion (Kirk & Deacon, 1987a; 1987b).

Inconsistent performance is a major limitation to the widespread use of biocontrol agents in commercial agriculture. Therefore, combining various biocontrol agents having different, ideally complementary, suppressive mechanisms may result in increasing the amount and consistency of disease control (Dandurand & Knudsen, 1993; Duffy & Weller, 1995; Lemanceau & Alabouvette, 1991; Pierson & Weller, 1994). Duffy and Weller (1995) reported that combination treatments consisting of G. graminis var. graminis applied to the soil and fluorescent pseudomonads applied to the seed were significantly more suppressive to take-all than either treatment used alone.

In the current study, we utilized a fungus and a bacterium isolated from a Montana soil suppressive to take-all to determine their biocontrol activity against take-all alone and their compatibility and effectiveness when applied together. The fungus (I-52), which has been identified as a Phialophora sp., has previously been shown to control take-all in greenhouse (Andrade, 1992) and field experiments (unpublished). Phialophora spp. from

wheat or other grasses have been reported to suppress take-all in Australia and Europe (Deacon, 1976; Wong, 1994) but, to our knowledge, not in the U.S.A. Phialophora spp. may suppress take-all by inducing host resistance, by competing with Ggt for substrates and infection sites, or by increasing exudate leakage thus enhancing antagonistic rhizosphere microflora. The bacterium, a Bacillus sp. isolated from Larslan soil, showed antibiotic activity in vitro against Ggt. The hypothesis in this study is that the combination of these two organisms, perhaps with different biocontrol mechanisms, would result in enhanced take-all suppression over that from either organism alone.

Materials and Methods

In vitro Inhibition by *Bacillus* L (Larslan)

Bacillus sp. strain L was isolated from Larslan soil by heating a soil suspension at 80°C for 30 min and plating soil dilutions on potato dextrose agar (PDA) and casein agar plus glucose (CAG). The strain that induced the highest inhibition of Ggt growth in vitro was selected for further use. Bacillus L was tested for its ability to inhibit Ggt on PDA and CAG plates. Bacillus L (10 µl of a $1x10^6$ - 10^7 bacterial suspension grown in potato dextrose broth, PDB) was spotted at equidistant points at the edge of each plate and incubated at room temperature. Forty-eight hours earlier, immediately, or 48 h later, a 7-mm

diameter plug of a fresh culture of Ggt grown on PDA was placed in the center. The diameter of Ggt was measured 4 days later. Possible inhibition of Phialophora sp. (I-52) by Bacillus L was also tested in the same way.

To further test antagonism in vitro, a technique involving wells made in half-strength PDA (½ PDA) and filled with sterile soil was used (Andrade, 1992). For Bacillus L, 30 µl of a heavy bacterial suspension (> $1x10^9$ cfu/ml) of a 24-h-old culture grown in PDB was placed into wells made in PDA plates in which autoclaved soils (Larslan, PostFarm, Toston soils) were added. Four 10-mm diameter wells per plate were made equidistant from each other and 15 mm from the edge of the plates. A 7-mm plug of Ggt was then placed in the center of each plate. Sterile soil-containing wells with only the addition of PDB were used on each plate as a check.

To test if other mechanisms, in addition to antibiosis, were involved in antagonism to Ggt, a single drop of a heavy bacterial suspension taken from a 24-h-old culture grown in PDB was placed on the hyphae from the border of a 7-day-old Ggt culture grown on ½ PDA. Three drops from Bacillus L and one drop of sterile PDB as a check were used per plate. After 4 to 6 days of incubation at room temperature, hyphal lysis, protoplasm condensation, cessation of growth, or any other abnormality that was microscopically visible was recorded.

In vitro inhibition by *Phialophora* sp. (I-52)

To test the ability of I-52 to inhibit the growth of Ggt, 7-mm agar plugs of the antagonist were placed on 2% malt extract agar (MEA) and on ½ PDA plates at equal distance from a central 7-mm disc of Ggt. Ggt growth and the percentage of inhibition were determined after 7, 14, and 21-days incubation. I-52 was added to the plate at the same time as Ggt or 3 days earlier. Sterile soil-containing wells were also used to test antagonism of I-52 toward Ggt.

Metabolite extraction and bioassay. Phialophora sp. (I-52) extracts were tested for in vitro inhibition of Ggt. The extraction and testing of metabolites was determined as described in Worasatit et al. (1994). Briefly, a broth culture of I-52 was prepared by inoculation of twenty-five 7-mm mycelial plugs into 500 ml of sterilized 1/5 strength PDB. The cultures were incubated at room temperature for 26 to 28 days as standing cultures. The mycelium was removed by filtration through cheesecloth and the broth was extracted sequentially with equal volumes (500 ml) of methylene chloride (fraction 1) and ethyl acetate (fraction 2); and, following acidification of the aqueous layer with concentrated hydrochloric acid (pH 1), again with ethyl acetate (fraction 3) to ensure that acidic metabolites

would not be lost. Once dried, all three fractions were dissolved in 10 ml of methanol, filter-sterilized by passing through a filter membrane (pore size 0.22 µm, Nalgene) and 20 µl of each solution placed in the middle of a PDA plate. Uninoculated sterile 1/5 PDB was also incubated for 26 days and later extracted in the same way as the I-52 filtrate and was used as a control. The lids of the Petri dishes were left slightly opened under a sterile air flow to allow the methanol to evaporate. A 7-mm mycelial plug of Ggt, Phialophora spp. (I-52 or I-58), or other fungi (Fusarium graminearum, Rhizoctonia solani AG 8) taken from the growing margin of a colony was then inoculated where the metabolite had been spotted on the PDA plate and incubated at 25°C in the dark for 4 days. The diameter of each colony was measured and the percent inhibition calculated using the formula:
% inhibition = $((D_1 - D_2)/ D_1) \times 100$ where D_1 (diameter of control plates) was taken from the colony of Ggt growing on plates to which 20 µl of the extract fraction from 1/5 PDB had been added and D_2 was the diameter of Ggt growing on plates to which the respective metabolites extracted from I-52 broth culture had been added.

Assay of siderophore production. Detection of siderophore production by Phialophora sp. isolates and Bacillus L was based on the universal chrome azurol S (CAS) assay (Schwyn & Neilands, 1987). Siderophores are

observed readily on CAS blue agar plates by the appearance of orange halos around colony growth.

Greenhouse Tests

Soil. Larslan soil was used in this experiment. This soil has been shown to have suppressive characteristics to take-all (Andrade et al, 1994a). All treatments were tested in autoclaved or natural soil. Soil to be used was sieved (2 mm), placed in a 2 cm thick layer on large plastic trays, moistened with tap water, and air-dried for 48 h in the greenhouse prior to autoclaving. The soil was autoclaved at 121°C for 1 h in plastic containers on three consecutive days, covered with aluminum foil, and then plated on PDA after autoclaving to confirm its sterility.

Inoculum preparation. A single pathogenic isolate of Ggt isolated from artificially inoculated plants in the field was used. Ggt and I-52 were cultured on PDA. Young cultures (5-7 days) were transferred to autoclaved oat or canola seed jars (about 150 g of canola or oats per 1 L jar to which 125 ml of distilled water was added prior to autoclaving for 90 min), respectively, as described by Weller and Cook (1983). After 30 days incubation, followed by drying, dry Ggt-infested oat kernels were blended in a commercial blender (Waring) for 12 s at low speed and 10 s at high speed, sieved to a particle size of 0.5-1.0 mm, and stored in sterile flasks at 4°C until use. Phialophora

sp. (I-52) was grown on sterilized canola seed. When almost all the canola substrate was colonized (10 days to 2 weeks), the inoculum was dried under a sterile air flow and stored at 4°C.

Seed preparation and bacterial inoculation. Wheat seed (cv. 'Pondera') was surface sterilized by immersion for 3 min in a solution of 2.5% sodium hypochlorite, rinsed in sterile distilled water, and dried overnight under a sterile air stream. Bacillus sp. strain L was grown on PDA in Petri dishes that were inoculated by flooding with 2 ml of a turbid bacterial suspension. Plates were incubated for 48 h at room temperature. Four milliliters of a 1.5% solution of methyl cellulose were added to the plate and the bacteria were scraped into a test tube, shaken for 30 sec, and then mixed with 5 g of surface sterilized wheat seed. Treated seeds were dried overnight under sterile air. Immediately thereafter, the number of colony forming units (CFU) per seed was determined by macerating 10 seeds in a sterile mortar and pestle with 10 ml of phosphate buffer (0.05 M, pH 7.0) and plating 10 µl of appropriate dilutions of the homogenate on PDA plates. Coated seed were stored in plastic petri dishes and sampled periodically for 5 weeks for viable CFUs.

