CHAPTER 1

OBJECTIVES AND LITERATURE REVIEW

History. Take-all or a variety of older names such as whiteheads, dead-heads, shriveled heads, and in turf, Ophiobolus patch, is a root and foot rot disease of cereals and grasses (Nilsson, 1969) caused by *Gaeumannomyces graminis* (Huber and McCay-Buis, 1993). Specifically, take-all of wheat (*Triticum aestivum* L.) is caused by *G. graminis* (Sacc.) Arx and Oliver var. *tritici* Walker (= *Ophiobolus graminis* Sacc.) (Cook and Rovira, 1976, Asher and Shipton, 1981, Huber and McCay-Buis, 1993). Although possibly not the most appropriate name, take-all has become the accepted English common name for this disease (Nilsson, 1969), and may be the most important root disease of wheat world-wide (Weller and Cook, 1983).

Take-all was first described as a disease of grasses in Sweden in 1823 (Mathre, 1992). Since the middle of the 1800's, the causal organism was thought to be a fungus. It was not until 1890 that Prillieux and Delacroix established a link between a disease of wheat in France, and the fungus *G. graminis* (Nilsson, 1969). The fungus had first been described by Berkeley and Broome in 1861 as *Sphaeria cariceti,* and later by Saccardo (1875) as *Rhaphidophora graminis* (Nilsson, 1969). In 1881, Roumeguére and Saccardo changed the name of the fungus to *O. graminis* (Nilsson, 1969). In 1922 *O. cariceti* was proposed by Fitzpatrick et al., and since then several other proposals have been made, such as *Plagiosphaeria* sp. or *Linocarpon* sp. (Nilsson, 1969). Arx and Oliver

felt in 1952 that since typical species of the genus *Ophiobolus* are members of the Pseudosphaeriales, and *O. graminis* was placed in the Diaporthales in the Gnomoniaceae, this fungus could be placed in the genus *Gaeumannomyces* (Nilsson, 1969), and is reviewed in Mycologia memoir number 7 (Barr, 1978).

The disease. Infection by *Gaeumannomyces graminis* only occurs in plants belonging to the *Gramineae* (Nilsson, 1969). Nilsson (1969) lists 402 referenced host plants of *O. graminis* (*G. graminis*). Listed are areas in which the disease is of agronomic interest: New Zealand, Australia, Japan, Chile, The United States, Canada, South Africa, Kenya, Italy, France, Belgium, The Netherlands, Germany, Poland, Russia, Ireland, Great Britain, Norway, Denmark, and Sweden (Nilsson, 1969).

Spread of the disease and infection from the ascospores or microspores is unlikely (Nilsson, 1969). Germinating ascospores penetrated and infected wheat seedlings only in sterile soil, and at extremely high densities (Nilsson, 1969). It is believed that spread of the fungus and root infection occurs predominantly by the brown to dark brown, 3-6µm diameter mycelia (Nilsson, 1969). The thicker, colored external (to the plant) hyphae are termed macrohyphae or runner-hyphae, whereas thinner, hyaline hyphae are termed microhyphae or infection-hyphae. In 1928 it was noted that penetration of the root epidermis was primarily by microhyphae, but either type could penetrate the plant. Penetration of a root seems to be mechanical (Nilsson, 1969). Near the tip of the microhypha, structures called hyphopodia pierce the outer wall of epidermal cells without development of distinct apressoria (Nilsson, 1969). Hyphae then grow intracellularly in a radial manner, not parallel to the root, and then enter the stele where

disintegration of tissue occurs (Nilsson, 1969). As might be expected, young roots are easily infected and colonized, whereas penetration is potentially impeded by the maturation of older roots (Nilsson, 1969). However, plants may be infected and colonized at any stage of growth (Huber and McCay-Buis, 1993). Infection is favored by soil temperatures ranging from 12-20 C (CMI Descriptions # 383).

Strains and Pathogenicity. The "wheat" strain of *O. graminis, i.e., G. graminis* (Sacc.) Arx and Oliver var. *tritici* Walker readily causes disease in wheat. This strain does not cause disease in oats (*Avena sativa* L.), whereas the "oat" strain, *G. graminis* (Sacc.) Arx and Oliver var. *avenae* Turner, has a wider host range, and can cause disease in wheat as well as oats (Nilsson, 1969). A third strain, found to cause disease in turf is *G. graminis* (Sacc.) Arx and Oliver var. *graminis* , and causes less disease in wheat (Huber and McCay-Buis, 1993). The three varieties can be distinguished by aminopeptidase profiles (Huber and McCay-Buis, 1993).

In Britain, twenty five *Ggt* isolates from each of several sites were tested for pathogenicity by Asher in 1980. Variation existed for each of the characters tested (virulence, growth rate, and coloration). One important finding was that there was not a statistically significant difference between the damage inflicted by isolates obtained from fields that had undergone short (several years) and long (approximately 15 years) cropping sequences. Dewan and Sivasithamparam tested the virulence of *Ggt* isolates from wheat and ryegrass, *Lolium rigidum* L., and found that most *Ggt* isolates from wheat were more damaging to wheat than to ryegrass, and on wheat specifically, the *Ggt* isolates from wheat were more damaging than rye isolated *Ggt* (Dewan, 1989). *Ggt*

isolated from rye was more damaging to wheat than to ryegrass indicating that rye may be somewhat resistant to *Ggt* (Dewan, 1989).

Fungal Vegetative Compatibility. Heterokaryon formation among individuals is an important component of many fungal life cycles (Leslie, 1993). The anastomosis of hyphae and sharing of nuclei is not only important in the formation of heterokaryons for the sexual phase of the organism, but also in the transfer of viruses. Strains of a fungus that are vegetatively compatible (VC) are said to be in the same VCG or vegetative compatibility group. There are often many genes that control vegetative compatiblity. This recognition system is controlled by mating-type genes and other genes designated as *vic*, or vegetative incompatibility genes. Generally speaking, vegetative compatibility or incompatibility involves the restriction of the transfer of nuclear and cytoplasmic material among fungi (Leslie, 1993).

In *Aspergillus*, *Cryphonectria*, *Fusarium*, and *Neurospora,* fusion of hyphae occurs naturally, even if they are incompatible. In incompatible reactions, however, there is a death of the heterkaryon. A direct test for the formation of a heterokaryon involves the creation of a situation under which neither component could survive individually. Obtaining *nit* (nitrate non-utilizing) mutants is a method used to create such markers. With *Fusarium*, spontaneous *nit* mutants can be can be recovered as chlorate-resistant sectors in such high frequency that they can be used for population studies (Leslie, 1993).

Barrages or the "barrage zone" is formed under incompatible reactions, and is considered an antagonistic situation. The barrage requires hyphal fusion after which

death occurs. A central region of dead and dying cells forms, and a dark layer of pigmented hyphae is laid down, often accompanied by the formation of perithecia. Vegetatively compatible strains do not interact in this manner, but simply grow together without an alteration in fungal morphology (Leslie, 1993).

VCGs permit the study of fungal populations and their diversity. Strains within the same VCG are capable of exchanging genetic information. It is felt that if selection maintains a large number of VCGs, it is quite possibly due to the spread of pathogenic agents such as viruses. In general, a large number of VCGs indicates a sexually reproducing population. In an asexual population, members of each VCG will form a sub-population of organisms subjected to the forces of natural selection. Over time, VCGs are lost by chance making the population less diverse. However, if a particular VCG is more fit than others, it will predominate over many geographic locations. Overall, there is no correlation between pathogenicity and VCG (Leslie, 1993).

Variation among isolates of *Ggt* has been reported by Nilsson (1969). Anastomosis between isolates is only successful in pathogenically similar isolates from the same area, *i.e.,* Western Australian isolates from oats were incompatible with South Australian isolates from oats, both of which were incompatible with wheat isolates. Jamil et al (1984) tested thirty-one isolates of *G. graminis* from a single field in Britain and were classified into eighteen vegetative compatibility (VC) groups. VC groups were determined by placing isolates opposite each other and noting the presence of a striking barrage zone, indicating incompatibility, with the presence of a zone of clearing with adjacent pigmented zones, or compatibility with the absence of this reaction. Double-

stranded RNAs found among the many VC groups indicate that anastomosis among VC groups actually may occur.

Asher (1981) notes that in several studies, heterokaryon formation between isolates was rarely achieved. One study reported that only six of forty-five pairs were apparently compatible while selfed pairs were compatible. A different study indicated that the frequency of heterokaryon formation was extremely low and noted that heterokaryon formation was even low between an auxotrophic mutant and a wildtype (WT) strain of itself.

Agronomy. Extensive field studies have been conducted, and results are often conflicting or equivocal (Werker and Gilligan, 1990). The disease is extremely variable, and thus repeatability of experimental effects and detection of treatment effects within experiments is difficult (Werker and Gilligan, 1990). As with many pathogens and the disease they cause, disease symptoms appear in patches within fields containing the take-all pathogen. Patches of diseased plants can be as small as several plants or as large as several square meters. Setting up plots with *Ggt* present in all of the treatment locations is very difficult, and therefore the data obtained often contains large variation.

Crop rotation is currently the only recognized method of control (Werker and Gilligan, 1990), however here in Virginia, growers find more profit in double cropping wheat and soybeans (*Glycine max* (L.) Merrill or barley (*Hordeum vulgare* L. emend. Bowden) and soybeans every year, setting up conducive conditions for take-all of wheat (E. L. Stromberg, personal communication, 1994). Treatments such as fungicide seed dressings, form, timing, and quantity of nitrogen fertilizer, chloride, and manganese, have

been investigated as possible methods of control (Weste, 1971, Werker and Gilligan, 1990, Huber and McCay-Buis).

An experiment in the United Kingdom showed that planting date had a significant affect on disease (Werker and Gilligan, 1990). There was more disease initially in early sown plots, but disease increased faster in later sown plots. Overall, later sown plants had more roots per plant than did early sown plants. Although dependent on season, Autumn applied nitrogen increased disease, and the effect of chloride application was variable (Werker and Gilligan, 1990).

In an Oregon study, early seeded plants (4 October) showed a greater incidence and severity of take-all, versus later seeded plants (27 October) (Taylor et al., 1983). Except for NH_4Cl + NaCl, and NH_4Cl + KCl + P treatments, yields were significantly increased by delaying seeding (Taylor et al, 1983). A lower soil pH (5.6) had significantly higher yields than a higher soil pH (6.2) due to liming (Taylor et al., 1983). Plants receiving NH_4Cl had significantly higher yields than those given either $(NH_4)2SO_4$ or $Ca(NO_3)_2$ (Taylor et al, 1983). The combination of NH_4Cl + KCl + P, late seeding date, and lower pH seemed to be superior (Taylor et al., 1983).

Christianson et al., 1987, felt that, theoretically, disease may be minimal at an NH_4^+ to NO_3^- soil ratio that is best suited to the plant. It has been shown in Oregon, however, that NH_4^+ fertilizers, low soil pH (5.5), and use of chloride-containing fertilizer resulted in the highest NH_4^+ to NO_3^- soil ratio, lowest disease severity, and highest grain yields (Christianson et al., 1987).

In the United States, much of this work has been conducted in the midwest and West where growing conditions, soils, and cropping practices are different from those here in Virginia. Typically the mid-western and western wheat growing regions use anhydrous ammonia fertilizer at approximately 120 pounds of nitrogen per acre in a single application, and expect approximately 50 bushels per acre. Virginia winter wheat growers typically strive for a total of 80-100 bushels of wheat per acre, and have learned that wheat responds to aggressive management. Approximately 120 total pounds of nitrogen per acre are applied over the growing season. Typically, 15-20 pounds are applied at planting with two more split applications of approximately 40-60 pounds each. The number of tillers are determined in January or February, and the number of pounds of nitrogen to be applied are determined. Fewer tillers mean more nitrogen should be used, with the grower striving for 5-8 tillers for optimal yield. The final fertilizer application occurs just prior to growth stage 30. A 30% UAN (urea-ammonium nitrate) solution is generally used because it is less expensive than other forms of nitrogen fertilizers.

Huber and McCay-Buis (1993) found that soybeans in a double-cropped field in which take-all had been present showed symptoms of manganese (Mn) deficiency. Tissue analysis and alleviation of the symptoms with a foliar manganese sulfate spray confirmed this. Soil tests indicated that adequate Mn was available. A ten-fold higher population of Mn-oxidizing organisms was found in the rhizosphere of deficient plants in the take-all soil. The pathogen, *G. graminis* var. *tritici* (*Ggt*) is also a Mn-oxidizer (Huber and McCay-Buis, 1993).

Huber and McCay-Buis (1993) feel that conditions which increase Mn availability (Mn^{++}) generally reduce the severity of take-all, while the reverse is also true. The activity of Mn as a cofactor in the shikimic acid pathway could be an aspect of its involvement in plant resistance, i.e., formation of phenolic compounds (Huber and McCay-Buis, 1993). Ammonium is involved in the formation of amino acid intermediates and shikimic acid pathway products which could be an aspect of its involvement in reduced take-all incidence and severity (Huber and McCay-Buis, 1993). Root development is positively correlated with Mn availability and ammoniacal nitrogen, possibly permitting disease escape and the exudation of siderophores by roots for the uptake of Mn (Huber and McCay-Buis, 1993). Seed containing high levels of Mn had increased seedling vigor, more extensive root systems, less severe disease, and consistently higher yields than seeds with lower Mn levels (Huber and McCay-Buis, 1993). Application of Mn as a seed treatment reduced take-all and increased yields (Huber and McCay-Buis, 1993). Broadcasting $MnS0_4$ requires high rates, and is readily oxidized by soil organisms, limiting the time of its availability, and foliar application of Mn is not effective below ground (Huber and McCay-Buis, 1993).

Table 1 is modified from that of Huber and McCay-Buis, 1993, showing overall conditions influencing take-all severity vs. nitrification and Mn availability.

