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.