Experimental design. Experiments were carried out in the Plant Growth Center (PGC, Montana State University) by

using the tube assays as described by Wilkinson et al. (1985) and modified by Weller et al. (1985), except that no vermiculite was added. Each tube had a cotton ball in the bottom and was filled with 50 g of the test soil. Ggt inoculum was mixed with the soil at 1% (w/w). I-52 inoculum was added at a ratio of 9/1 (w/w, I-52/Ggt). Seven treatments were prepared, in autoclaved and natural soil, as follows: (1) noninoculated control, (2) Ggt alone, (3) Ggt and I-52, (4) Ggt with I-52 and Bacillus L, (5) Ggt and Bacillus L, (6) killed I-52 inoculum, and finally (7) seed treated with killed Bacillus L. Both I-52 and Bacillus L were autoclaved twice at 121°C for 90 min and plated immediately to confirm that they were dead. One pregerminated wheat kernel (48 h on wet filter paper) was planted per tube after 8 ml of water was added. The seed was then covered with a 1 cm layer of sterilized sand and watered again with 2 ml of sterile water. The tubes were covered with a plastic wrap until emergence, and subsequent irrigations consisted of 10 ml sterile distilled water three times weekly. Plants from each treatment were sampled 10, 25, 35, 45, 55, and 65 days after planting. The shoots were dried at 70°C for 48 h and weighed. The roots were washed thoroughly under running tap water to remove adherent soil and rated for take-all infection on a 0-5 scale (0 = no disease, 1 = one or two lesions on the roots, 2 = 50-100% of the roots with one or

more lesions each, 3 = all roots with lesions and some evidence of infection on the stem, 4 = lesions abundant and moving up the tillers, 5 = plants severely stunted or dead) (Weller et al., 1985). Root weight was determined after drying at 70°C for 48 h. The experiment was conducted twice.

Isolation procedure. For enumeration of bacteria and fungi in the rhizoplane, roots were dried on filter paper and weighed, sampled for dry matter determination and either macerated in 10 ml of phosphate buffer (0.05 M) in a mortar for 2 min and appropriate dilutions plated on PDA and 1/10 tryptic soy agar (TSA), or small sections (5-10 mm-length) were surface disinfected in 10% Sodium hypochlorite for 2 min and plated on PDA amended with streptomycin (100 mg/L).

Root Preparation for Scanning Electron Microscopy (SEM) Observation

Wheat seedlings were harvested at different intervals after planting (10, 25, 35, 45, 55, 65 days). They had been either inoculated with Ggt alone, Ggt and/or I-52, Bacillus L, or not inoculated as controls. The first seminal root was cut off, the proximal 2-3 cm were placed into a fixative of 2.5% glutaraldehyde, buffered to pH 7.0 with 0.2 M sodium cacodylate buffer for 2 h. For observation with the SEM, the fixed root pieces were

washed for 3 min in distilled water, immersed in 1% osmium tetroxide (OsO_4) for 10 min, rinsed in 5 changes of distilled water over a period of 2 h, and stored in absolute methanol until use. Roots were placed in metal baskets filled with methanol, dried in a critical-point drier with liquid carbon dioxide and attached to stubs with double-sided adhesive tape. A small drop of silver paint was drawn up from the stub to the root to increase electrical contact. The root was sputter-coated with a 50 nm layer of gold in an Aragon-filled 'Polaron E300' coating unit. Stubs were examined using a scanning electron microscope.

Statistical Analyses

Analyses of variance and multiple comparisons were performed by using the SAS or MSUSTAT programs.

Results

Survival of *Bacillus* L in Storage

Bacillus L on the treated seed stored at room temperature dropped from 10^9 cfu to a 10^5 cfu over a 5 wk period. When stored at 5°C, the concentration decreased to 10^6 cfu after five weeks.

In vitro inhibition by I-52 and *Bacillus* L

Maximum inhibition (84%) of Ggt growth by Phialophora sp. (I-52) was obtained on PDA when Ggt was placed three

days after I-52. When Bacillus L was plated on PDA 48 h earlier than Ggt, linear mycelial growth of the latter was reduced by 78% (Table 14). Bacillus L inhibited I-52 growth by 61% on PDA when added 48 h earlier and by 42% when both were added at the same time (Table 14).

Table 14. In vitro growth inhibition of Gaeumannomyces graminis var. tritici by Phialophora sp. (I-52) and Bacillus L.

Phialophora sp. (I-52)	PDA	WA
Ggt added 3 d earlier	51.8[a]	49.5
Ggt added same time	55.2	41.9
Ggt added 3 d later	84.4	52.8
Bacillus L	**PDA**	**CAG**
Ggt added 48 h earlier	57.5[b]	49.8
Ggt added same time	57.3	57.8
Ggt added 48 h later	77.7	60.2
I-52 added 48 h earlier	40.6[c]	46.1
I-52 added same time	42.1	42.6
I-52 added 48 h later	60.8	54.5

a: Percent inhibition of Ggt growth by I-52 after 6-day incubation at 20°C.
b: Percent inhibition of Ggt growth by Bacillus L after 5-day incubation at 20°C.
c: Per cent inhibition of I-52 growth by Bacillus L after 5-day incubation at 20°C.

Antibiotic Activity

Metabolites extracted from I-52 filtrate slightly, but significantly, reduced Ggt growth on ½ PDA but did not

show any noticeable antibiotic activity against the other fungi tested (Table 15). The ethyl acetate fraction (fraction 2) inhibited Ggt growth by over 50% (Figure 16).

Mycelial Inhibition in Dual Cultures

Paired cultures on PDA or WA-coated glass slides did not manifest any hyperparasitic hyphal interaction between I-52 and Ggt. Protoplasm condensation and growth cessation were, however, visible when a Bacillus L suspension was placed on the surface of actively growing Ggt hyphae. Phialophora sp. I-52 strongly inhibited the growth of Ggt mycelium but no hyphal lysis was noted.

Siderophore Production

On CAS blue agar, I-52 exhibited visible siderophore production after 48 h whereas Bacillus L did not produce any after 5 day-incubation (Figure 17). After one week, however, Bacillus L appeared to produce siderophores illustrated by an orange halo around the colonies which expanded with time.

Glasshouse Experiment

The analysis of variance of the root infection and the shoot dry weight data of experiment 1 showed highly significant differences ($p < 0.001$) among soils (sterile and nonsterile), treatments, sampling dates as well as highly significant interactions among soils*treatments,

Table 15. Assay of antibiotic activity of metabolites toward various fungi extracted from Phialophora sp. (I-52) filtrate.

Treatment	Ggt	I-52	I-58[a]	Fusarium graminearum	Rhizoctonia solani AG8
Control (Methanol)[b]	25.7d*	41.3 cd	31.0 b	59.7 d	74.3 b
Methylene chloride (check)[c]	25.3 d**	42.3 d	32.3 b	47.7 a	73.7 b
Methylene chloride (I-52)	18.7 c	39.0 bc	31.3 b	49.7 ab	73.0 b
Ethyl Acetate (check)[d]	24.7 d	38.7 b	34.3 c	55.3 abcd	74.7 b
Ethyl Acetate (I-52)	12.0 a	34.3 a	26.3 a	56.0 bcd	66.7 a
Ethyl Acetate (pH1)[e] (check)	25.7 d	38.7 b	34.7 c	58.7 cd	75.0 b
Ethyl Acetate (pH1)[e] (I-52)	16.3 b	41.3 cd	31.3 b	51.0 abc	73.3 b
LSD (0.05)	2.1	2.5	2.0	8.2	2.6

a: Phialophora sp. (I-58).
b: 20 µl of methanol were placed in the center of a ½ PDA plate, allowed to dry for ½ h and thereafter a 7-mm agar disc of the test fungus was placed on the same spot.
*: Mean diameter of three plates (mm) after 4 day-incubation in the dark on ½ PDA.
c: Methylene chloride fraction of 1/5 PDB incubated for 26-28 days without I-52 (control) and extracted in the same way as I-52 filtrate.
d: Ethyl acetate fraction of 1/5 PDB.
e: Ethyl acetate fraction of 1/5 PDB or I-52 filtrate after acidification (pH 1).
**: Values followed by the same letter are not significantly different according to LSD test (p = 0.05).