Biological control. A review on biological control is presented in Ecology of Soil-borne Plant Pathogens, Prelude to Biological Control (Baker and Snyder, 1965). It has been noted that some soils have been considered to have a general or specific antagonism toward *Ggt* (Cook and Rovira, 1976). This group found that the antagonism

studied by them was effective at dilutions as high as 1 in 1000, was transferrable from soil to soil, was operable on or near wheat roots, is destroyed by 60C moist heat for 30 min or desiccation, and was fostered by wheat monoculture but may be lost during fallow or rotation, especially into legume or pasture crops (Cook and Rovira, 1976). They felt that fluorescent *Pseudomonas* bacteria were involved because: a) of 100 bacterial and actinomycete fungi tested for suppressive activity, the eight that had equal or greater activity than the suppressive soil were pseudomonads (seven were fluorescent), b) the temperatures tested would kill pseudomonads but not spore-forming bacteria, c) the numbers of fluorescent pseudomonads (10^4/g) were higher than a nearby conducive soil (10/g), d) "protected" roots had asporogenous, Gram negative, small rod shaped bacteria, fitting the description of pseudomonads, e) these were also noted using scanning electron microscopy, f) fluorescent pseudomonads are common inhabitants of the rhizosphere and rhizoplane, g) dilution plate counts using a selective medium indicated that fluorescent pseudomonads are 100 to 1000 times more numerous on diseased roots, and h) it has been shown that 70% of fluorescent pseudomonads isolated from soil are suppressive to *Ggt* compared to 20% of bacteria from non-selective media, and 7% of bacillus species (Cook and Rovira, 1976). In 1983, Weller studied the colonization of wheat roots by a fluorescent pseudomonad, strain 2-79 (PF 2-79) (Weller, 1983). Winter wheat seed were colonized with 10^8 CFU/per seed, and were sown in the field. Greater than 10^6 CFU of the bacterium per 0.1 g root were present up to 1 month after planting, and the bacteria were present along the entire length of the root (7 cm at 1 mo). PF 2-79 numbers declined slightly during the winter, but increased in the spring up until maturity of the

wheat. Numbers of PF 2-79 were higher on roots colonized with *Ggt*, and aggressively competed with native bacteria on wheat roots. Virtually 100% of the bacteria on the primary (seminal) roots were PF 2-79, while only 10% of the bacteria on the secondary (nodal,crown) roots were PF 2-79.

Weller and Cook in 1983 applied *P. fluorescens* Migula strain 2-79 and 13-79 alone and in combination to wheat seed. The combination of PF 2-79 and PF 13-79 was more suppressive than PF 13-79 alone, and slightly more suppressive than PF 2-79 alone in three of six field tests. Suppression of take-all by the bacteria was expressed as fewer plants with foliage symptoms, taller plants, more heads, greater yield, and less root necrosis than plants from non-treated seed. It should be noted, however, that the health of the plants grown from bacteria-treated seed and *Ggt* inoculated more closely resembled that of the *Ggt* inoculated non-bacteria treated control than the non-*Ggt* infested non-bacteria inoculated control. In other words, bacteria-treated plants still were not nearly as healthy as the healthy, non-challenged control plants. The population of bacteria on the seed was stable for 21 days at 5C or 15C, but declined rapidly at 25C (Weller and Cook, 1983).

In 1988, Thomashow and Weller showed that PF strain 2-79 produced the antibiotic phenazine-1-carboxylate (PCA) which is active against *Ggt*. Six mutants defective in phenazine synthesis (Phz^-), derived by Tn5 insertion, were non-inhibitory to *Ggt* in vitro, and provided significantly less control than PF 2-79 on wheat seedlings ($P \leq 0.05$). Bull, Weller, and Thomashaw in 1991 demonstrated for the first time that phenazine was a major factor in the suppression of *Ggt* citing that PF 2-79-B46 (Phz^-)

was unable to suppress lesion formation. There was not an inverse correlation between the population size on seeds or roots, and number of root lesions (Bull, Weller, and Thomashaw,1991). Although PCA seems to be the major suppressive factor produced by *P. fluorescens*, minor factors, such as a fluorescent siderophore and another iron regulated factor, have a minor role (Ownley, Weller, and Thomashow, 1992). *In vitro*, suppression of *Ggt* by PF strain 2-79RN10 was greatest at pH 6.0-6.6, intermediate at pH 6.8-8.0, and least at pH 4.9-5.8. In soil, two *P. fluorescens* strains used significantly suppressed take-all at all pHs tested (4.9-8.0) (Ownley, Weller, and Thomashow, 1992).

In 1987, Howie, Cook, and Weller determined that *P. fluorescens* suppressive to take-all were distributed along the root at progressively lower cell densities with increasing distance from the seed (10^7-10^3 over the root length). Bacteria were not washed down the root due to the experimental system, and motility was not responsible for the downward colonization of roots due to the use of three non-motile mutants.

In 1993, Hornby et al. assayed the effectiveness of two bacilli (*B. cereus* var. *mycoides* Flugge and *B. pumilis* Meyer and Gottheil) and two *P. fluorescens* isolates. The bacilli added as soil drenches in Autumn or Spring were usually ineffective. Of those in which an affect was noted, half were associated with a disease increase, the other half with a disease decrease. The two pseudomonads applied as a seed treatment were ineffective (Hornby et al., 1993).

In 1985, Weller and Zhang developed an assay to rapidly screen bacteria for their ability to suppress take-all. The assay involved the use of tapered plastic tubes (2.5 X 16.5 cm). Five grams *Ggt* infested soil was placed onto a 6.5 cm column of vermiculite.

Bacteria-infested seed were placed on top and then covered with 1.5 cm vermiculite. Plants were assayed for root damage after 3-4 weeks. Of the 121 *Pseudomonas fluorescens* strains tested, 71 and 41% were suppressive in a Puget silt loam at 0.15 and 0.45% inoculum level respectively (oat kernals on a w/w basis). A significantly larger number ($P \leq 0.05$) of the isolates were suppressive if their origin was from suppressive soil wheat roots.

Ryder, Brisbane, and Rovira isolated bacteria from the rhizospheres of wheat plants, and tested them in a glasshouse assay (Ryder, Brisbane, and Rovira, 1992). Both *Ggt* -inhibitory and non-inhibitory *in vitro* isolates were tested in pots containing a non-sterilized soil-sand mixture. One of a group of non-fluorescent pseudomonads, *Pseudomonas corrugata* Roberts and Scarlett emend. Sutra et al., suppressed disease and stimulated plant growth.

More recent studies show that using combinations of bacterial isolates is probably the most effective method for controlling take-all. Duffy and Weller used many different fluorescent pseudomonads alone and in combination, and found the combination to be best (Duffy and Weller, 1995). Pierson and Weller have also used combinations (Pierson and Weller, 1994). Duffy et al. similarly found that combinations of fluorescent pseudomonads and the fungus *Trichoderma koningii* Oudemans were more effective than several bacterial combinations (Duffy et al. 1996).

Antifungal agents. Fungicides and other compounds have been used to suppress and or kill fungi. Some of these compounds have also been used as molecular markers. Listed are major groups of antifungal agents based on mode of action (Ware, 1989).

Inhibitors of electron transport. Sulfur, Organotins such as fentin hydroxide, Oxathiins such as carboxin; Dinitrophenols such as dinocap.

Enzyme inhibitors. Copper; Mercury; Dithiocarbamates such as maneb, ferbam, manzate, and dithane; Thiazoles, such as ethazole; substituted aromatics such as chlorothalonil, and PCNB; Dicarboximides, such as captan and folpet; imides, such as iprodione and vinclozolin; Quinones, such as dichlone and chloranil.

Inhibitors of nucleic acid metabolism and protein synthesis. Benzimidazoles such as benomyl, thiabendazole, and carbendazim; Antibiotics such as cycloheximide and streptomycin; aliphatic nitrogens such as dodine; Triazines, such as anilazine; Phenylamides such as metalaxyl.

Sterol synthesis inhibitors. Imidazoles, such as imazalil and prochloraz; Triazoles, such as difenoconazole, triadimafon, propiconazole and myclobutanil; Pyrimadines, such as ethirimol; the Piperazine, triforine; Morpholines, such as tridemorph and fenpropimorph;

Several chemicals used in this study include benomyl, nystatin, and cycloheximide. Benomyl is a known mitotic inhibitor meaning benomyl (actually its breakdown product, MBC) does not allow microtubules to effectively operate which is necessary for mitosis. Fungal resistance to benomyl is conferred by single base changes in the beta-tubulin gene, thus altering the amino acids which make up beta-tubulin (Yarden and Katan, 1993).

Nystatin, a polyene antibiotic, requires the presence of sterol in the membrane for toxicity. It is hypothesized that pores are created in membranes by multiple polyene

molecules forming hydrogen bonds with sterol molecules in the membrane. Additional hydroxyl groups of the polyene molecules face toward each other forming a pore, and the amino sugar found on each molecule is pushed inward (inside the cell) or outward (toward the cell wall) away from the membrane. Cells are killed by the subsequent leakage of cellular constituents (Holz, 1979, and Pratt and Fekety, 1986). Nystatin resistant fungi have a marked decrease in their ergosterol content (Vanden Bossche, 1997). Beezer (1986), however, suggests that resistance may involve changes in cell wall components, specifically the amino-acid content and fatty acid chain length.

Cycloheximide, specific for cytosolic ribosomes, is a protein synthesis inhibitor (Oleinick, 1975). Protein production is inhibited by preventing the amino acids bound to RNA into protein (Sisler and Siegel, 1967). Resistance is conferred by one to many genes (Sisler and Siegel, 1967).

Research Objectives

1) Develop a greenhouse bioassay to evaluate the effectiveness of agents to suppress take-all of soft red winter wheat caused by *Ggt*.

2) Determine the ability of a bioassay to predict field activity of agents to suppress take-all of soft red winter wheat caused by *Ggt*.

3) Determine the efficacy of i) mineral management (nitrogen source and reduced manganese), ii) Registered chemicals and the experimental chemical Mon 65500 and iii) three bacterial isolates to suppress take-all of soft red winter wheat caused by *Ggt*.

4) Determine any differences that may exist among selected isolates of *Gaeumannomyces graminis* varieties through a bioassay and growth studies.

Table 1. Influence of soil conditions on take-all*.

Condition	Nitrification	Mn availability	Take-all
Liming	Increase	Decrease	Increase
Manure	Increase	Decrease	Increase
Loose seedbed	Increase	Decrease	Increase
Soybean prior	Increase	Decrease	Increase
Alfalfa prior	Increase	Decrease	Increase
NO_3^-	X	Decrease	Increase
Short monocrop	X	Decrease	Increase
Plant stress	X	Decrease	Increase
Early seeding	X	Decrease	Increase
High moisture	Increase	Decrease	Increase
Alkaline pH	Increase	Decrease	Increase
Nitrif. inhib.	Decrease	Increase	Decrease
Acid pH	Decrease	Increase	Decrease
Oats prior	X	Increase	Decrease
NH_4^+	X	Increase	Decrease
Tolerant cult.	X	Increase	Decrease
Late seeding	X	Increase	Decrease
Lupine prior	Decrease	Increase	Decrease
Cl^-	Decrease	Increase	Decrease
Firm seed bed	Decrease	Increase	Decrease

*modified from Huber and McCay-Buis 1993 .

XUnknown or effect is dependent on environmental conditions

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CHAPTER 2

Mineral and Chemical Management of Take-all in a Virginia Soil

J. B. Crozier[1], E. L. Stromberg[1], and M. M. Alley[2]. [1]Department of Plant Pathology, Physiology, and Weed Science, Virginia Polytechnic Institute and State University, Blacksburg, Virginia, 24061-0331. [2]Department of Crop Soil and Environmental Sciences, Virginia Polytechnic Institute and State University, Blacksburg, Virginia, 24061-0403.

ABSTRACT

Take-all of wheat, caused by the fungus *Gaeumannomyces graminis* var. *tritici* (*Ggt*) is a problem wherever wheat is grown. Take-all has been observed in many of the intensively managed fields in Virginia. Crop rotation is currently the only recognized method for control of this pathogen. Our objective was to determine the effectiveness of nitrogen source, reduced manganese, and several common registered chemicals on the suppression of take-all of wheat in Virginia. Ammonium and manganese were evaluated in a greenhouse and in the field for disease suppression. 'Jackson' soft red, winter wheat seeds were planted in the greenhouse in plastic cones in a Kempsville loam field soil (fine-loamy, siliceous, thermic Typic Hapludult) containing millet seed infested with *Ggt* or sterile millet seed. Plants were grown approximately one month, and watered with distilled water as needed. A root necrosis rating, and root and dry shoot weight were determined as a measure of disease severity. Root necrosis developed in all plants in

artificially *Ggt*-infested soil. Contrary to published data from other regions in the USA, plants did not significantly gain root and shoot weight due to either ammonium or manganese in the presence of the pathogen, ($P \leq 0.05$), nor was root necrosis consistently affected by treatment. In the field over three seasons using a randomized complete block design, ammonium sulfate and manganese did not consistently suppress take-all (significant differences at the 0.1 confidence level). Four common registered chemicals tested in the field did not statistically significantly control take-all at the 0.1 confidence level in two out of three seasons.

INTRODUCTION

Take-all of wheat (*Triticum aestivum* L.), caused by the ascomycete fungus *Gaeumannomyces graminis* (Sacc.) Arx and Oliver var. *tritici* Walker (= *Ophiobolus graminis* Sacc.) is a problem where wheat is grown, and may be the most important root disease of wheat world-wide (Cook and Rovira, 1976; Asher and Shipton, 1981; Weller and Cook 1983; and Huber and McCay-Buis, 1993). Penetration of wheat roots is mechanical by the formation of hyphopodia (Nilsson, 1969). As might be expected, young roots are easily infected and colonized, whereas the process is delayed or impeded by root maturation (Nilsson, 1969). Plants may be penetrated and colonized at any stage of growth, however (Huber and McCay-Buis, 1993), and yield losses are due to the reduction of water and nutrient movement as the root tissue is colonized (Colbach et al., 1997).