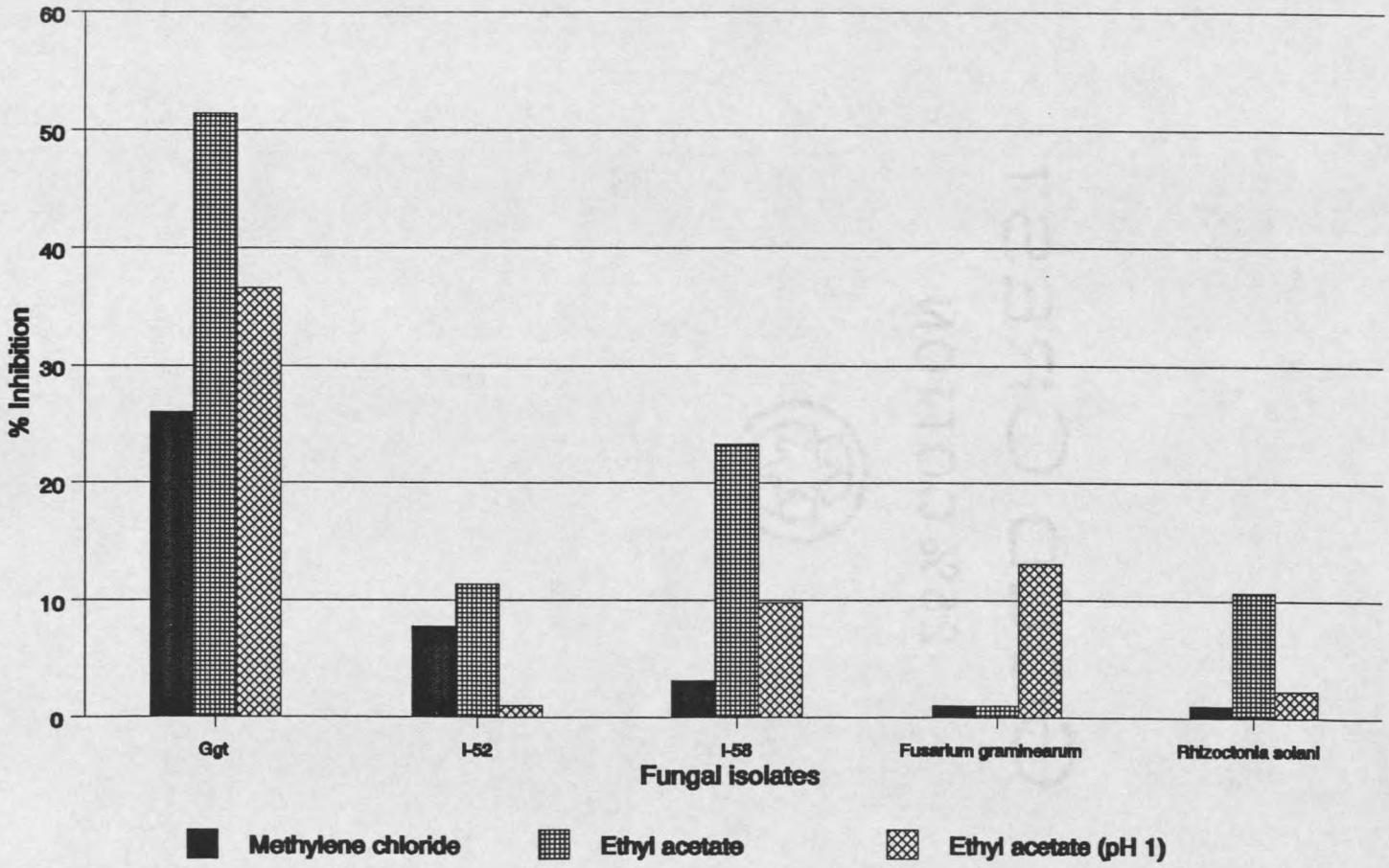

Figure 16. Percent inhibition of fungal radial growth by metabolites extracted from Phialophora (I-52).

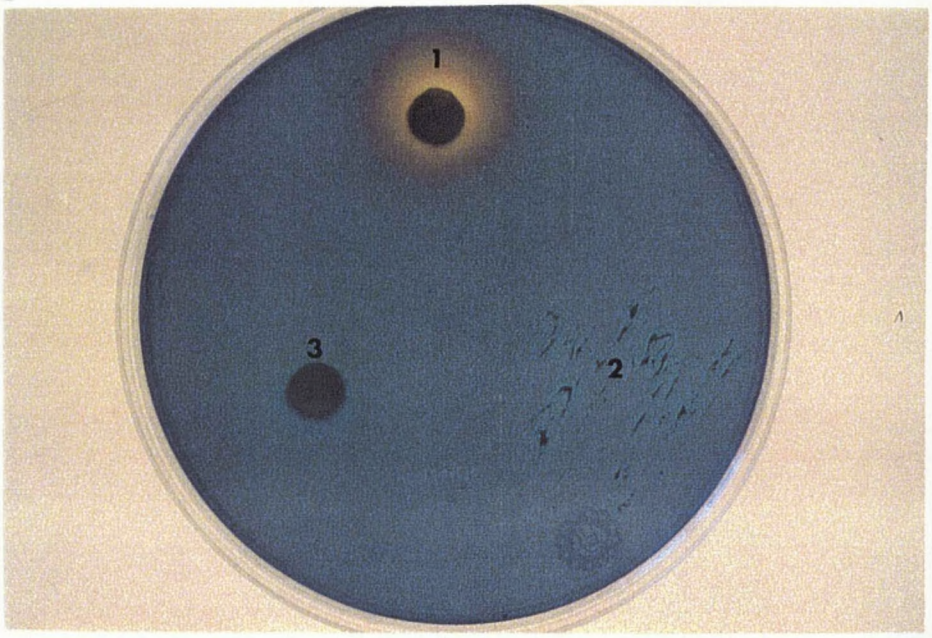

Figure 17. Siderophore production by Phialophora sp. I-52 (1), Bacillus sp. strain L (2), and Gaeumannomyces graminis var. tritici (Ggt 698) (3) on CAS blue agar after 4 days.

soils-sampling dates, treatments-sampling dates, and soils-treatments-sampling dates.

The average disease rating on the roots, over all the treatments and sampling dates, was much lower in nonsterile (0.3) than in sterile soil (0.7) which confirms the suppressive properties of Larslan soil. By autoclaving it the biologically suppressive components were eliminated. However, across all treatments, the shoot dry weight was higher in nonsterile (101.4 mg) than in sterile soil (83.1 mg).

Concerning the various treatments, I-52 was associated with the lowest root infection (0.1), which was

lower, yet not statistically significant, than the level of take-all in Larslan soil which developed from naturally occurring inoculum (0.2) (the control to which nothing was added). In contrast, the Bacillus L treatment gave a much higher root infection (1.0) (Figure 18). When averaged across sterile and nonsterile soils, the treatment in which the soil was amended with Ggt and I-52, and the seed coated with Bacillus L resulted in the highest shoot dry weight (104.1 mg) followed by the Ggt/I-52 treatment (99.1 mg), while the treatment Ggt/Bacillus L resulted in the lowest shoot dry weight (80.8 mg) and was in the same group (SNK,0.05) as the Ggt control (82.4 mg) (Figure 19). When averaged across sterile and nonsterile soils, the treatment where I-52 and Bacillus L were combined resulted in higher shoot weight than that of the healthy control plants 65 days after planting. I-52 alone was associated with higher shoot weight than the Ggt control throughout the experiment, and higher than the healthy plants 55 days after planting (Figure 20). Figure 21 shows that the treatments gave different results in sterile and nonsterile soils. In nonsterile soil, root infection was 0.6, 0.4, 0.2, 0.1, and 0.3 for Ggt, Ggt/I-52/Bacillus L, Ggt/Bacillus L, Ggt/I-52, and the control where nothing was added, respectively. However, in the sterile soil, these same treatments were associated respectively with root infection values of 2.2, 0.2, 1.8, 0.2, and 0.1.