Effort to determine if fertilizers predominantly containing the ammonium ion, or if reduced manganese may suppress take-all have been reported in various studies (Asher

and Shipton, 1981; Taylor et al, 1983; Christianson et. al., 1987; Cotterill and Sivasithamparam, 1988; Werker and Gilligan, 1990; Huber and McCay-Buis, 1993; and Colbach, Lucas, and Meynard, 1997). Researchers report that ammonium ions may stimulate antagonistic miroflora or lower pH, making manganese more available (Colbach, Lucas, and Meynard, 1997). Manganese may play a role in suppression of disease in that manganese is a cofactor in the shikimic acid pathway leading to antifungal phenolic compounds (Huber and McCay-Buis, 1993). Huber and McCay-Buis (1993) reported that few diseases respond as dramatically as take-all to nutrition, with ammonium nitrogen decreasing disease severity. Huber gave twenty-eight instances where ammonium-nitrogen reduced take-all, twenty-two where nitrate nitrogen increased take-all, and eight cases where nitrate-nitrogen reduced yield-loss. (Asher and Shipton, 1981).

Dr. B. Cunfer (Plant pathologist, University of Georgia) stated that in the Southeast, take-all has become a problem, possibly due to the double-cropping of soybeans (*Glycine max* (L.) Merrill.) (Hollis, 1998). Double-cropping with soybeans is not a true rotation, and his work suggests that soybeans maintain the fungus well (Hollis, 1998). The closely related variety *Gaeumannomyces graminis* var. *graminis* has been isolated from soybean pods (Roy et al., 1982) Virginia growers have increased acreage of intensively managed wheat, made common the practice of double-cropping with soybeans, and have noted an increased presence of take-all in Virginia's intensively managed wheat fields. Virginia growers already under normal intensive management practices use approximately 120 lbs of nitrogen on a winter wheat crop over an entire

season. A 30% urea-ammonium nitrate (UAN) solution is often the fertilizer source of choice. The ammonium ion is immediately available from ammonium nitrate (approximately 42% of total UAN solution by weight) and ammonium ions are eventually liberated by the microbial breakdown of urea (approximately 33% of total UAN solution by weight).

A greenhouse bioassay was developed and field studies were conducted to determine the effectiveness of the ammonium ion and manganese on the suppression of take-all in a typical Virginia coastal plain sandy loam soil (Kempsville loam). Current ammonium concentrations have not effectively suppressed take-all in Virginia under the current management level. In the field, common registered chemicals, difenoconazole, triadimenol/captan, tebuconazole/thiram, and carboxin, were also tested in combination with the two nitrogen sources and presence or absence of manganese.

MATERIALS AND METHODS

Bioassay

Soil. A sample of Kempsville loam soil (Kempsville loam, fine-loamy, siliceous, thermic Typic Hapludult) typical of soils used for wheat production was obtained from a freshly plowed field (Robinette and Hoppe, 1982). The soil was dried, sieved through a 0.7 cm (0.25 inch), mixed for 5 min and prior to further use. The soil was analyzed at A & L Eastern Agricultural Laboratories (7621 Whitepine Road, Richmond VA 23237). Selected chemical and physical properties are shown in Table 1. Calcium phosphate monobasic and potassium chloride were mixed into the soil for five min (25 μg P /g soil

and 25 µg K /g soil). Fifteen grams of calcium carbonate per 30 kg soil were added to raise the pH to approximately 6.5.

Inoculum. Autoclaved German Foxtail millet (*Setaria italica* L) seed (152 g) in a 1000 ml flask was inoculated with ten, 8mm discs of fungal mycelium from the leading edge of colonies grown on 20 ml potato dextrose agar (PDA). Millet inoculum was incubated at 25 C for 30d with mixing once after 7d. Individual *Ggt* -infested millet seeds were capable of producing approximately 13.6 mm growth after 2 d or 32.8 mm growth after 4 d on 20 ml PDA (average radial growth from ten seeds at 25 C). One hundred percent of the infested millet seeds developed a colony. At the time of planting, either sterile millet or *Ggt* -infested millet were mixed into the soil at a rate of 10 g millet inoculum per 1 kg air dried soil. A cotton ball was inserted into the bottom of each Conetainer® [Ray Leach Conetainers®, Portland, Oregon (4 X 21 cm)], and 150 g soil mix was added to each cone.

Solutions and planting. Mineral solutions were prepared such that 40 ml of solution could be added to 150g mixed soil in each cone to yield the desired µg N/g soil concentration. The sulfate ion was balanced for each appropriate test with calcium sulfate. 'Jackson' soft red winter wheat seeds were planted in cones in 150g of the air-dried Kempsville loam coastal plain soil artificially infested with *Ggt* - or sterile- millet (*S. italica* L.) seed inoculum (10g inoculum / kg soil). An initial 40ml watering with an NH_4NO_3, $(NH_4)_2SO_4$, or a $Ca(NO_3)_2$ solution yielded 30 µg N/g soil. . Ammonium nitrate at 30µg N / g soil was used for all greenhouse bioassay tests because this was a realistic nitrogen rate with a source that contains both ammonium and nitrate. Thirty µg

N / g soil (30 ppm) is the equivalent of 60 lbs nitrogen per acre. This is half of the total nitrogen normally used on a Virginia winter wheat crop. Since the bioassay only tested plants against the pathogen for one month, there certainly could be differences in the field, but the purpose of the bioassay was to quickly determine any potential treatment effects. Plants were watered with $MnSO_4$ at a given concentration if testing the effect of manganese on the suppression of take-all in an NH_4NO_3 solution yielding 30 µg N/g soil. Wheat seeds were planted approximately 1.5 cm deep and the hole was covered with soil.

Greenhouse and treatments. Plants were grown in a greenhouse with an average temperature of approximately 24 C with a one-recorded high of 29.0 C and low of 16.0 C. Lighting was aided with artificial lights on a 12 hour cycle (Sylvania Lighting Equipment, 21 Penn Street, Fall River, MA 02724, 1000 Watt, lamp type M47). Light intensity was approximately 143.2 µmol $M^{-2}S^{-1}$ PAR (945 ft-c) under lower lighting conditions, and 193.8 µmol $M^{-2}S^{-1}$ PAR (1279 ft-c) under higher lighting conditions. Plants were grown for one month and were watered with 10ml distilled water as needed such that no water was leached from the soil. Tests were performed using a completely randomized block design with seven replications. Plants were thinned to one plant per cone. Plots contained *Ggt* millet inoculum or sterile inoculum at 20, 30, or 40 µg N/g soil nitrogen from ammonium sulfate, ammonium nitrate, or calcium nitrate, and 30 or 150 µg manganese/g soil adding 30 µg N/g soil from ammonium nitrate.

Data collection. Plants were removed intact from cones, and the soil was rinsed from the roots. Roots were excised and placed into 70% ethanol, and shoots were placed into paper bags. Roots were given a root necrosis severity rating (0 = no lesions, 1 = one

to several lesions on roots, 2 = extensive lesions on roots, or several entire roots necrotic, 3 = lesions on roots as well as any darkening of crown, 4 = extensive darkening of crown, 5 = plant dead). Roots were then weighed after being blotted with paper towels and air dried for 10-12 min, and in some cases scanned (HP ScanJet 3C, Hewlett Packard, Boise, Idaho 83707) to obtain root area (Analyzer, 1991, non-commercial software). Shoots were dried at 70 C overnight and weighed after cooling to room temperature.

Field plots

Field tests were conducted for three seasons on the Kempsville soil at the Eastern Virginia Agricultural Research and Extension Center (EVAREC), Warsaw, Virginia. Plots were 25 ft long (7.62 m) x 49 inches wide (1.25 m) (6, 7 inch (18 cm) rows). Chemicals used in combination with field mineral treatments included: Vitavax® (carboxin, Uniroyal Chemical Company, Middlebury, CT 06749), Dividend® (difenoconazole, Novartis, Greensboro NC 27419), Raxil-Thiram® (tebuconazole and thiram, Gustafson, Dallas, TX 75266), Baytan® (triadimenol, Gustafson), and Captan 400® (captan, Gustafson). Seed was treated with a motorized centrifuge-type seed treater (Hans-Urich Hege Maschinenbau, D7112 Waldenburg/Wurttemberg, Hohebuch, Germany).

RESULTS

Greenhouse Bioassay

Nitrogen source. The data obtained through the greenhouse bioassay tests indicate that under these conditions, take-all as measured by disease incidence and

severity, shoot weight, and root weight, was not affected by nitrogen source or with the addition of manganese (Tables 2-4). Plants grew better when an ammonium containing fertilizer was used (ammonium sulfate or ammonium nitrate) regardless of the presence of the pathogen. (Tables 2 & 3). Calcium nitrate yielded smaller shoots and less dense roots. From these data it is clear that under these conditions, ammonium nitrogen from ammonium sulfate or ammonium nitrate allowed for a trend in better overall plant growth than did nitrate nitrogen from calcium nitrate regardless of the presence of the pathogen.

Root necrosis in all of the nutrients studied was low to moderate (ranged from approximately 1.0 to almost 4.0). In two cases in the greenhouse tests (calcium nitrate 20 and 40, ammonium nitrate 20 and 40, and ammonium sulfate 20 and 40) root necrosis was worse due to the use of nitrate (Table 2). In the following two tests (Table 3) in the presence of ammonium nitrate at 30µg N / g soil, root necrosis, root area, and root weight were not affected by the source of nitrogen. Only shoot weight was better in the second test, but again, this was also the case in the absence of the pathogen.

It should be noted that under controlled greenhouse conditions, *Gaeumannomyces graminis* caused root disease as expected (Tables 2-4). Average root weights were lower in plants which were exposed to the pathogen than in those that were not exposed to the pathogen. Interestingly, average shoot weights were variable among tests regardless of the presence or absence of the pathogen under light disease pressure (Test 2, Table 3). Under greater disease pressure (Test 1, Table 3) average shoot weights were generally smaller in the presence of the pathogen except for calcium nitrate-fertilized plants.

Under heavy disease pressure (Test 3, Table 4) both root weights and shoot weights were much lower in the presence of the pathogen.

Manganese. Necrosis was significantly worse with the use of manganese at 150 μg Mn / g soil (Table 4). This is a very unrealistic rate of manganese (300 lbs per acre). Manganese at the rate of 60 lbs per acre (30 μg Mn / g soil) had no affect on the suppression of take-all (Table 4). Only in one test was shoot weight significantly higher due to the presence of manganese, and root weight and root area were not affected or lowered the presence of manganese (Table 4).

Bioassay. The greenhouse bioassay was useful in determining the effectiveness of these mineral management strategies under controlled conditions. Rapidity was also a benefit, as numerous experiments are potentially possible in a single year regardless of temperature or weather conditions.

Field Tests

The yields in the first season were very high regardless of treatment (Table 5). Ironically, the largest yield (113 bu/acre) was from the calcium nitrate/no manganese/with difenoconazole plots while the lowest yields (105 bu/acre) came from the ammonium sulfate/with manganese/tebuconazole and thiram plots. For seasons one and two, there were statistically significant differences among several treatments at an alpha level 0.1 whereas in season three, no differences were present. There were no consistent results regarding nitrogen source. Using a factorial analysis to pool the data, nitrogen source was not significant in any of the three seasons. In two out of the three seasons (seasons one and three), the highest yields were from plots fertilized with

calcium nitrate. The factorial analysis also indicated that neither level of manganese (either with or without) affected yield. There were few differences among the four chemical treatments in the three seasons of field tests at an alpha level of 0.1 (Table 6). Plots planted with difenoconazole-treated seed significantly out-yielded plots planted with carboxin-treated seed in season one. Difenoconazole-plot yields as well as carboxin-plot yields were not significantly different from triadimenol/captan and tebuconazole/thiram plot yields. In seasons two and three, there were no significant differences among chemical-treated plot yields.

The number of whiteheads per plot, an indication of root health, was determined in the first season. The number of whiteheads ranged from 5 to 200, and all but two of the plots had fewer than approximately 18 whiteheads per plot. This indicates that the pathogen was scattered widely across the field in patches. Even at 200 whiteheads per plot there was not a significant average yield loss. The area affected was determined in seasons two and three. Again, there were no significant differences among plots. A large F-statistic in seasons two and three indicate that there were differences among the plots (within the same treatment). Although this is not a statistically significant finding, the results indicate the patchy nature of the pathogen in the field.

DISCUSSION

Nitrogen source. Throughout the literature it has been suggested that specific forms of nitrogen have different affects on the severity of take-all (Asher and Shipton, 1981; Taylor et al, 1983; Christianson et al., 1987; Werker and Gilligan, 1990; and Huber and McCay-Buis, 1993; Colbach et al., 1997). Researchers have suggested that nitrate

nitrogen may allow an increase in take-all severity while ammoniacal nitrogen may decrease take-all severity. Much of this work has been conducted in the mid-western and western United States where growing conditions, soils, and cropping practices are different from those in Virginia and the mid-Atlantic region.

Under controlled greenhouse conditions, *Gaeumannomyces graminis* could effectively infect, colonize, and cause disease in wheat. Regardless of the presence of the pathogen, a trend toward greater yields was noted with the use of ammonium containing fertilizers. As would be expected with a root pathogen, greater root damage as noted by root necrosis ratings resulted in less dense roots and less shoot mass. Under conditions in which there was less root damage, root weights were still smaller than from non-pathogen exposed plants, but shoot weights were variable. This indicates that under lighter disease pressure the plant response may be to compensate for a mild root reduction by increasing shoot and leaf area.

Current management practices. Virginia's winter wheat growers typically strive for 80-100 bushels of wheat per acre, and have learned that wheat responds to aggressive management. Approximately 120 total pounds of nitrogen per acre are applied over the growing season. Typically 15-20 pounds of nitrogen are applied at planting with two split applications of approximately 40-60 pounds each applied in late winter and early spring. A 30% UAN solution is generally used because it is a lower cost fertilizer.

In Indiana, wheat is currently fertilized with approximately 90-100 lb UAN (urea-ammonium nitrate) added at the breaking of dormancy and strive for 80-100 bu/acre (C.