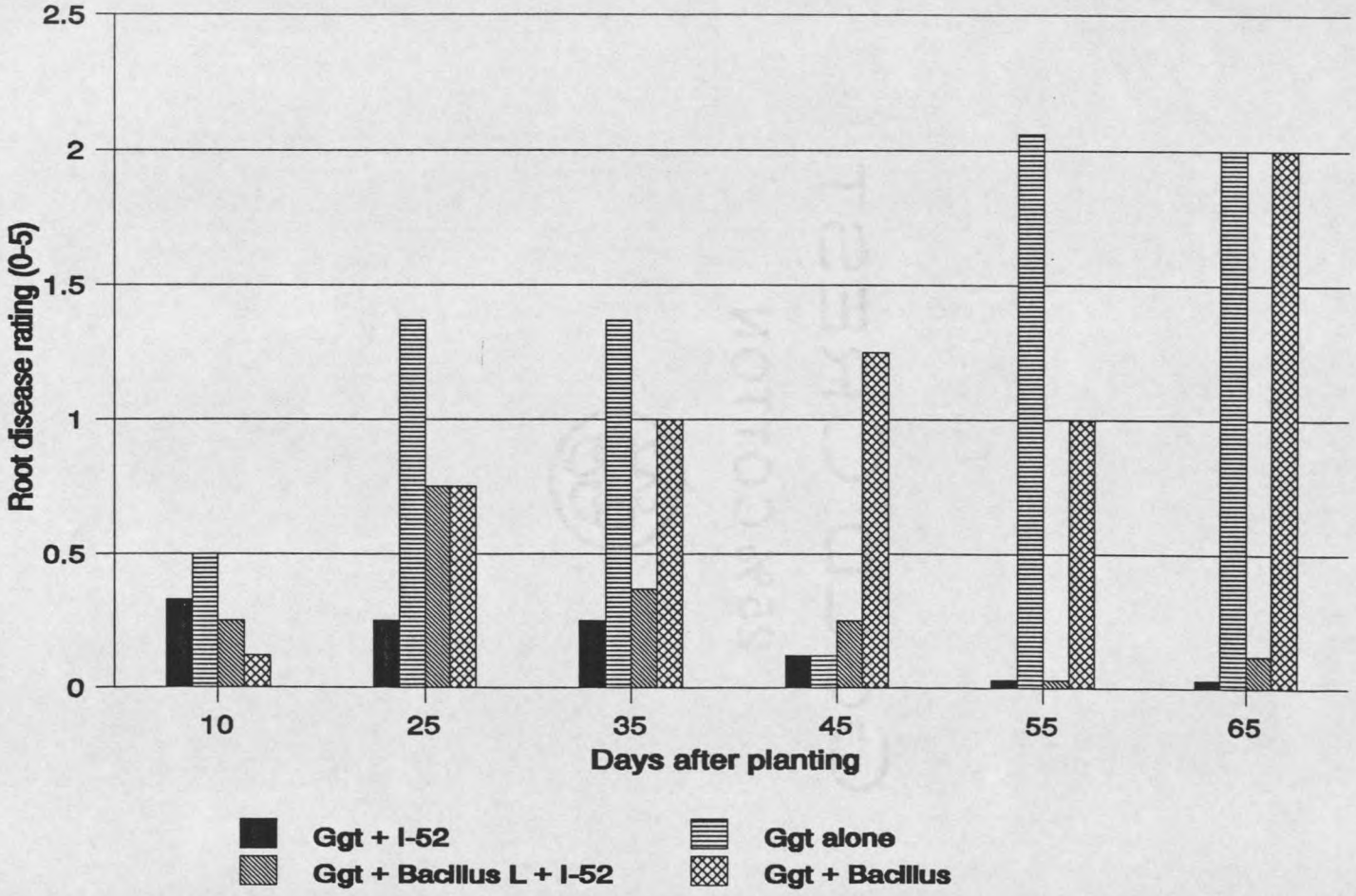

Figure 18. Effect of Phialophora (I-52) and Bacillus L on control of take-all in wheat under greenhouse conditions.

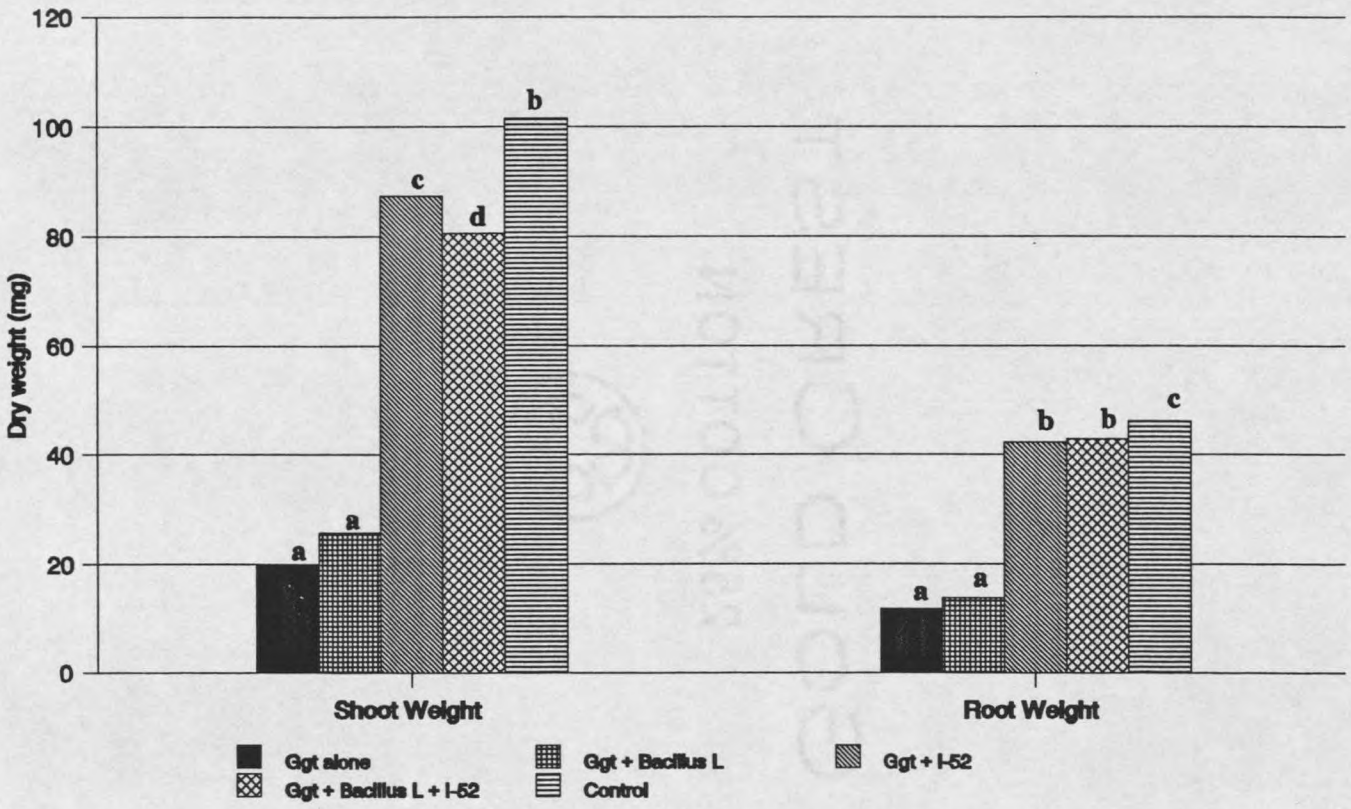

Figure 19. Effect of Phialophora (I-52) and Bacillus L on wheat seedling growth averaged across autoclaved and natural soils under greenhouse conditions.