Mansfield, Purdue University Extension Agronomist, personal communication). In Huber and McCay-Buis' review (1993) which described the benefit of ammoniacal fertilizers, it was shown that at the highest rate of 118 lb N per acre as anhydrous ammonia yielded only approximately 39-69 bu/acre depending on soil type. This indicates that even with an ammoniacal nitrogen source, in the mid-west, any reduction of take-all symptoms is still not increasing yields to current intensive management standards. In an earlier paper (Huber et al., 1968) it was shown that under dryland conditions in Idaho using 40 lb/acre ammonium sulfate in the fall, 58 bu/acre per acre were achieved. The take-all index was lower with the use of ammonium sulfate, but this was against the use of no fertilizer at all and a yield of 28 bu/acre. Under irrigation in Idaho, all ammonium sulfate was added at once (0-300 lbs/acre) in the fall. Whiteheads decreased at 120 lbs N, but overall there were no differences in the yields which were very high. Also, high yields were obtained even though there were 40-45% whiteheads and the roots in all treatments looked very necrotic. Ammonium sulfate only reduced whiteheads where the N used exceeded 180 lbs. In an Australian report by Cotterill and Sivasithamparam (1988), it is noted that in the presence of no fertilizer, ammonium sulfate, or ammonium nitrate, no significant differences were obtained for incidence,take-all severity, or thousand kernel weight in one study while the use of nitrogen increased dry shoot weight in a pot experiment. The studies indicated relate the differences in past research and current Virginia intensive wheat management.

Due to Virginia's increased acreage of intensively managed wheat and an increased presence of take-all in Virginia's intensively managed wheat fields, it was felt

that it was of much importance to determine the suppressiveness of the ammonium ion and manganese in a Virginia soil and under Virginia management practices. Many of Virginia's growers double-crop soybeans, and therefore do not rotate out of wheat or barley (*Hordeum vulgare* L. emend Bowden, possibly setting up conducive conditions for take-all. Rotation is, however, the only recommended control for take-all. Work by Cunfer also suggests that rotation is extremely important in the control of take-all (Hollis, 1998). It has been noted that soybeans maintain the fungus well (Hollis, 1998). It is possible that the common practice of double-cropping with soybeans is maintaining high levels of fungal inoculum.

Our work does not support the work of others that suggest that ammonium and manganese suppress take-all. The source of nitrogen used by Virginia growers for winter wheat is already routinely from a 30% UAN solution, which contains ammonium nitrate at approximately 42% (by weight), and urea at approximately 33%, with the remaining component as water. Both field tests and greenhouse tests (at the equivalent of 60 lbs N per acre) indicated that in this soil, take-all was not suppressed effectively or consistently by ammonium or manganese.

Nitrogen uptake by wheat. In the literature it has been shown that wheat usually grows better with mixtures of NH_4^+ and NO_3^- (Wang and Below, 1992; Feil, 1994). There is little information about the variation in the NH_4^+ NO_3^-uptake ratio between cereal cultivars, but a screening of sixteen German spring wheat cultivars in a 1:1 mixture of NH_4^+ and NO_3^- showed diversity in this trait (Feil, 1994). In a study conducted be Feil, (1994), cv. Star clearly favored NH_4^+ over NO_3^-, while cv. Remus

exhibited a more balanced uptake ratio. In studies conducted by Wang and Below (1992, 1998), it was noted that the cultivars tested produced more dry weight, tillers, and accumulated more N when grown with mixed N that either NH_4^+ or NO_3^- alone. Plants grown with mixed N or with NH_4^+ only had higher shoot to root ratios than those grown with NO_3^- alone (Wang and Below,1998). Even in a study by Cotterill and Sivasithamparam (1988), it is noted that both ammonium sulfate and ammonium nitrate were equally effective in their study.

It is our contention that in this soil under the controlled conditions of the greenhouse, 'Jackson' soft red winter wheat responded better to ammoniacal nitrogen not because of suppression of the pathogen, but possibly due to a physiological ability to gain more shoot and root weight in the presence of ammoniacal nitrogen whether from ammonium sulfate or ammonium nitrate.

We feel that the past results concerning the suppression of take-all by nitrogen source or by manganese needs to be revisited. As greater numbers of wheat growers intensively manage their wheat, with greater fertilizer inputs and greater yields, it is imperative to re-evaluate past results concerning the actual effectiveness of nitrogen source on take-all.

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Table 1. Selected chemical and physical properties of a Kempsville loam (fine-loamy, siliceous, thermic Typic Hapludult) soil utilized in greenhouse bioassays.

Parameter	Analyses	Method†
Textural Classification	Sandy Loam	
Sand %	59.7‡	
Silt %	24.7	
Clay %	15.7	
Organic matter %	1.4	29-3.5.2
CEC (meq/100g)	3.9	(sum of cations)
pH	5.7	12-2.6, 12-3.4.4
NO_3 (µg/g)	9.0	33-3.2.1, 33-8.3
P (µg/g)	49.0 (Bray 1)	24-5.1
K (µg/g)	91.0	13-3.5.2
Mn(µg/g)	25.7	19-3.4

†All methods are listed in Methods of Soil Analyses Part 2. 1982. Chemical and Microbiological Properties 2nd Edition. American Society of Agronomy and Soil Science Society of America.

‡ Average of three separately analyzed samples

Table 2. Nitrogen source and rate influence on the severity of take-all in a greenhouse experiment.

Treatment	Root Necrosis Rating (0-5)‡	Root Area (cm^2)	Root Weight (mg)	Shoot Weight (mg)
AN20 +†	2.0 b*	0.210 b	50.1 ab	57.9 a
AS20 +	2.1 ab	0.221 b	57.5 a	66.8 a
CN20 +	2.5 a	0.147 a	30.7 b	41.8 b
AN30 +	2.1 a	0.191 ab	58.6 a	68.0 b
AS30 +	2.1 a	0.259 b	71.2 a	78.4 a
CN30 +	2.0 a	0.159 a	38.4 b	50.1 c
AN40 +	2.0 b	0.197 ab	56.3 ab	65.3 a
AS40 +	1.9 b	0.257 b	69.0 a	75.5 a
CN40 +	2.6 a	0.172 a	38.0 b	46.0 b

*Treatment means in each column within the same test followed by the same letter are not significantly different at the 0.05 confidence level. Note that each concentration was analyzed separately.

† AN = ammonium nitrate, AS = ammonium sulfate, CN = calcium nitrate, number = μg N/g soil, + = with *Ggt* millet inoculum.

‡ Rating: 0 = no root lesions, 1 = 1 to several root lesions, 2 = extensive root necrosis, 3 = lesions on roots as well as any darkening of crown, 4 = extensive darkening of crown, 5 = plant with extensive necrosis.

Table 3. Nitrogen source and the influence of the presence or absence of *Gaeumannomyces graminis* var. *tritici* on severity of take-all in a greenhouse experiment.

	Root Necrosis Rating (0-5) ‡		Root Area (cm^2)		Root Weight (mg)		Shoot Weight (mg)	
			Test #					
Treatment	1	2	1		2		1	2
AN30 +†	3.3 a*	1.3 a	0.314 a		62.3 a		70.5 a	69.2 b
AS30 +	3.0 a	1.0 a	0.289 a		61.2 a		69.6 a	79.6 a
CN30 +	3.6 a	1.0 a	0.266 a		60.0 a		61.0 a	59.4 b
AN30 -	0.1 a	0.7 a	0.607 a		74.3 a		84.5 a	69.2 b
AS30 -	0.1 a	0.6 a	0.569 a		71.7 a		90.5 a	77.5 a
CN30 -	0.3 a	0.6 a	0.586 a		76.7 a		58.7 b	56.8 c

* Treatment means in each column within the same test followed by the same letter are not significantly different at the 0.05 confidence level. Note that tests with and without artificial inoculum were analyzed seperately.

† AN = ammonium nitrate, AS = ammonium sulfate, CN = calcium nitrate, Mn = manganese, number = μg N or Mn/g soil, +=with *Ggt* millet inoculum, - = sterile millet inoculum.

‡ Rating: 0 =no root lesions, 1 = 1 to several root lesions, 2 = extensive root necrosis, 3 = lesions on roots as well as any darkening of crown, 4 = extensive darkening of crown, 5 = plant killed or nearly dead with extensive necrosis.

Table 4. Nitrogen source and the influence of manganese on the severity of take-all in a greenhouse experiment.

	Root necrosis rating (0-5) ‡			Root area (CM^2)			Root weight (mg)			Shoot weight (mg)		
	Test #											
	1	2	3	1		2	3	1		2	3	
AN30 +†	3.3 a	1.3 a	3.8 a	0.314 a		62.3 a	29.8 a	70.5 a		69.2 a	45.0 b	
AN30 Mn 30 +	3.3 a	0.5 a	3.1 a	0.252 a		38.2 a	41.2 a	72.3 a		52.3 a	59.4 a	
AN30 -	0.1a	0.7 a	0.5 a	0.607 b		74.3 a	48.1 a	86.6 a		69.2 a	71.8 a	
AN30 Mn 30 -	0.4a	0.7 a	0.5 a	0.516 a		72.7 a	36.3 a	75.9 a		74.9 a	67.0 a	
AN30 +	...	1.3 b	...	...		62.3 a	...	...		69.2 a	...	
AN30 Mn 150+	...	2.3 a	...	...		45.6 b	...	...		67.5 a	...	
AN30 -	...	0.7 b	...	...		74.3 a	...	...		69.2 a	...	
AN30 Mn 150-	...	1.7 a	...	...		62.1 a	...	...		73.5 a	...	

* Treatment means in each column within the same test followed by the same letter are not significantly different at the 0.05 confidence level. Note that tests with and without artificial inoculum were analyzed separately.

† AN = ammonium nitrate, Mn = Manganese, number = µg N or Mn / g soil, +=with *Ggt* millet inoculum, - = sterile millet inoculum, … = no data

‡ Rating: 0 =no root lesions, 1 = 1 to several root lesions, 2 = extensive root necrosis, 3 = lesions on roots as well as any darkening of crown, 4 = extensive darkening of crown, 5 = plant killed or nearly dead with extensive necrosis.

Table 5. The influence of nitrogen source, manganese, and chemical seed dressings for the suppression of take-all in three field experiments.

	Season 1	Season 2	Season 3
Treatment	Yield (bu/acre) 13.5% H_2O	Yield (bu/acre) 13.5% H_2O	Yield (bu/acre) 13.5% H_2O
AS +Mn + carboxin †	111 abc*	78 ab	70 a
AS +Mn + difenoconazole	110 abc	82 ab	74 a
AS +Mn + tebuconazole-thiram	105 abc	84 ab	70 a
AS +Mn + triadimenol + captan	108 c	87 a	72 a
AS -Mn + carboxin	109 abc	84 ab	74 a
AS -Mn + difenoconazole	110 abc	82 ab	75 a
AS -Mn + tebuconazole-thiram	111 abc	80 ab	73 a
AS -Mn + triadimenol + captan	113 a	84 ab	73 a
CN +Mn + carboxin	107 abc	75 b	77 a
CN +Mn + difenoconazole	112 abc	80 ab	74 a
CN +Mn + tebuconazole-thiram	110 abc	86 a	75 a
CN +Mn + triadimenol + captan	111 abc	79 ab	72 a
CN -Mn + carboxin	106 bc	86 a	71 a
CN -Mn + difenoconazole	113 ab	85 a	79 a
CN -Mn + tebuconazole-thiram	107 abc	84 ab	76 a
CN -Mn + triadimenol + captan	111 abc	80 ab	75 a

* Treatment means in each column within the same test followed by the same letter are not significantly different at the 0.1 confidence level.
†AS=ammonium sulfate, CN=calcium nitrate, Mn=manganese, carboxin = Vitavax, Uniroyal Chemical Co, Inc., difenoconazole = Dividend, Novartis, Raxil-Thiram = tebuconazole-thiram, Bayer, triadimenol = Baytan, Gustafson, Inc. Captan, Gustafsonm=, Inc.

Table 6. The influence of chemical seed dressings on the suppression of take-all in three field experiments.

	Season 1	Season 2	Season 3
Seed Treatment	Yield (bu/acre) 13.5% H_2O	Yield (bu/acre) 13.5% H_2O	Yield (bu/acre) 13.5% H_2O
Difenoconazole†	111 a*	82 a	75 a
Triadimenol/captan	111 ab	83 a	73 a
Tebuconazole/thiram	108 ab	83 a	74 a
Carboxin	107 b	81 a	73 a

* Treatment means in each column within the same test followed by the same letter are not significantly different at the 0.01 confidence level

† carboxin = Vitavax, Uniroyal Chemical Co, Inc., difenoconazole = Dividend, Novartis, Raxil-Thiram = tebuconazole-thiram, Bayer, triadimenol = Baytan, Gustafson, Inc. Captan, Gustafsonm=, Inc. .

CHAPTER 3

Evaluation of Chemical and Biological Seed Dressings for the Suppression of Take-all of Wheat

J. B. Crozier[1], E. L. Stromberg[1], G. H. Lacy[1], D. P. Roberts[2]. [1]Department of Plant Pathology, Physiology, and Weed Science, Virginia Polytechnic Institute and State University, Blacksburg, Virginia, 24061-0331. [2]Biocontrol of Plant Diseases Laboratory, USDA-ARS, Beltsville MD 20705-2350.

ABSTRACT

Take-all of wheat, caused by the fungus *Gaeumannomyces* var. *tritici* (*Ggt*) is a problem wherever wheat is grown. Take-all has been observed in many of the intensively managed wheat fields in Virginia. Crop rotation is the only method recommended for control of this pathogen. A greenhouse bioassay was developed to predict efficacy of performance in the field. An experimental chemical (MON 65500), registered chemicals, and biological control agents were tested either alone or in combination in the presence and absence of *Ggt*. MON 65500 was tested at four rates in a greenhouse for suppression of take-all. Treated 'Jackson' soft red winter wheat seeds were planted in plastic cones in Kempsville loam (fine-loamy, siliceous, thermic Typic Hapludult) field soil containing millet seed infested with *Ggt* or sterile millet seed. Plants were grown approximately one month and watered as needed with distilled water.

A root necrosis rating and root and a dry shoot weight were determined as a measure of disease severity. Root necrosis developed in all plants in *Ggt*-infested soil, but plants from seeds treated with MON 65500 and in combination with difenoconazole, had significantly greater root and shoot weight ($P \leq 0.05$). Root necrosis did not progress to the crown with MON 65500 treatments. In field trials, plants treated with MON 65500 out-yielded the control plots by 4262 kg/ha (significantly greater at $P \leq 0.05$). In the greenhouse, two *Bacillus* spp. isolates and one fluorescent Pseudomonad, shown to produce a zone of inhibition against *Ggt* in vitro, were used as a seed dressing or applied to cones as a drench. Root necrosis developed in all plants in *Ggt* -infested soil, but plants from bacteria-treated seeds gained statistically significantly more root and shoot weight ($P \leq 0.05$) in only one of several tests conducted. Six USDA-maintained bacterial isolates did not perform well in the greenhouse bioassay or in the field, and plants from Gustafson-product-treated seed, including several biocontrol agents, yielded only 3799.0 kg/ha (56 bu/acre) at best over two seasons in the field. The greenhouse bioassay was predictive of field results for those treatments tested in both locations.

INTRODUCTION

Take-all of wheat (*Triticum aestivum* L.) caused by the ascomycete fungus *Gaeumannomyces graminis* (Sacc.) Arx and Oliver var. *tritici* Walker (= *Ophiobolus graminis* Sacc.) occurs wherever wheat is grown and may be the most important root disease of wheat world-wide (Cook and Rovira, 1976, Asher and Shipton; 1981; Weller and Cook, 1983; Huber and McCay-Buis, 1993). Plants may become infected at any stage of growth and yield losses are due to the reduction of water transpiration and

nutrient movement (Huber and McCay-Buis, 1993; Colbach et al., 1997). Penetration of wheat roots is mechanical by the formation of hyphopodia (Nilsson, 1969). Young roots are easily infected and colonized, whereas the process is delayed or impeded by root maturation (Nilsson, 1969).

There has been an increased presence of take-all noticed in Virginia's intensively managed wheat fields. Virginia growers already under normal management practices use one of several chemical seed dressings, including Dividend® (difenoconazole, Novartis, Greensboro NC 27419) and Baytan® (triadimenol, Gustafson, Dallas, TX 75266) as well as Raxil-Thiram® (tebuconazole and thiram, Bayer, Kansas City, MO 64120) and Vitavax 200® (carboxin, Uniroyal Chemical Company, Middlebury, CT 06749). The Dividend® label indicates it is suppressive to take-all. Current chemical usage, however, does not control take-all in Virginia. A new product by Monsanto, MON 65500 (Monsanto Life Sciences Company, St. Louis, MO 63198), is specific to *Ggt* and has shown promise in China (Halsey et al., 1998).

It has been noted that some soils have been considered to have a specific antagonism toward *G. graminis* var. *tritici* (*Ggt*), and could be fostered by wheat monoculture but could be lost during fallow or rotation, especially into legume or pasture crops (Cook and Rovira, 1976). It was noted that fluorescent pseudomonad bacteria were involved in the suppression of take-all (Cook and Rovira, 1976).

Weller and Cook in 1983 applied *P. fluorescens* (Migula) strain 2-79 and 13-79 alone and in combination to wheat seed. The combination of PF 2-79 and PF 13-79 was more suppressive than PF 13-79 alone, and slightly more suppressive than PF 2-79 alone

in three of six field tests. The health of the plants grown from bacteria-treated seed and *Ggt* -inoculated treatments more closely resembled that of the *Ggt* –inoculated, non-bacteria treated control than the non-*Ggt* infested, non-bacteria inoculated control. The population of bacteria on the seed was stable for 21 days at 5C or 15C, but declined rapidly at 25C (Weller and Cook, 1983). Howie, Cook, and Weller noted that the numbers of bacteria decreased exponentially with increasing distance from the seed (Howie, Cook, and Weller, 1987). Weller and Zhang (1985) developed an assay to rapidly screen bacteria for their ability to suppress take-all and involved the use of tapered plastic tubes (2.5 X 16.5 cm).

In 1993, Hornby et al. assayed the effectiveness of two bacilli (*Bacillus cereus* var. *mycoides* Flugge and *B. pumilis* Meyer and Gottheil) and two *P. fluorescens* isolates. The Bacilli added as soil drenches in Autumn or Spring were usually ineffective. The seed treatments were also ineffective.

More recent studies show that using combinations of bacterial isolates is probably a more effective method for controlling take-all. Duffy and Weller (1995) as well as Pierson and Weller (1994), used many different fluorescent pseudomonads alone and in combination, and found that combinations were best. It has also been shown that pseudomonads can stimulate plant growth (Ryder and Rovira, 1992).

A greenhouse bioassay was developed and field studies were conducted to determine the efficacy of experimental and registered chemical seed dressings, as well as several bacterial isolates on the suppression of take-all in a Virginia soil. The experimental compound, MON 65500, had excellent greenhouse and field activity against

take-all while a registered chemical seed dressing, difenoconazole, suppressed *Ggt* very little. None of the biological agents tested effectively suppressed take-all in the greenhouse or in the field. Carboxymethylcellulose used in one test as a seed treatment in the greenhouse as well as in the field increased take-all severity.

MATERIALS AND METHODS

Microorganisms

Fungi. The isolate of *Ggt* used for the greenhouse inoculations was from Dr. Mathre, Montana State University, Boseman, Montana, 59717, and was grown on potato dextrose agar (PDA). The fungus was hyphal-tipped and stored both at 1 C in slants (PDA) and at –87 C in 15 % glycerol. For field inoculations, plants with take-all symptoms were taken from a naturally *Ggt*-infested field intended for use in this study (Eastern Virginia Agricultural Experiment Station, Warsaw VA), and an isolate of *Ggt* (hereafter called the Warsaw isolate) was obtained using the selective medium SM-Ggt3 (Juhnke et al., 1984), hyphal-tipped and stored as was the Montana isolate.

Biologicals. All of the bacteria used were obtained from the source listed in the tables (USDA or Gustafson). The USDA isolates were isolated from corn roots in Virginia *Bacillus.* sp isolate II originated as a contaminant on a plate in the laboratory which visibly suppressed *Ggt* mycelial growth. *Bacillus* sp. isolate 202-10 B and fluorescent pseudomonad 407-7 were obtained from wheat roots. Wheat plants were selected that were apparently healthy but surrounded by plants with severe take-all symptoms and thus were considered to have potentially *Ggt*-suppressive organisms associated with the roots. Plants were brought back to the lab in coolers. Most of the soil

was shaken from the roots, and root pieces were placed into sterile distilled water and shaken. Dilutions of the suspension were plated onto PDA, and single colonies were isolated and re-streaked. Each isolate was tested against *Ggt in vitro*, with *Bacillus.* sp. 202-10 B fluorescent pseudomonad 407-7 selected due to their ability to produce a zone of inhibition. Isolates supplied by Gustafson were Kodiak® (*B. subtilis*, Gustafson), *B. cereus* , and several experimental biological control agent candidates.

Greenhouse Bioassay

A sample of a Kempsville sandy loam soil typical of soils used for wheat production was obtained from a freshly plowed soil (Robinette and Hoppe, 1982). Selected chemical and physical properties are shown in Table 1. The soil was dried and sieved through a 0.7 cm (0.25 inch), mixed, and stored prior to the greenhouse experiments. Calcium phosphate monobasic and potassium chloride were mixed into the soil for 5 min prior use (25 µg P/g soil and 25 µg K/g soil) as well as fifteen grams of calcium carbonate per 30 kilograms soil to raise the pH to approximately 6.5.

Sterile German foxtail millet (*Setaria italica* L.) seed (152 g in a 1000 ml flask) was inoculated with ten, 8mm discs of *Ggt* grown on 20 ml PDA in 100 X 15 mm Petri dishes, and subsequently incubated at 25 C for one month. At the time of planting, either sterile millet or *Ggt*-infested millet was mixed into the soil at a rate of 10 g per 1 kg air dried soil. A cotton ball was inserted into the bottom of each Conetainer® [Ray Leach Conetainers®, Portland, Oregon, (4 x 21 cm)] and 150 g soil mix added to each cone.

An ammonium nitrate solution was prepared such that 30 ml of solution was added to 150 g the Kempsville loam soil in each cone to yield 30 µg N/g soil. Two

'Jackson' soft red winter wheat seeds were sown approximately 1.5 cm deep, and covered. Tests were performed using a completely randomized block design with seven replications. Plants were thinned to one plant per cone after approximately one week.

Plants were grown in the greenhouse at approximately 24 C with a one-recorded high of 29 C and low of 16 C. Lighting was aided with artificial lights on a 12 hour cycle (Sylvania Lighting Equipment, 21 Penn Street, Fall River, MA 02724, 1000 Watt, lamp type M47). Light intensity was approximately 143.2 µmol M^2S^{-1} PAR (945 ft-c) under lower lighting conditions, and 193.8 µmol $M^{-2}S^{-1}$ PAR (1279 ft-c) under higher lighting conditions. Plants were grown for one month and watered when needed with 10ml distilled water such that no water was leached from the soil.

Plants were removed intact from cones, and the soil was rinsed from the roots under running water. Roots were excised and placed into 70% ethanol, and shoots placed into paper bags. Roots were given a root necrosis severity rating (0 = no lesions, 1 = one to several lesions, 2 = extensive lesions, or several entire roots necrotic, 3 = lesions on roots and darkening of crown, 4 = extensive darkening of crown, 5 = plant dead). Roots were weighed after being blotted with paper towels and air-dried for 10-12 min. Shoots were, dried at 70 C overnight and weighed after cooling to room temperature.

Chemical analysis. Treatments included non-treated seed plots as well as chemically treated seed. MON 65500 was tested at four rates, 6.25, 12.5, 25.0, and 50.0 g ai/kg seed and with difenoconazole at rate of 12.5 g. Seeds were treated with a motorized centrifuge-type seed treater, Hans-Urich Hege Maschinenbau, D7112 Waldenburg/Wurttemberg, Hohebuch, Germany.

Biological agent analysis. Bacteria were grown overnight in potato dextrose broth (PDB) or tryptose soy broth (TSB), and centrifuged into a pellet. The cells were re-suspended into a total volume of 10 ml of the same broth. Cells were either counted visually with a hemocytometer or were diluted and plated onto PDA. Seeds were either shaken (10 g seed) in the concentrated bacterial suspension and planted two seeds per hole, or the suspension pipetted directly into the hole with two seeds. The hole was closed using sterile forceps.

In the single test in which seeds were coated with a peat carrier (USDA-maintained biologicals), a loopful of cells were transferred to 100 ml nutrient broth and incubated for 24 h at 30 C such that the concentration of cells was 10^8-10^9/ml. One-hundred milliliters nutrient broth culture was added to 200 g sterile peat and mixed. A 12% carboxymethylcellulose solution was prepared, and a slurry was prepared using 10 ml 12% carboxymethylcellulose solution to 20 g peat inoculum. The slurry was mixed with the wheat seeds to yield a uniform coating. Dry peat was used to bind the coating on each seed. Non-used seed was stored at 1C until counting could be performed (2 days).

Field trials

Plots and inoculum. Flasks of sterile millet seed were inoculated with the Virginia Warsaw isolate and allowed to colonize the millet for approximately one month. The *Ggt*-millet inoculum was dispensed along with the 'Jackson' soft red winter wheat seed at planting. Plots were 25 ft long (7.62 m) x 49 inches (1.25 m) wide (6, 7 inch (18 cm) rows), and 200 g *Ggt* inoculum was used in each plot. Plots were set up using a

completely randomized block design and seed was planted using a custom seed planter made by Almaco.

Chemical. Treatments included non-treated seed plots as well as chemically treated seed. MON 65500 was tested at four rates, 6.25, 12.5, 25.0, and 50.0 g ai/kg seed, and with difenoconazole at rate of 12.5 g and 26.0 ai/kg seed. The experimental chemical TTZ 804742J was also tested at 5.0 and 10.0 g ai/kg seed in season two. Gaucho® (imidacloprid, Bayer, Kansas City MO 64120) was used in all seed treatments in season two. Seeds were treated with a motorized centrifuge-type seed treater, Hans-Urich Hege Maschinenbau, D7112 Waldenburg/Wurttemberg, Hohebuch, Germany. Chemicals used for the Gustafson tests: imidacloprid, Raxil-Thiram® (tebuconazole and thiram, Bayer), Captan 400® (captan, Gustafson Inc., Dallas TX 75266), and Epic 500® (furmecyclox, Gustafson).

Biological. USDA-maintained bacterial isolates were grown as above and seed was coated with the peat/carboxymethylcellulose mixture with or without a biological agent. Similarly, seeds were treated with compounds and bacteria specified by Gustafson, Inc.

RESULTS

Greenhouse chemical study. In the greenhouse over three tests, MON 65500 treated seed yielded statistically significantly greater root and shoot weight and statistically significantly reduced root necrosis (Table 2). Disease incidence was 100% in all treatments with *Ggt*. Plants treated with MON 65500 developed necrotic roots in the presence of *Ggt*, but the first several millimeters of root tissue around the seed were still

white and non-necrotic. Some darkening of the root tissue did occur however (note root necrosis ratings, Table 2). Plants were healthier when the seed was treated with both MON 65500 and difenoconazole than when they were treated with difenoconazole alone (Table 3).

Greenhouse biologicals study. In the greenhouse, the six USDA-maintained bacterial isolates tested showed no statistically significant suppressiveness to take-all (Table 5). All plants in the greenhouse exhibited severe root necrosis (see root necrosis rating, Table 5). Though not statistically significant, it appeared that the peat and carboxymethylcellulose seed coating may have enhanced disease. Further testing should include carboxymethylcellulose-caoted seed and non-carboxymethylcellulose-coated seed to specifically tests if this coating statistically significantly enhances disease.

The *Bacillus.* sp. isolate BII, *B.* sp. isolate 202-10B and fluorescent pseudomonad 407-7 did not suppress take-all well in the greenhouse. In each case in the single significant test (one out of two tests for BII, and one out of four tests each for 202-10B and 407-7, $P \leq 0.05$), the roots from treated seed had a greater root weight and lower root necrosis rating, but were still very necrotic and resembled the non-bacteria treated control roots more than the non-*Ggt* inoculated roots (Appendix B).