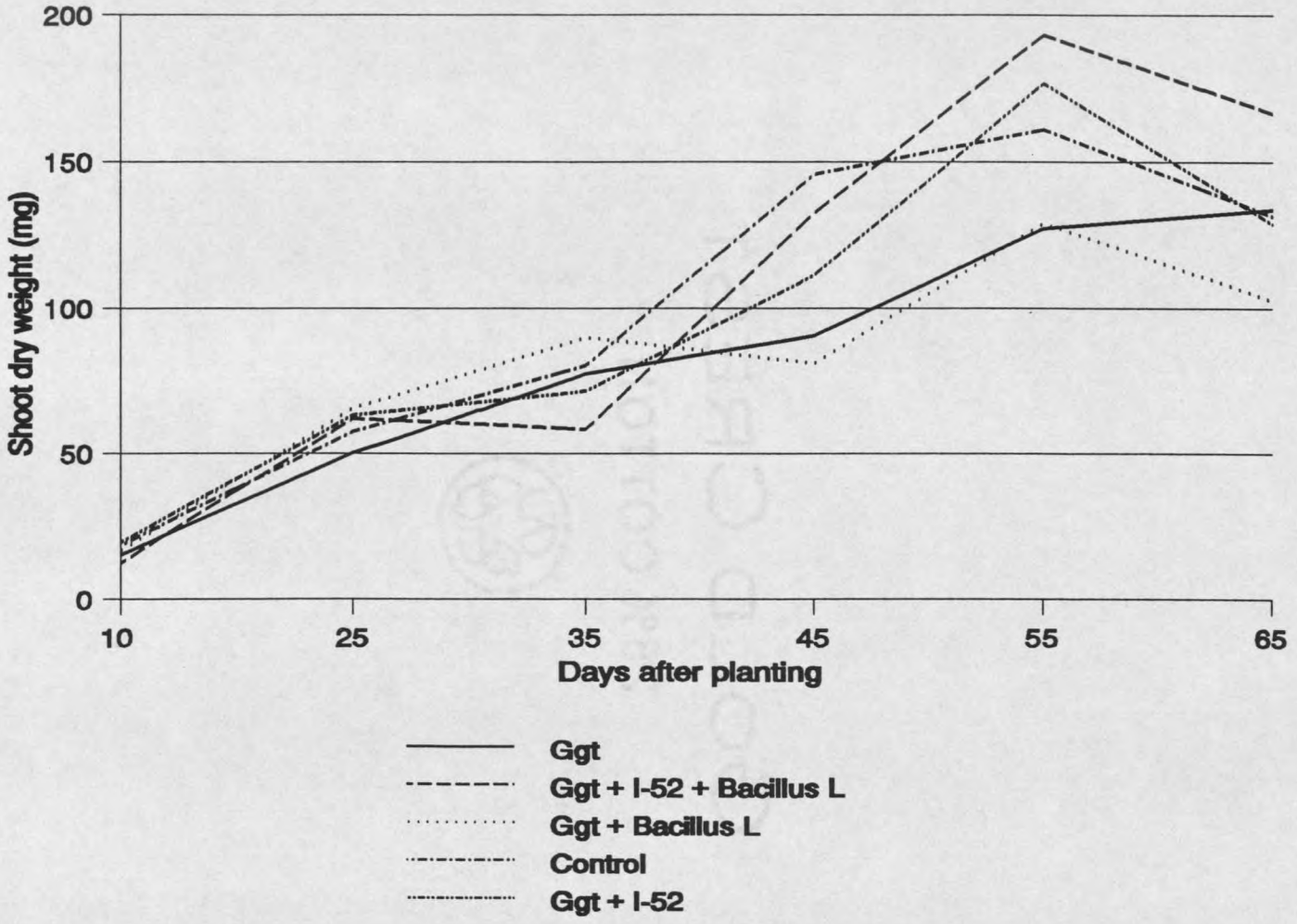

Figure 20. Effect of Phialophora (I-52) and Bacillus L on shoot weight of wheat seedlings under greenhouse conditions.

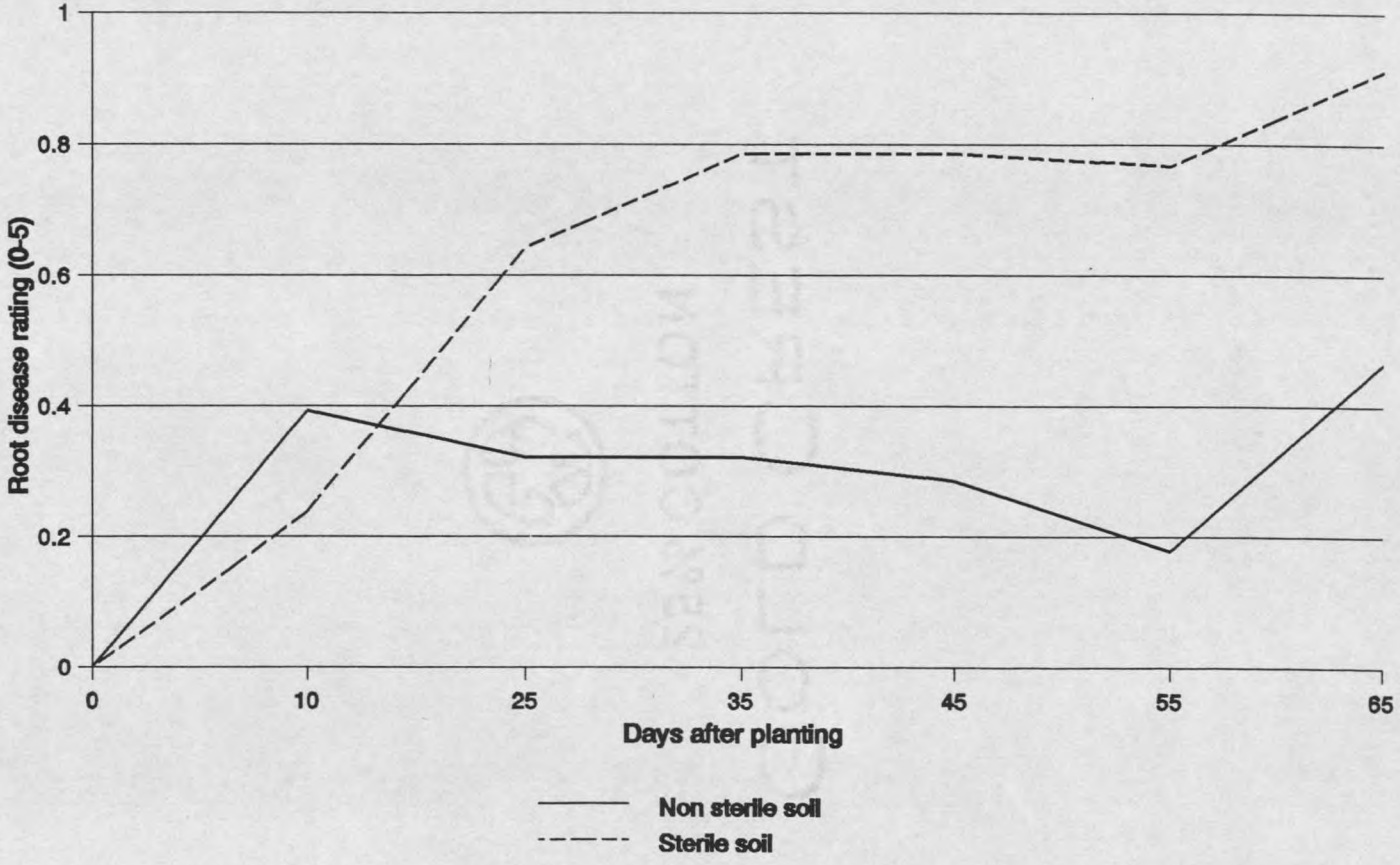

Figure 21. Progression of take-all infection (averaged across treatments) on wheat seedling roots under greenhouse conditions.

There is a good indication that take-all infection is severe in sterile soil amended with Ggt and that I-52/Bacillus L and I-52 alone reduced the infection in both soils by antagonizing the take-all fungus. In general, shoot weight as well as the root infection by Ggt increased with time. However, sterile and nonsterile soils behaved differently. In nonsterile soil, the shoot weight increased steadily from the first sampling date (10 d after planting) to the fifth sampling date (55 d after planting) and the root infection decreased during the same period of time. In contrast, in sterile soil the root infection increased and the increase in shoot dry weight was lower than in nonsterile soil.

In experiment 2, generally, when I-52 and Bacillus L were added separately or combined, in presence of Ggt, they significantly reduced root infection. When I-52 was introduced either alone or with Bacillus L, both shoot and root weights were significantly increased (Table 16). Ten days after planting, I-52 was recovered from wheat roots in natural and autoclaved soils but not when Bacillus L was present. I-52, alive or dead, was associated with an increase of bacteria isolated from the roots both in natural and autoclaved soils. After 35 days, I-52 was recovered from treatments where it was introduced alone or with Bacillus L. In the presence of dead I-52 or dead

Table 16. Effect of Phialophora (I-52) and Bacillus L on root infection, shoot and root weights of wheat plants grown in Larslan soil in the greenhouse.

Treatment	Root infection (0-5)[a]	Shoot dry weight (mg)[a]	Root dry weight (mg)[a]
Ggt[b]	4.2 a*	19.9 e	11.8 c
Ggt + Bacillus L	3.5 b	25.7 e	13.8 c
Ggt + I-52[c]	0.4 c	87.4 c	42.2 b
Ggt + I-52 + Bacillus L	0.4 c	80.6 d	42.8 b
Autoclaved I-52	0.1 c	114.8 a	47.8 a
Autoclaved Bacillus L	0.1 c	101.5 b	46.1 ab
Control	0.2 c	99.3 b	49.0 a

a: Values are averaged across sterile and nonsterile Larslan soil and the 6 sampling dates.
b: Rate of Ggt inoculum: 1% w/w.
c: Rate of Phialophora (I-52) inoculum: 9:1 I-52/Ggt, w/w.
*: Values followed by the same letter are not significantly different according to LSD test (p = 0.05).

Bacillus L, high populations of bacteria were isolated from the roots (Tables 17 and 18). Ggt was not recovered from roots protected by I-52. Sixty-five days after planting, I-52 was still recovered from the roots at high frequency and continued to protect the roots against colonization by Ggt. Bacterial populations continued to increase on roots in both autoclaved and natural soils, particularly in treatments where I-52, alive or dead, or Bacillus L had been introduced (Tables 17 and 18).