Chemical field study. In the field, plants treated with MON 65500 developed some root necrosis, but yielded exceptionally well under intense disease pressure in season one, and out-yielded the non-treated plots in season two, $P \leq 0.05$ (Table 4). At best in season one, chemically treated seed (MON 65500 at 50.0 g ai/100 kg seed, plus difenoconazole at 12.0 g ai/100 kg seed and metalaxyl at 18.0 g ai/100 kg seed) yielded

6566 Kg/Ha (97 bu/acre). This was 4261 kg/ha greater than the control plots, or 61% better. The use of difenoconazole and metalaxyl (Apron®) alone (difenoconazole at 12.0 g ai/100 kg seed and metalaxyl at 18.0 g ai/100 kg seed) yielded only 4096 kg/ha (61 bu/acre). In the second season, a year in which weather conditions were very conducive to take-all, yields were highest with MON 65500/difenoconazole/metalaxyl combination 4216 kg/ha (63 bu/acre) while the high rate of difenoconazole plus metalaxyl performed poorly yielding only 2275 kg/ha (34 bu/acre). Yields overall were lower than in season one, and were below what is expected under current intensive wheat management expectations. It is felt that heavy rains in season two leached nitrogen from the soil as well as washed chemical away from the position of the seed. It is also possible that due to a mild winter, *Ggt* may have had a greater opportunity to cause damage over the entire growing period.

Plants were given a visual plant health rating four times prior to harvest in season one. On all four rating dates (data not shown), the visual assessment yielded the same results as the information gained by the harvest data. In each case, the control plants were the least healthy, followed by the difenoconazole/metalaxyl plants. The healthiest plants were those treated with MON 65500. Therefore, difenoconazole had some suppressiveness toward take-all, but in the field there was a statistically significant difference between the yields in the plots in which these two chemicals were used, indicating that the activity of MON 65500 (90-95 bu/acre, season one, 58-63 bu/acre season two) was superior to that of the conventional seed dressing, difenoconazole (61 bu/acre, season one, 33-45 bu/acre, season two). The difenoconazole-plot yields were

statistically significantly greater than the non-chemically treated plots in season one, and statistically significantly worse than the non-treated plots in season two, $P \leq 0.05$ (34 bu/acre, season one, 49 bu/acre, season two).

Biologicals field study. As in the greenhouse, the USDA-maintained isolates tested in the field did not suppress take-all (Table 5). Plots with seed treated with isolate BCB2 did have a greater yield than all other plots, but this difference was not statistically significant, $P \leq 0.05$.

Yields were very low by Virginia intensive wheat management standards with all Gustafson products tested (Tables 6 and 7). The greatest yield was 3799 kg/ha (56 bu/acre) in season one, which was a tebuconazole and thiram, imidacloprid, captan, *B. subtilis* (Ehrenberg) Cohn, and colorant seed treatment. This was only 2189 kg/ha (32.5 bu/acre) over the non-treated control plots (1610 kg/ha or 24 bu/acre). The second highest yield was from a tebuconazole-thiram , and imidacloprid seed treatment only (3601 kg/ha or 54 bu/acre). Overall, yields were very low in comparison to the chemically treated seed previously discussed in another experiment, and grown within the same field. In season two (Table 7), there were no statistically significant differences among the yields. Again, yields for all treatments were below expected intensive-management standards.

DISCUSSION

In Virginia, wheat is double-cropped with soybeans (*Glycine max* (L.) Merrill), or the field is planted in barley (*Hordeum vulgare* L. emend Bowden.) which is then

double-cropped with soybeans. Therefore no true rotation into a non-host crop occurs. It has been suggested in the literature that nitrogen source (ammonium vs nitrate nitrogen) or manganese may suppress take-all. Studies suggest that in Virginia, nitrogen source and manganese do not effectively suppress disease under Virginia management practices (Phytopathology, 1996, 86:S120). Since take-all has been noted as increasing in Virginia wheat fields even with the use of urea-ammonium nitrate (UAN), mineral management is not recommended to Virginia growers. An increase in take-all indicates current management practices are possibly conducive to the development of take-all, and current chemical usage is not suppressive.

Chemical. Dividend® (difenoconazole) states on the label that it is suppressive to take-all. In our field plots, the difenoconazole-treated-seed plots did yield significantly more than non-chemically-treated-seed plots, but the actual yield was small in comparison to the extremely high-yielding MON 65500-treated-seed plots in season one. In the greenhouse, seed treated with MON 65500 and with difenoconazole were healthier than those treated with difenoconazole alone or non-chemically treated plants. Plants in field plots in which MON 65500 was used had some root necrosis, indicating the survival and colonization by *Ggt*. If this is the case, natural *Ggt* inoculum may survive and actually increase in a field in which seed treated with MON 65500 is being used. Because this chemical is not fungicidal, wheat yields could be increased, but *Ggt* inoculum may also be sustained or increased. One potential combination could be the use of both MON 65500 and difenoconazole. Bioassay tests (Table 3) and field tests (Table 4) show that this combination is a viable option. Rotation into a non-host crop in

the absence of graminaceous weeds, however, is undoubtedly still the best way to decrease *Ggt* inoculum in the field. It has been noted in our lab, however, that several *Ggt* isolates produced perithecia on sterile soybean pods. This is an indication that double-cropping with soybeans as is practiced commonly in Virginia may support a *Ggt* population within the field.

Biological. Some suppression was noted with high numbers of bacteria in the greenhouse bioassay, but this was in only one of several tests conducted. In the field under heavy disease pressure, none of the isolates tested effectively suppressed disease.

Bacilli and pseudomonads may be responsible for antagonism toward *Ggt*, the take-all fungus (Cook and Rovira, 1976; Weller, 1983; Weller and Zhang, 1985; Howie, Cook, and Weller, 1987; Ownley, Weller, and Thomashow, 1992; Ryder and Rovira, 1992; Hornby et al., 1993). Tested here were multiple bacterial isolates for the suppression of take-all in both a greenhouse bioassay and in the field in an attempt to find biologically *Ggt* -suppressive agents. Take-all is currently controlled only by rotation into a non-host crop. In the literature (Asher and Shipton, 1981; Taylor et al, 1983; Christianson et. al., 1987; Cotterill and Sivasithamparam, 1988; Werker and Gilligan, 1990; Huber and McCay-Buis, 1993; and Colbach, Lucas, and Meynard, 1997) as mentioned previously, it has been suggested that mineral management of take-all, ie. by the use of ammonium-containing fertilizers or application of manganese may be beneficial. This is not the case in Virginia, where chemical control, as noted in this study, was superior. Biological control agents are attractive because they are a non-chemical seed treatment that may colonize the roots that need protection from a soil-

borne pathogen such as *Ggt*. Since biological control agents are living, however, they must be capable of surviving long enough in high enough numbers to suppress disease. Unlike pseudomonads, bacilli are attractive due to the formation of endospores which can withstand periods not conducive to growth. In future testing in Virginia, it will be imperative to test many bacterial strains in combination as has been suggested by previous researchers. Each population of bacteria may grow best in a slightly different niche in the rhizosphere and therefore roots may be effectively colonized over longer periods over more surface area. Such combinations are more likely to offer a long-term suppressive rhizosphere for the control of take-all. It may also be appropriate to test biological control agents in combination with MON 65500 to determine if chemicals such as this will allow the colonization of *Ggt*-antagonistic bacteria.

For both greenhouse and field studies, a good seed treatment technique will need to be determined. It was evident in the USDA-biologicals tests that the carboxymethylcellulose/peat mixture possibly enhanced disease. This is similar to what was found by Huber (Huber, D. M et al., 1989), where whiteheads were in greater number and there were reduced grain yields in plots in which peat-treated seed was used. It is possible that the peat carrier retained moisture in close proximity to the seed and subsequently to the emerging roots allowing early root infection and colonization. This environment may be good for the colonization of the biocontrol bacteria, but may also have promoted the growth of *Ggt*.

Bioassay. Field tests are the best way to evaluate possibly suppressive agents. However, if the bioassay can be predictive of field activity, more chemical or biological

agents could be screened for their potential field activity. The bioassay was most predictive in the testing of the experimental chemical Mon 65500. The Dividend® label suggests that this product is suppressive to take-all. Under the controlled conditions of the greenhouse Dividend® was not suppressive unless it was combined with Mon 65500. In the field, however, yields with Dividend® were higher than the non-treated controls. Yields with Dividend® were not even close to the yields obtained with Mon 65500. Yields obtained with Dividend® were below that considered acceptable by intensive wheat management standards and could not suppress disease in the greenhouse. It is evident that the greenhouse bioassay can be used to quickly screen potential chemical and biological seed dressings prior to employing more expensive and time consuming field tests.

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Table 1. Selected chemical and physical properties of a Kempsville loam (fine-loamy, siliceous, thermic Typic Hapludult) soil utilized in greenhouse bioassays.

Parameter	Analyses	Method†
Textural Classification	Sandy Loam	
Sand %	59.7‡	
Silt %	24.7	
Clay %	15.7	
Organic matter %	1.4	29-3.5.2
CEC (meq/100g)	3.9	(sum of cations)
pH	5.7	12-2.6, 12-3.4.4
NO_3 (µg/g)	9.0	33-3.2.1, 33-8.3
P (µg/g)	49.0 (Bray 1)	24-5.1
K (µg/g)	91.0	13-3.5.2
Mn(µg/g)	25.7	19-3.4

†All methods are listed in Methods of Soil Analyses Part 2. 1982. Chemical and Microbiological Properties 2nd Edition. American Society of Agronomy and Soil Science Society of America.

‡ Average of three separately analyzed samples

Table 2. MON 65500 tested for suppression of take-all in the greenhouse.

	Test Number								
	1	2	3	1	2	3	1	2	3
Trt†	Root Necrosis Rating (0-5) ‡			Root Weight (mg)			Shoot Weight (mg)		
0.0+	4.3 a	4.6 a	4.6 a	26.2 b	33.2 b	21.2 b	32.2 b	36.9 b	19.5 d
0.0+	4.4 a	4.7 a	5.0 a	21.7 b	18.8 b	10.6 c	28.6 b	23.8 c	12.4 e
6.25+	2.6 b	2.7 b	2.4 b	40.8 a	73.0 a	31.8 a	49.8 a	54.0 a	30.5 c
12.5+	2.3 b	2.7 b	2.1 b	46.8 a	71.7 a	30.8 a	51.1 a	57.7 a	31.4 bc
25.0+	2.0 b	2.1 c	2.0 b	44.3 a	69.9 a	32.9 a	53.0 a	59.0 a	37.5 a
50.0+	2.1 b	2.1 c	2.1 b	42.1 a	66.0 a	32.0 a	48.8 a	56.9 a	35.7 ab
0.0-	0.4 a	0.7 a	0.6 a	44.6 a	97.4 a	41.2 ab	65.0 a	71.4 a	46.1 ab
0.0-	0.3 a	0.4 a	0.7 a	51.4 a	70.5 b	48.6 a	66.0 a	61.8 b	49.6 a
6.25-	0.6 a	0.7 a	0.6 a	45.8 a	86.3 ab	43.7 ab	49.8 a	54.0 a	30.5 c
12.5-	0.6 a	0.3 a	0.3 a	56.2 a	88.4 ab	40.0 ab	66.3 a	70.4 a	44.4 b
25.0-	0.4 a	0.4 a	0.4 a	51.8 a	81.7 ab	36.8 b	64.0 a	68.2 ab	45.9 ab
50.0-	0.4 a	0.6 a	0.4 a	46.7 a	91.5 ab	35.2 b	68.3 a	70.8 a	45.3 ab

* Means in columns followed by the same letter do not significantly differ ($P \leq 0.05$). Inoculum had been mixed into the soil as one month old millet seed inoculum (10 g/kg soil) or as sterile millet seed used as a control with plant health measured by dry shoot weight, root weight, and a root necrosis rating after being grown in a greenhouse for one month. Tests with and without *Ggt* inoculum are analyzed separately.

† Treatment as a dry seed dressing in g ai/100 kg seed, + = *Ggt* millet inoculum, - = Sterile millet inoculum. Note that two separate non-treated controls were used (0.0 g ai/kg seed). Line 1 = non-treated seed from the lab, line 2 = seed run in blank seed treater and taken to the field for field tests as were the chemically treated seed.

‡ Rating: 0 =no root lesions, 1 = 1 to several root lesions, 2 = extensive root necrosis, 3 = lesions on roots as well as any darkening of crown, 4 = extensive darkening of crown, 5 = plant killed with extensive culm and crown necrosis.

Table 3. Influence of Difenoconazole and MON 65500 on the suppression of take-all in the greenhouse.

Treatment†	Root Necrosis Rating ‡ (0-5)	Root Weight (mg)	Shoot Weight (mg)
NT +	4.4 a	25.0 a	40.4 b
difenoconazole 12.5 +	4.4 a	27.3 a	42.5 b
difenoconazole 12.5 + MON 65500 12.5	2.3 b	37.8 a	55.7 a
NT -	0.3 a	40.8 a	58.7 a
difenoconazole 12.5 -	0.1 a	39.2 a	48.4 a
difenoconazole 12.5 - MON 65500 12.5	0.0 a	34.6 a	53.2 a

* Means in columns followed by the same letter do not significantly differ ($P \leq 0.05$). Inoculum had been mixed into the soil as one month old millet seed inoculum (10 g/kg soil) or sterile millet seed used as a control with plant health measured by dry shoot weight, root weight, and a root necrosis rating after being grown in a greenhouse for one month. Tests with and without *Ggt* inoculum are analyzed separately.

† Treatments were dry seed dressings at the listed rate in g ai/100kg seed. NT = non-treated seed. + = with *Ggt* millet inoculum, - = with sterile millet seed inoculum.

‡ Rating: 0 =no root lesions, 1 = 1 to several root lesions, 2 = extensive root necrosis, 3 = lesions on roots as well as any darkening of crown, 4 = extensive darkening of crown, 5 = plant killed with extensive culm and crown necrosis.

Table 4. Influence of MON 65500 and difenoconazole on take-all severity in an anually artificially infested field over two seasons.