Table 17. Fungal and bacterial isolations from wheat seedling roots 10 days after planting under greenhouse conditions.

Autoclaved soil	Ggt	Antagonist	Organisms isolated	
			Fungi	Bacteria*
+	-	-	-[a]	3.7
+	+	I-52	I-52	14.0
+	+	I-52+*Bacillus* sp. L	-	16.0
+	+	*Bacillus* sp. L	Ggt	2.2
+	+	-	-	4.6
+	-	Dead I-52	-	13.0
+	-	Dead *Bacillus* sp. L	-	5.0
-	-	-	-	0.6
-	+	I-52	I-52	7.6
-	+	I-52+*Bacillus* sp. L	*Fusarium* spp.	7.5
-	+	*Bacillus* sp. L	-	3.9
-	+	-	-	4.0
-	-	Dead I-52	white + yellow mycelia	7.6
-	-	Dead *Bacillus* sp. L	-	0.6

*: cfu/mg roots X 10^4.
a: no fungal mycelium isolated (I-52, Ggt, or others).

Table 18. Fungal and bacterial isolations from wheat seedling roots 65 days after planting under greenhouse conditions.

Autoclaved soil	Ggt	Antagonist	Organisms isolated	
			Fungi	Bacteria*
+	-	-	I-52	4.1
+	+	I-52	I-52	78.0
+	+	I-52+*Bacillus* sp. L	I-52	70.0
+	+	*Bacillus* sp. L	-[a]	32.0
+	+	-	Ggt	45.0
+	-	Dead I-52	*Fusarium* spp.	34.0
+	-	Dead *Bacillus* sp. L	white fungi	4.1
-	-	-	white fungi, *Fusarium* spp.	5.0
-	+	I-52	I-52, white fungi, *Fusarium* spp.	51.0
-	+	I-52+*Bacillus* sp. L	I-52, white fungi	96.0
-	+	*Bacillus* sp. L	-	87.0
-	+	-	white fungi	41.0
-	-	Dead I-52	*Fusarium* spp.	66.0
-	-	Dead *Bacillus* sp. L	*Fusarium* spp., white fungi	2.7

*: cfu/mg roots X 10^4.
a: no fungal mycelium isolated (I-52, Ggt, or others).

SEM Observations

In an attempt to investigate how I-52 and *Bacillus* L caused the reduction in root infection by Ggt, roots were studied by SEM. Wheat plants were grown in autoclaved soil and watered with sterile water in the presence or absence of Ggt. Uninoculated wheat plants were healthy with no macroscopic root lesions or deformities. Very high densities of bacteria became established in the rhizoplane

of those plants inoculated with Bacillus L. The latter did not seem to cause any damage to the roots. When Ggt was inoculated alone there were extensive macroscopic lesions on the roots and profuse mycelial growth along with yellowing of leaves. After 25 days, the foliar symptoms disappeared in I-52 treatments but persisted in Ggt alone. Root symptoms were much less visible in the former than in the latter. Figure 22 illustrates the extensive growth of Ggt hyphae over the lesions which developed in the first days of growth. In treatments where Bacillus L was introduced, in some regions of the roots the hyphae were colonized by bacteria, and extensive lysis of hyphae often occurred (Figure 23). The remnants of the lysed hyphae were usually associated with bacteria held together by filamentous material (Figure 24). This filamentous material may have been undigested parts of the fungus or products of the bacteria involved in the lysis of the fungus cell wall. Abundant bacterial growth was also noticed in the treatment where Bacillus L and I-52 were combined (Figure 25). Lysis of hyphae was not observed when only Ggt was introduced into the sterile soil. Roots of plants grown in autoclaved soil where Ggt and I-52 were introduced exhibited an extensive mycelial growth but with no clear distinction between the two fungi. Penetration of the hyphal walls has not been seen by SEM though breakage of the hyphal walls was seen in a few instances. It is

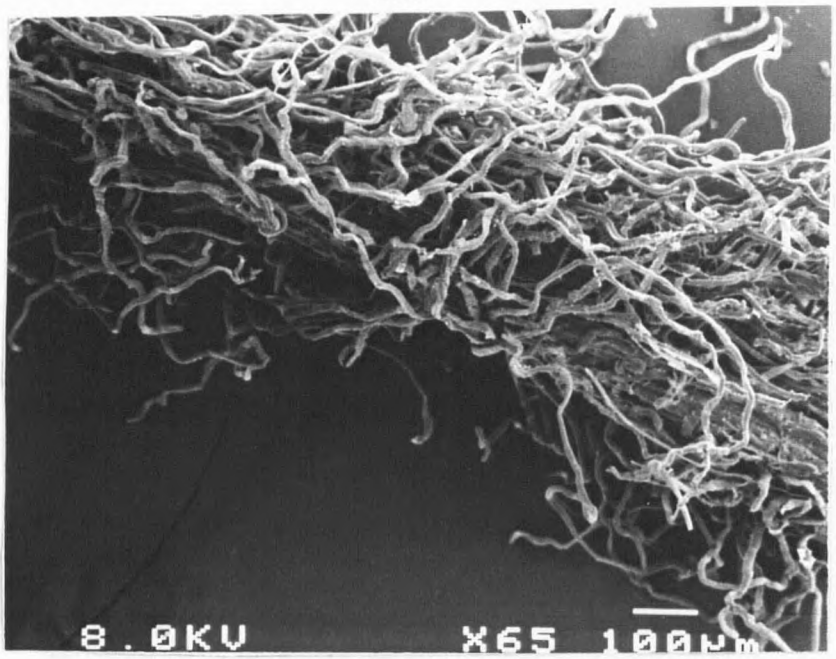

Figure 22. Extensive colonization by Gaeumannomyces graminis var. tritici (Ggt 698) of a wheat seedling root grown in autoclaved soil in a control treatment where Ggt 698 was introduced alone (x 84).

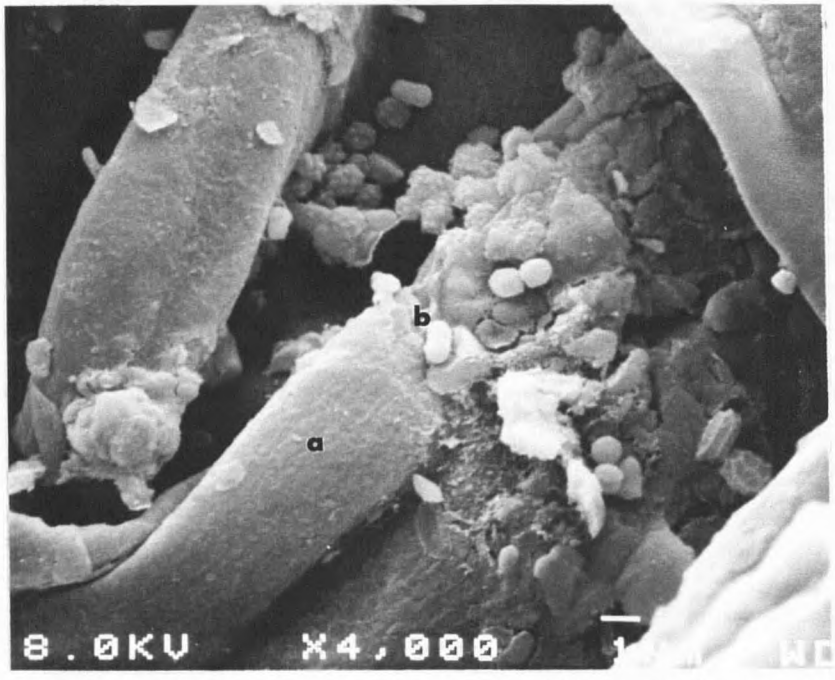

Figure 23. Ggt hyphae (a) on wheat seedling roots grown in autoclaved soil. Ggt hyphae are colonized by Bacillus sp. strain L (b) and some hyphal lysis is visible (x 5,200). In this treatment Ggt 698 was introduced into the soil and seeds were coated with Bacillus sp. strain L.

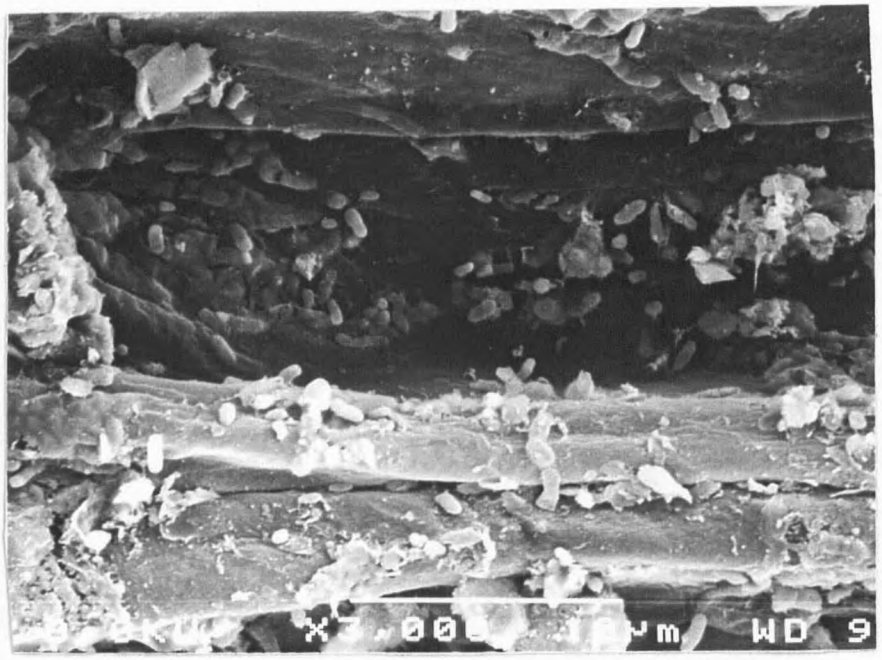

Figure 24. Association of Bacillus sp. strain L with wheat seedling roots (x 3,900). In this treatment, Ggt 698 was introduced into the soil and seeds were coated with Bacillus sp. strain L.

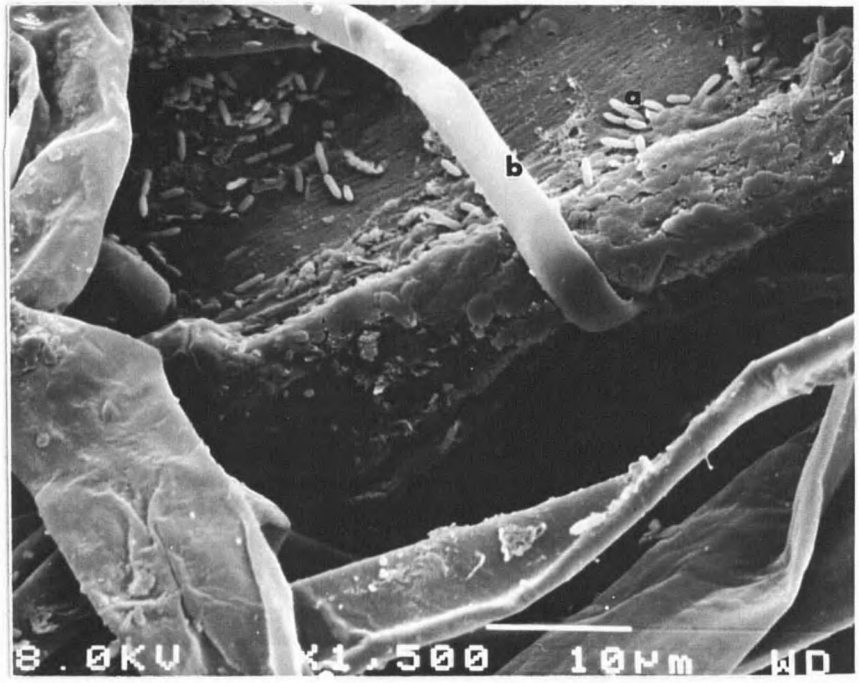

Figure 25. Association of Bacillus sp. strain L (a) with wheat seedling roots. In this treatment, Ggt 698 and Phialophora sp. I-52 (b) were introduced into the autoclaved soil and seed were coated with Bacillus sp. strain L (x 1,950).

possible that the breaks in the hyphal wall could have been caused during preparation of the material and were not causally connected with the presence of either Ggt, Phialophora sp.(I-52) or Bacillus L.

Discussion

The results of this study support previous evidence that soil suppressiveness to take-all could involve effects of antagonistic microorganisms, particularly fungi and bacteria. The isolates used in this study originated from a Montana soil suppressive to take-all (Larslan soil). When reintroduced into the same soil, Phialophora sp. (I-52) duplicated the effects of a suppressive soil. The present Bacillus strain was selected based on a strong in vitro inhibition of Ggt growth, but a lack of a good correlation between antagonism in vitro and in the soil is not uncommon. In addition, this study demonstrates that a combination of a Phialophora sp. (I-52) and a strain of Bacillus sp. might have potential for biological control of take-all. The health of the plants, as measured by root infection and root weight, grown from bacteria-treated wheat seeds was generally closer to that of the infected nontreated control than to that of the noninfected, nontreated control, yet the shoot dry weight was higher in the former than in the latter treatment.

However, when I-52 was added to the soil, both the health of the plants and the shoot dry weight were better than the infected nontreated check. Furthermore, the shoot weight was higher than that of the noninfected, nontreated check which suggests that I-52 might have a dual beneficial action by suppressing Ggt and promoting the growth of the plants. Take-all suppression was more consistent in natural soil where the natural population of I-52 and Bacillus spp. is present than in the same soil sterilized.

The Phialophora sp. I-52 appears to give a good control of Ggt in natural nonsterile soil perhaps because of its association with other microorganisms, especially with other sterile fungi that abound in Larslan soil, or due to some physico-chemical modifications of the soil properties caused by the sterilization process. The enhanced growth of wheat from addition of I-52 is attributed to suppression of Ggt and stimulation of the growth of the plant. Indeed, the plants grown in soil where I-52 was added alone or in combination with Bacillus L showed an earlier heading, a greener and healthier looking vegetative system, and non- or less-infected roots with more fine root hair development, and longer roots than the plants infected with Ggt.

The addition of dead I-52 and dead Bacillus L enhanced the growth of the plants, in the absence of Ggt,

only in natural soil, which suggests that this growth promotion is mediated by indirect effects on the microflora of the soil which in turn could enhance the growth of the plants by excreting growth hormones.

Contrary to what was expected, Phialophora sp. I-52 alone seems to have an advantage over its combination with Bacillus L or Bacillus L alone. This latter finding suggests that in fact some of the in vitro inhibition of I-52 growth by Bacillus L is also taking place in the rhizoplane. Some possibilities to explain why control has only been slightly successful with Bacillus L might be that the bacterium may have mutated in storage so that, even if it was good in initial in vitro studies, it now is no longer effective. However, this explanation is not very plausible since Bacillus L was stored for only a short period before use and further testing confirmed its antibiosis in vitro. Another possibility to consider is the lack, in general, of a good correlation between in vitro and field antagonism. Thus, bacterial action that depends exclusively on antibiotic production controlled by plasmids may not be suitable. Pseudomonads, for example, have been widely reported to control take-all (Cook & Baker, 1983; Weller & Cook, 1983), yet their disadvantage is that they may be less stable genetically and less tolerant of environmental extremes than the spore-forming bacilli. Pseudomonas spp. may not be suitable for take-all

control in cold, wet, and alkaline soils in Montana where their antagonistic activity and their persistence in the rhizosphere and on the roots would be reduced. Thus, selecting biological control agents should be specific to the soil and environmental conditions of the area where they will be used.

Kim et al. (1994) reported that a Bacillus strain suppressed rhizoctonia root rot, pythium root rot, and take-all of wheat in growth chamber tests and increased the yield of wheat by 26.6% in field tests. However, Capper and Campbell (1986) found that two Bacillus species did not survive very well under field conditions so that, although the dose may be sufficient at the start, it does not maintain itself. Other experiments with these bacteria have shown some early effects which did not persist until harvest (Campbell & Faull, 1979). Under dry conditions, bacilli can become dormant and they do not then function as antagonists (Campbell & Clor, 1985). This pattern of initial protection and subsequent failure to protect against an increasing challenge was observed by Capper and Higgins (1993) and might have occurred in the present study under glasshouse conditions as Bacillus L was associated with a lower root infection than in the Ggt control for only 10 to 25 days after planting. In one study Stephens et al. (1993) observed that there was not necessarily any relationship between the ability of a

bacterium to inhibit a fungal pathogen when the bacterium was grown in the laboratory on media that favored the production of either antibiotics or siderophores, and the biocontrol activity of the bacterium *in vivo*.

Gaeumannomyces graminis var. *tritici* on roots of wheat grown axenically without added bacteria showed no lysis of the hyphae, whereas extensive colonization of the hyphae and lysis was observed in the presence of the antagonistic *Bacillus* L (Figures 22, 23, 24, and 25). Since we could not distinguish between hyphae of *Gaeumannomyces* and *Phialophora* with the SEM, we only assumed that the latter competed with the former for early colonization of the roots based on extensive hyphal growth on the roots and absence of take-all lesions. The roots were yellow to light brown compared to dark brown with black lesions reaching the crown in roots where Ggt alone was added to the sterile soil.