		Season 1		Season 2	
Treatment †		Yield kg/ha 13.5% H_2O	Yield bu/acre 13.5% H_2O	Yield kg/ha 12.5% H_2O	Yield bu/acre 12.5% H_2O
Non-treated		2304 c	34 c	3301 bc	49 bc
MON 65500‡	6.25	6209 a	92 a	…	…
MON 65500	12.5	6007 a	89 a	…	…
MON 65500	25.0	6372 a	95 a	…	…
MON 65500	50.0	6070 a	90 a	…	…
MON 65500	6.25				
difenoconazole	12.0				
metalaxyl	18.0	6244 a	93 a	4216 a	63 a
MON 65500	12.5				
difenoconazole	12.0				
metalaxyl	18.0	6059 a	90 a	3913 ab	58 ab
MON 65500	25.5				
difenoconazole	12.0				
metalaxyl	18.0	6254 a	93 a	3924 ab	58 ab
MON 65500	50.0				
difenoconazole	12.0				
metalaxyl	18.0	6566 a	97 a	…	…
difenoconazole	12.0				
metalaxyl	18.0	4096 b	61 b	3024 c	45 c
difenoconazole	26.0				
metalaxyl	18.0	…	…	2275 d	34 d
TTZ EXP804742J	5.0				
mefanoxam	1.0	…	…	2861 cd	43 cd
TTZ EXP804742J	10.0				
mefanoxam	1.0	…	…	2321 d	35 d

* Means followed by the same letter do not significantly differ ($P \leq 0.05$).

† g ai/100 kg seed. In season 2, imidacloprid (Gaucho®) at 1.0 g ai/100 kg seed used in all treatments. No metalaxyl was used as in season 1.

‡ MON 65500, … = not-tested

Table 5. Influence of USDA-maintained bacterial isolates on the suppression of take-all in the greenhouse and in the field (1996-1997).

	Greenhouse			Field		
Treatment	Root Rating (0-5)†	Root Weight (mg)	Shoot Weight (mg)	Yield bu/acre	Yield kg/ha	$\%H_2O$
Non-treated‡	3.5 a	40.7 a	37.4 a	33.5 a	2254.3 a	14.1 a
Peat control	3.3 a	26.8 a	27.8 a	32.5 a	2184.2 a	15.6 a
VA 218	3.3 a	41.6 a	34.8 a	33.0 a	2227.2 a	15.3 a
VA 163	3.4 a	40.4 a	36.3 a	31.9 a	2145.5 a	15.3 a
VA 144	3.4 a	37.1 a	36.4 a	...	...	...
VA 156	3.3 a	38.5 a	35.5 a	...	...	...
BCB	3.0 a	41.5 a	35.3 a	36.6 a	2466.3 a	16.2 a
BCB2	...	...	...	51.5 a	3497.4 a	13.0 a
BC1	3.4 a	36.3 a	33.9 a	...	...	...
BCB/VA 163	...	...	...	43.2 a	2909.4 a	14.4 a

* Means in columns followed by the same letter do not significantly differ ($p \leq 0.05$). Inoculum mixed into the soil as one month old millet seed inoculum (10 g/kg soil) with plant health measured by dry shoot weight, root weight, and a root necrosis rating after being grown in a greenhouse for one month, or evaluated in the Eastern Virginia Agricultural Research and Extension Center) for suppression of take-all by the yield (kg/ha and bu/acre), 100K weight in grams, and %. Carboxymethylcellulose was applied to all seed.

† Rating: 0 =no root lesions, 1 = 1 to several root lesions, 2 = extensive root necrosis, 3 = lesions on roots as well as any darkening of crown, 4 = extensive darkening of crown, 5 = plant killed with extensive necrosis.

‡ For greenhouse study, Non-Treated = 2.0×10^3, Peat Control = 2.5×10^3, VA 218 = 1.0×10^6, VA 163 = 8.0×10^6, VA 144 = 3.4×10^6, VA 156 = 1.4×10^6, BCB = 1.1×10^6, BC1 = 7.5×10^5 CFU / seed.

Table 6. Influence of Gustafson products on the suppression of take-all in the field; Season 1.

Treatment	g ai/100 kg Seed	Yield kg/ha 13.5% H_2O	Yield bu/acre 13.5% H_2O
imidacloprid†	1.0	1610.1 c	23.9 c
tebuconazole/thiram	3.5	3600.6 a	53.5 a
imidacloprid	1.0		
triadimenol	1.25	3088.2 abc	45.8 abc
captan	2.0		
imidacloprid	1.0		
colorant	0.24		
tebuconazole/thiram	3.5	1751.9 bc	26.0 bc
imidacloprid	1.0		
Bacillus subtilis	0.1		
tebuconazole/thiram	3.5	2113.6 abc	31.4 abc
furmecyclox	2.8		
tebuconazole/thiram	3.5	3063.3 abc	45.5 abc
imidacloprid	1.0		
GB29 ASC 66570	2.8		
tebuconazole/thiram	3.5	2280.7 abc	33.8 abc
imidacloprid	1.0		
GB33	2.8		
tebuconazole/thiram	3.5	2405.0 abc	35.7 abc
imidacloprid	1.0		
Bacillus cereus GB37	2.8		
triadimenol	1.25	3799.0 a	56.4 a
captan	2.0		
imidacloprid	1.0		
Bacillus subtilis	0.1		
colorant	0.24		
triadimenol	1.25	3424.8 ab	50.9 ab
captan	2.0		
furmecyclox	2.8		

imidacloprid	1.0			
colorant	0.24			
triadimenol	1.25		3600.1 a	53.4 a
captan	2.0			
GB29 ASC 66570	2.8			
imidacloprid	1.0			
colorant	0.24			
triadimenol	1.25		3024.5 abc	44.9 abc
captan	2.0			
GB32	2.8			
imidacloprid	1.0			
colorant	0.24			
triadimenol	1.25		3517.4 a	52.2 a
captan	2.0			
imidacloprid	1.0			
GB33	2.8			
colorant	0.24			
triadimenol	1.25		2929.3 abc	43.5 abc
captan	2.0			
imidacloprid	1.0			
Bacillus cereus		2.8		
colorant	0.24			

*Means followed by the same letter do not significantly differ ($P \leq 0.05$).
† imidacloprid = Gaucho®, tebuconazole/thiram = Raxil-Thiram®, triadimenol = Baytan®, captan = Captan 400®, *Bacillus subtilis* = Kodiak®, furmecyclox = Epic®.

Table 7. Influence of Gustafson products on the suppression of take-all in the field; Season 2.

Treatment	g ai/100 kg Seed	Yield kg/ha 13.5% H_2O	Yield bu/acre 13.5% H_2O
imidacloprid†	1.0	3100.1 a	46.0 a
tebuconazole/ metalaxyl imidacloprid	0.16 1.0	3214.2 a	47.6 a
tebuconazole/ metalaxyl imidacloprid *Bacillus subtilis*	0.16 1.0 0.1	3012.2 a	44.7 a
tebuconazole/ metalaxyl imidacloprid *Bacillus* sp. LS108	0.16 1.0	2656.7 a	39.4 a
tebuconazole/ metalaxyl *Bacillus* sp. LS178	0.16	3130.6 a	46.5 a
tebuconazole/ metalaxyl imidacloprid *Bacillus* sp. LS182	0.16 1.0	2664.0 a	39.6 a
tebuconazole/ metalaxyl imidacloprid *Bacillus* sp. LS124	0.16 1.0	3148.3 a	46.8a
tebuconazole/ metalaxyl imidacloprid *Bacillus* sp. LS151	0.16 1.0	3060.2 a	45.4 a
triadimenol/thiram imidacloprid	9.0 1.0	3330.3 a	49.5 a

triadimenol/thiram imidacloprid *Bacillus subtilis*	9.0 1.0	3351.8 a	49.8 a
triadimenol/thiram imidacloprid *Bacillus* sp. LS108	9.0 1.0	3127.6 a	46.4 a
triadimenol/thiram imidacloprid *Bacillus* sp. LS178	9.0 1.0	3314.7 a	49.2 a
triadimenol/thiram imidacloprid *Bacillus* sp. LS182	9.0 1.0	3433.8 a	51.0 a
triadimenol/thiram imidacloprid *Bacillus* sp. LS124	9.0 1.0	3587.4 a	53.3 a
triadimenol/thiram imidacloprid *Bacillus* sp. LS151	9.0 1.0	3053.3 a	45.3 a

*Means followed by the same letter do not significantly differ ($P \leq 0.05$).
† imidacloprid = Gaucho®, triadimenol/thiram = Baytan-Thiram®, *Bacillus subtilis* = Kodiak®. All treatments included acid red # 33 colorant.

CHAPTER 4

Characterization of Virginia Isolates of *Gaeumannomyces graminis*

J. B. Crozier, E. L. Stromberg, and G. H. Lacy. Department of Plant Pathology, Physiology, and Weed Science, Virginia Polytechnic Institute and State University, Blacksburg, Virginia, 24061-0331.

ABSTRACT

Take-all of wheat caused by the fungus *Gaeumannomyces graminis* var. *tritici* (*Ggt*) occurs wherever wheat is grown and take-all has been observed in many of the intensively managed wheat fields in Virginia. Crop rotation is recognized as the only effective method for control of this pathogen. Little information is available on the variability of this fungus, with most information coming from British and Australian reports. Isolates of *Ggt* from Virginia and Montana, and Isolates of *Gga* and *Ggg* from ATCC (British isolates), were tested for pathogenicity and virulence against 'Jackson' soft red winter wheat in a greenhouse bioassay. A root necrosis rating, and root and dry shoot weight were determined as an estimate of disease severity. Colony and hyphopodia morphology, as well as growth rates were examined for a series of isolates. Growth rate per day and total growth on PDA was related to the relative wheat damage individual isolates caused in the greenhouse. Most isolates produced simple hyphopodia including several which genotypically appeared to be *G. graminis* var. *graminis* (*Ggg*). This is the first report from this region of the world on the production of putative

vegetative compatibility groups and the first use of chemical markers as a means to determine the sharing of nuclei between "incompatible" isolates after anastomosis. Two parents and five progeny were examined using RAPDs including for the first time bacterial Repetitive Extragenic Palindrome sequence (REP) primers. The effect of double-cropping soybeans and no-tillage practices on take-all incidence and severity are examined. We used soybean pods as a primary means of producing perithecia, and subsequently crossed wildtype and benomyl resistant strains on the pods to determine the stability of the benomyl resistance.

INTRODUCTION

Take-all of wheat (*Triticum aestivum* L.) is caused by the ascomycete fungus *Gaeumannomyces graminis* (Sacc.) Arx and Oliver var. *tritici* Walker (*Ggt*) = *Ophiobolus graminis* Sacc. Take-all occurs wherever wheat is grown, and may be the most important root disease of wheat world-wide (Cook and Rovira, 1976, Asher and Shipton; 1981; Weller and Cook, 1983; Huber and McCay-Buis, 1993). Young roots are easily penetrated by means of hyphopodia, infected, and colonized, whereas the process is delayed or impeded by root maturation (Nilsson, 1969). Plants may be infected at any stage of growth, and yield losses are due to the reduction of water and nutrient movement due to the proliferation of the fungus in root tissue and subsequent root and culm necrosis (Huber and McCay-Buis, 1993, Colbach, Lucas, and Meynard, 1997). Spread of the disease and infection from the ascospores or microspores (conidia) is unlikely (Nilsson, 1969). Ascospores caused take-all in wheat seedlings only in sterile soil, and at extremely high densities (Nilsson, 1969). Ascospores are produced in perithecia which

may be found on diseased tissue in the field. This fungus is homothallic meaning sexual reproduction is not required for the production of perithecia. In the laboratory, it has been difficult and laborious to produce perithecia consistently. Perithecia are generally not produced on common media such as PDA. The literature suggests that perithecia are produced on developing wheat seedlings from surface disinfested seed (this however is very labor intensive and contamination is a problem), or in special media containing wheat leaf extract, or a medium containing 1% glucose and 2% asparagine (Mathre, 1992). For optimal production of perithecia, it is suggested that one incubate cultures in the light at approximately 13-24 C at between 700-3875 lux, with an optimum of 2135 lux (Asher, 1981). Roy et. al. (1982) showed that there was a natural incidence of *Ggg*-produced perithecia on soybean pods in the field.

Thick pigmented brown to dark brown 3-6µm diameter hyphae termed macrohyphae or runner-hyphae and are believed to be responsible for the spread of the fungus (Nilsson, 1969). Thinner hyaline hyphae are termed microhyphae or infection-hyphae which allow penetration of wheat roots mechanically by the formation of hyphopodia (Nilsson, 1969). The morphology of the hyphopodia has traditionally thought to vary depending on the variety of *G. graminis*. The "wheat" strain of *G. graminis* (*Ggt*) readily causes disease in wheat. This strain does not cause disease in oats (*Avena sativa* L.), whereas the "oat" strain, *G. graminis* (Sacc.) Arx and Oliver var. *avenae* Turner (*Gga*), has a wider host range, and can cause disease in wheat as well as oats (Nilsson, 1969). A third strain, found to cause disease in turf is *G. graminis* var. *graminis* (*Ggg*), and is less virulent, causing less disease on wheat (Huber and McCay-

Buis, 1993). Traditionally, *Ggt* and *Gga* have been thought to produce hyphopodia which are hyphal swellings formed in aggregations, whereas *Ggg* has been thought to produce more complex hyphopodia which are lobed.

Studies have shown that there is variation in virulence among *Ggt* isolates. Asher tested 25 *Ggt* isolates from each of several sites for pathogenicity and virulence (Asher, 1980). Variation existed for each of the characters tested (virulence, growth rate, and coloration). Dewan and Sivasithamparam tested the pathogenicity and virulence of *Ggt* isolates from wheat and ryegrass (*Lolium rigidum* L.) and found much variability (Dewan, 1989).

In Australia, anastomosis between isolates was only successful in isolates with similar host ranges from the same area (Nilsson, 1969). Western Australian isolates from oats were incompatible with South Australian isolates from oats, both of which were incompatible with wheat isolates (Nilsson, 1969).

The anastomosis of hyphae and sharing of nuclei is not only important in the formation of heterokaryons for the sexual phase of an organism, but is also implicated in the transfer of viruses (Leslie, 1993). Strains of a fungus that are vegetatively compatible (VC) are said to be in the same VCG or vegetative compatibility group. Vegetative incompatibility, therefore, involves the restriction of the transfer of nuclear and cytoplasmic material among fungi (Leslie, 1993).