Field trials have shown that selected bacteria, when added to soils infested with take-all, could reduce root lesions (Faull, 1978). Cook and Rovira (1976) suggested that such specific antagonism probably occurs in the rhizosphere or on the root surface. Rovira and Campbell (1975) studied with the scanning electron microscope interactions between *Gaeumannomyces* and bacteria grown *in vitro* and showed that perforation of the hyphal wall and lysis of the hyphae were associated with a decrease in

disease symptoms. Faull and Campbell (1979) reported that in the presence of Bacillus mycoides the hyphae of G. graminis were lysed and it was suggested that the lysis was the reason for the control of the disease.

Another facet of the competitiveness of bacilli as plant growth promoting rhizobacteria (PGPR) is their ability to persist both free in the soil and in the rhizosphere. This is greatly influenced by a number of different factors including soil composition (Heijnen & van Elsas, 1994) and temperature (Sun et al., 1994). Juhnke (1987) reported that although the Bacillus isolates tested did not result in the highest bacterial counts, as a group they were the most consistent colonizers. The Bacillus L used in this study showed a high root colonization efficiency in addition to in vitro antibiotic and lytic activities against Ggt, yet it was not efficacious in suppressing take-all on wheat seedlings under glasshouse conditions.

One objective of this study was to test if a combination of a fungus and a bacterium would control take-all better than either antagonist alone. However, this was not clear here, and the Phialophora sp. alone suppressed Ggt as much as when combined with Bacillus L. On the other hand, the combination of the two organisms provided a much better control of the disease than Bacillus L alone. Leeman et al. (1996) reported that

combination of Fusarium oxysporum and Pseudomonas sp. only reduced disease significantly, compared with separate inoculation of the organisms, if both were applied at densities which were not individually able to reduce disease significantly. Future work may involve in vivo screening of other Bacillus strains from the same suppressive soil and testing them in combination with Phialophora spp. at various inoculum rates. These bacilli could be marked with antibiotic or fungicide resistance to study their persistence under experimental and field conditions, particularly their spread from seed and their multiplication in the rhizosphere.

In addition, it is important to have a screening system which uses the overall beneficial effect on the plant, in the presence of the pathogen, of potential biocontrol agents instead of selection based only on in vitro antagonism. This will simultaneously test for antagonism of any sort, colonization of the roots, and the ability to survive and grow in the soil environment. The use of combinations of competent bacteria and nonpathogenic antagonistic fungi might be very efficient in controlling take-all due to a potential complementarity of their antagonistic activities and their temporal and spatial colonization of the root surface and rhizosphere. In alkaline soils where iron is less available than in acid soils, Bacillus spp. and Phialophora spp. could be

engineered for high production of siderophores which would allow them to out-compete Ggt for iron uptake.

Fungi can grow at lower water potentials than bacteria, and once these fungi have entered the seminal roots they can further colonize the subcrown internode and the crown roots as they emerge. Once inside wheat tissues, they are not as subject to fluctuating water potentials as are rhizosphere bacteria.

Under irrigated conditions in Montana, a better control of take-all may be achieved with a combination of Bacillus spp. and Phialophora spp. that are present in suppressive soils. The problem with such mixed systems is that they may inhibit each other, or they may be synergistic, and the results are likely to be even more difficult to understand.

SUMMARY

The pathogens that cause take-all belong to the Gaeumannomyces-Phialophora complex of fungi that parasitize the roots and crown of graminaceous plants. Gaeumannomyces spp. have Phialophora spp. anamorphs. These fungi are ecologically obligate parasites that grow on the roots with similar-looking runner hyphae. The taxonomy of this complex is confused.

In this study, I report the identification of previously unknown fungi isolated from a Montana soil suppressive to take-all as Phialophora spp. and the results of a comparative study of morphological, cultural, and molecular characteristics of some of these fungi. These Phialophora spp. exhibited in vitro inhibition of Ggt growth. They were nonpathogenic on wheat and barley. However, they did not confer a substantial protection of wheat and barley seedlings against Ggt in glasshouse experiments.

Phialophora spp. presented a diverse array of cultural characteristics. Their growth rate ranged between 5.0 and 9.6 mm/day; the mycelium color varied among isolates and even within isolates depending on media, age of the colony, and subculturing. Most of the isolates produced simple to slightly lobed hyphopodia on wheat

coleoptiles and none produced perithecia in culture or on wheat roots. Sizes of phialospores and phialides were variable and overlapping between isolates. Some Phialophora spp. produced siderophores and/or diffusible antibiotic metabolites on agar plates.

The study of the rDNA sequences showed that some of the Phialophora spp. were very closely related to Gaeumannomyces spp. After comparing the molecular information from the rDNA regions with morphological characters, I conclude that Phialophora sp. I-52 is most similar to P. graminicola described by Deacon (1974).

A practical application of the nucleotide sequence comparison was the design of primers to be used in detecting Phialophora sp. I-52 in the soil and on cereal and grass roots.

Phialophora spp. were isolated from a Montana take-all suppressive soil and under field conditions showed good potential as biological control agents against Ggt both in a suppressive and a conducive soils. In particular, isolate I-52, whose inoculum was produced on canola seed and added to the soil at rates equivalent to 15 kg and 45 kg/ha, proved efficient in suppressing the disease generated by artificial soil infestation at the time of sowing. This was a more severe test on the biocontrol agent since take-all inoculum was relatively high and close to the seed, a situation that may not

always occur in naturally infested soils. Therefore, Phialophora sp. I-52 may be a good candidate for commercial introduction into fields infested with Ggt or to prevent take-all establishment in fields newly converted to wheat monoculture.

The mechanisms involved in the suppression of Ggt by Phialophora sp. I-52 seem to be diverse and complex. I-52 appears to achieve this role through competition for infection sites in the rhizoplane, through competition for nutrients most likely for iron via siderophore production, by promoting plant growth, and by producing antibiotics or fungistatic metabolites.

The need for diversity in biological control is strongly emphasized. Besides additive or synergistic effects, multiple agents could become active when others become inactive as environmental conditions change, or become active at various time intervals as host organs develop and concomitantly initiate different microniches and/or infection courts. Unfortunately, the results of combining I-52 and another Phialophora sp. (I-58) as well as I-52 and Bacillus sp. strain L were not conclusive. Combinations of antagonists did not confer any advantage over the use of I-52 alone in protecting wheat seedlings or mature plants under glasshouse and field conditions.

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APPENDIX

Media

Sigma products were obtained from Sigma Chemicals Co., St. Louis, MO. Bacto products were obtained from Difco Laboratories, Detroit, MI.

All media are autoclaved for 30 min. at 121°C.

1/10 Tryptic Soy Broth Agar

Tryptic Soy Broth, Sigma	3.0 g
Bacto Agar, Difco	15.0 g
Distilled H_2O	1.0 L

Lilly and Barnett Glucose Asparagine Agar (Lilly & Barnett, 1951)

Bacto Agar, Difco	20.0 g
Glucose	10.0 g
K_2HPO_4	1.0 g
$MgSO_4.7H_2O$	0.5 g
Asparagine	2.0 g
$FeSO_4$	0.2 mg
$ZnSO_4$	0.2 mg
$MnSO_4$	0.1 mg
Distilled H_2O	1.0 L

Casein Agar plus Glucose (Schaad, N.M. 1988)

Casein acid Hydrolysate	10.0 g
Yeast Extract	5.0 g
Glucose	5.0 g
K_2HPO_4	4.0 g
Bacto Agar, Difco	17.0 g
Double distilled H_2O	1.0 L

Chrome Azurol S Agar (Schwyn & Neilands, 1987)

CAS	60.5 mg
H2O	50.0 ml
Iron (III) Solution*	10.0 ml

Dissolved and mixed with:

HDTMA	72.9 mg
H2O	40.0 ml

Autoclave separately and add to:

Agar	15.0 g
Pipes	30.24 g
50% NaOH	12.0 g
Casamino acids (10%)**	30.0 ml
Glucose**	20%
H2O	850.0 ml

Phosphate Buffer (pH 6.8)

NaCl	8.5 g
K_2HPO_4	11.4 g
KH_2PO_4	6.8 g
Double distilled H_2O	1.0 L

*:1 mM FeCl3.6 H2O, 10 mM Hcl.

**: Casamino acids and glucose are added as sterile solutions after temperature drops to 50°C.