In species of *Aspergillus*, *Cryphonectria, Fusarium,* and *Neurospora,* fusion of hyphae occurs naturally, even if they are incompatible (Leslie, 1993). In incompatible reactions, however, there is a death of the heterokaryon. A direct test for the formation

of a heterokaryon involves the creation of a situation under which neither component could survive individually, such as the use of chemical genetic. Barrages are formed under incompatible reactions, and is considered an antagonistic situation. The barrage requires hyphal fusion after which cell death occurs (Leslie, 1993). A central region of dead and dying cells forms, and a dark layer of pigmented hyphae is laid down. This is accompanied in heterothallic fungi by the formation of perithecia. Vegetatively compatible strains do not interact in this manner, but simply grow together without an alteration in fungal morphology (Leslie, 1993).

VCGs allow the study of fungal populations and their diversity (Leslie, 1993). Strains within the same VCG are capable of exchanging genetic information due to their ability to anastomose. It is felt that if selection maintains a large number of VCGs, it is quite possibly due to the spread of pathogenic agents such as viruses (Leslie, 1993). A large number of VCGs can also indicate a sexually reproducing population (Leslie, 1993).

It has been noted that some pairings of *Ggt* isolates results in an "incompatible reaction". Jamil et al. (1984) noted that a definite zone of interaction was produced as a result of putatively incompatible crosses. The incompatibility was noted as the formation of pigmentation at the point of contact between two isolates with a zone of clearing directly between the merging hyphae. Self crosses and putatively compatible reactions did not result in the formation of dark bands or cleared zones.

It would be useful to be able to confirm whether "compatibility" or "incompatibility" and the formation of vegetative compatibility groups actually exist in

Gaeumannomyces graminis varieties. Using a set of chemical resistance genetic markers would leave the vast majority of the fungal genome intact as in the wildtype. Some groups have inserted resistance genes randomly into the *Ggt* genome by transformation. Genes inserted randomly may insert anywhere and in any number of loci. By creating spontaneous mutants one may keep the genetic difference between a wildtype and its mutant strain to a minimum. Molecular techniques may then be employed to determine the actual genetic makeup of "parents" and "progeny" of a putative anastomosis. Use of RAPDs may allow one to fingerprint the parents and progeny to determine the presence of the parent's DNA. What is required is the use of primers that indicate polymorphisms and is completely random.

Our work demonstrates that there is variability in the virulence among *Ggt* isolates collected from Virginia wheat growing regions. Crosses among isolates indicate the presence of many VC groups. Average saprophytic growth rate per day and average total growth on an artificial medium was related to an individual *Ggt* isolate's ability to cause damage. We also determined that culture on soybean (Glycine max [L.] Mer.) pods provides a very reliable and convenient method for the production of perithecia. We also have noted that not all isolates which are *Ggg* genotypically produce lobed hyphopodia as was once thought. This is also the first use of benomyl and propiconazole as chemical markers to determine if anastomosis had taken place and nuclei were shared in the same thallus. Molecular techniques were employed to determine the genetic makeup of two parents and five progeny with several primers, including bacterial Repetitive Extragenic Palindrome sequence (REP) primers.

MATERIALS AND METHODS

Isolation and storage. A single isolate from Montana was obtained from Dr. D. Mathre (Montana State University, Bozeman, MT, USA). Several isolates were obtained from ATCC (American Type Culture Collection, 12301 Parklawn Drive, Rockville MD 20852-1776), and all Virginia isolates were obtained from fresh material using a selective medium (Juhnke, 1984). Whole fresh wheat (*Triticum aestivum* L.) plants were washed under running tap water. Darkened wheat crowns were sliced into discs and roots were cut into small (several mm) pieces. Plant material was surface disinfested by placement into a 0.5 % sodium hypochlorite solution for several min with agitation. The material was rinsed with sterile distilled water, blotted on sterile paper towels, and plated onto SM-Ggt3 (Juhnke et al., 1984). Colonies which developed a dark appearance were transferred onto potato dextrose agar (PDA, Sigma, St. Louis, MO 63178).

All isolates were hyphal-tipped using the following procedure: One-hundred ml flasks containing approximately 25-50 ml sterile millet (*Setaria italica* L.) seed were inoculated with *Ggt*. Individual colonized seeds were placed onto water agar, or alternatively, small pieces of mycelium were placed directly onto water agar. As single hyphae emerged from the millet seeds or agar, hyphal tips were excised and transferred to PDA with the aid of a dissecting scope. Mycelial plugs of all isolates grown on PDA were frozen in 15% glycerol and maintained at approximately –87 C (Table 2).

Growth studies. One, 8mm dia. plug of mycelia from the leading edge of a colony growing on 20ml PDA (25C, in the dark) was placed either into the center or at the edge of a Petri dish (100 X 15 mm) containing 20 ml PDA. Cultures were grown at

25 C in the dark. Measurements for center-positioned cultures were taken from three cross-sections. The average amount of growth was determined. Measurements from edge-positioned cultures were taken at one position at the central outward-growing portion of the colony. One to three repetitions were evaluated for each isolate, and each test was conducted one to three times.

Pairing studies. Isolates were placed onto PDA such that there were two 4-5 mm plugs of one isolate paired against two plugs of a second isolate. The plugs were placed on the plate such that a long barrage zone, the region in which the hyphae of the paired isolates come into contact or close proximity, would be permitted as well as two control barrages. Plates were incubated in the dark at 25 C, and were examined for interaction between isolates.

Pathogenicity and virulence testing. A Kempsville sandy loam (Robinette and Hoppe, 1982) coastal plain soil typical of soils used for wheat production was obtained from a freshly plowed field at the Eastern Virginia Agricultural and Extension Center, Warsaw, Virginia. Selected chemical and physical properties are shown in Table 1. The soil was dried, sieved through an approximately 0.7 cm (0.25 inch) screen, mixed for 5 min and stored in covered plastic cans until further use. The soil was analyzed at A & L Eastern Agricultural Laboratories (7621 Whitepine Road, Richmond VA 23237), see Table 1. Calcium phosphate monobasic and potassium chloride were mixed into the soil for 5 min (25 µg P/g soil and 25 µg K/g soil). Fifteen g of calcium carbonate per 30 kg soil were required to raise the pH to approximately 6.5. A cotton ball was inserted into

the bottom of each Conetainer® [Ray Leach Conetainers®, Portland, Oregon (4 x 21 cm)], and 150 g soil mix was added to each cone.

An ammonium nitrate solution was prepared such that 30 ml of solution was added to 150 g Kempsville loam per cone to yield 30 µg N / g soil. Inoculum was grown on 20 ml PDA at 25 C in the dark. At the time of planting, either two sterile PDA or two *Ggt* inoculated PDA plugs cut from the margin of the PDA cultures were placed approximately 1.5 cm deep in the soil with two 'Jackson' soft red winter wheat seeds and then covered with soil. Cones in each test were arranged using a completely randomized block design with seven replications. Plants were thinned to one plant per cone.

The average greenhouse temperature was approximately 23 C with a one-recorded high of 32 C and low of 14 C. The relative humidity ranged from 21 to 90%, but was generally approximately 40% (Fisher Scientific Digital Relative Humidity/Temperature Meter, CAT # 11-661-13, Raleigh, NC 27629). Lighting was aided with artificial lights on a 12 hour cycle (Sylvania Lighting Equipment, 21 Penn Street, Fall River, MA 02724, 1000 Watt, lamp type M47). Light intensity was approximately 143.2 µmol $M^{-2}S^{-1}$ PAR (945 ft-c) under lower lighting conditions, and 193.8 µmol $M^{-2}S^{-1}$ PAR (1279 ft-c) under higher lighting conditions. Plants were grown for one month and watered with distilled water as needed.

Plants were removed intact from cones, and the soil was rinsed from the roots. Roots were excised and placed into 70% ethanol and shoots were placed into paper bags. Roots were given a root necrosis severity rating (0 = no lesions, 1 = one to several lesions, 2 = extensive lesions, or several entire roots necrotic, 3 = lesions on roots and

some darkening of crown, 4 = extensive root necrosis and extensive darkening of crown, 5 = plant dead). Roots were weighed after being blotted with paper towels and air dried for 10-12 min. Shoots were dried at 70C overnight, and weighed after cooling to room temperature.

Production of perithecia. Air-dried soybean pods and other soybean debris from mixed varieties of soybeans were collected at the Eastern Virginia Agricultural and Extension Center, Warsaw, Virginia. Approximately four dried pod halves were placed into a glass Petri dish (100 X 15mm) with two filter paper discs (Whatman #30, 90mm). Soybean pods were soaked in 20ml distilled water for 45-60 min. Excess water was poured off, and the Petri dishes were autoclaved for 30 min at 121 C. For experiments where non-sterile pods were used, Petri dishes and filter paper were used with soybean pods which had been soaked in sterile distilled water for approximately one hour. All plates were sealed with laboratory film.

Other methods for producing perithecia included surface disinfesting wheat seed and placing them onto a slant of ¼ strength PDA (10 g Sigma PDB and 15g agar per L) in a test tube (19 X 2.5 cm) which simultaneously had been inoculated with an 8mm dia. plug of *Ggt*. Several media were also tested for their perithecial production capabilities. Soybean pod medium (SPM) was produced by placing 5g soybean pods into a 1L flask and adding 500ml distilled water. The liquid was brought to a boil and steeped for 1.5 hours. The liquid was filtered through cheesecloth, and 7.5g agar was added to the 500ml liquid prior to autoclaving.

Malt extract peptone agar (MPA) similar to Speakman's medium was also used (Speakman, 1982). Ten grams of malt extract, 2.5g peptone, and 15 g agar per L was autoclaved. Lilly and Barnett's basal medium was also used (Lilly and Barnett, 1951). This medium contained 1% glucose and 0.2 % asparagine among other components as listed by Lilly and Barnett, pages 421 and 427, and thus was called GA medium.

Incubators used for perithecial production were set up for optimal conditions for the production of perithecia as noted in Asher, 1980, such that cool white fluorescent lights were placed approximately 52 cm above the Petri dishes yielding approximately 2150 lux (approximately 203 ft-c or 30.8 µmol $M^{-2}S^{-1}$ PAR). Lights were on for 16 hours per day with an incubator temperature of 20-22 C.

Production of perithecia by single ascospore isolates. Four perithecia formed by *Ggt* isolate M1 on sterile soybean pods as above were collected and agitated in 20ml sterile distilled water. A sterile pipette was used to place 0.2-0.5 ml ascospore suspension onto water agar. Ascospores were spread over the surface of the plate. Under a dissecting microscope, single ascospores were removed and placed onto PDA and incubated at 25C. Single ascospore isolates were placed onto sterile soybean pods to test for homothallism.

Production of perithecia and ascospores by benomyl resistant strains. Soybean pods were sterilized as above, and crosses were made on the pods to test perithecial production by benomyl resistant M1, CB1, and CD1. Three repetitions were made for each cross. As a preliminary test, a suspension of ascopores was pipetted onto PDA or PDA amended with 1.5 µg/ml benomyl. With successful results, approximately

15 single spores per cross from approximately 8-10 perithecia across the plate were plated onto PDA, or PDA amended with 1.5 µg/ml or 2.5 µg/ml benomyl.

Production of hyphopodia. A technique was developed similar to that of Epstein et al. (1994). A 4mm plug of mycelium from each isolate tested (from PDA) was placed onto V8 agar (200ml supernatant of V8 juice, Campbell Soup Co., Camden, New Jersey, 4 g $CaCO_3$, 17.5 g agar, and water to a total volume of 1 L). Plates of dilute V8 agar were also produced (Epstein et al., 1994) (8% supernatant from V8 juice and 4% agar). Upon cooling, half of the agar was removed from each plate, leaving the polystyrene Petri dish bottom exposed for one half of the plate. A 4mm plug of mycelium from V8 agar or from PDA was placed directly next to the cut edge of the agar (see Figure 7). Plates were sealed with laboratory film and incubated in the dark at 25C. Hyphopodia were produced as hyphae grew onto the bare surface of the Petri dish.

Photomicrographs were taken directly through the bottom of the plate, by placing a coverslip with sterile distilled water directly on the mycelium, or by lifting agar off of the plate leaving hyphopodia attached to the Petri dish and then using a coverslip.

Chemical-resistant marker strains. Wildtype (WT) isolates (three 4mm plugs used as inoculum for isolates CB1, CD1, and M1) were grown in 100 ml potato dextrose broth (PDB) in shake culture (250 ml flask at approximately 150 rpm). After 5-7 days the contents were blended for 30 seconds in a 1 L Waring blender (Waring Products Division, New Hartford, CT 06057). Four ml of the slurry was pipetted onto 40 ml agar in a 150 X 25 mm petri dish. The agar contained either benomyl or propiconazole (from Banner® mc, Novartis, Greensboro, NC, 27419) dissolved in ethanol (95%) or sterile

distilled water respectively. The appropriate concentration for obtaining mutants was determined by plating 1ml slurry onto 10 ml propiconazole- or benomyl-amended agar in 100 X 15 mm plates at varying concentrations. The concentration at which most of the mycelial fragments were apparently killed or injured by compound microscopy was chosen. An attempt was made to plate 1 X 10^9 nuclei per test based on average fragment size, number of cells per fragment, and an estimate of two nuclei per cell based on a report of 1-10 nuclei per *Ggt* cell (Asher, 1980). Tests were conducted repeatedly to obtain mutants which resembled the WT phenotype. Resistant strains were maintained on benomyl- or propiconazole-amended PDA at 1 C. Putative resistant strains were subjected to a series of dilutions of the appropriate chemical to determine the highest concentration at which each resistant strain could still grow but the WT could not grow. Four mm plugs of the resistant strain were paired on the same plate against its own WT isolate at varying dilutions. Successful markers were obtained for M1 (propiconazole) and CB1 (benomyl).

To determine if the two strains were capable of anastomosis, plugs of marked strains were paired on PDA and allowed to grow together. Plugs from the zone of interaction were excised and plated onto a medium containing the two different chemicals representing the resistance of the two paired isolates.

A second method was also used. Four mm plugs (one from each strain) were placed into a small volume of sterile PDB in a large flask (10ml PDB in a 250 ml flask). After approximately 3 weeks growth at 25 C, excess PDB was poured off, and mycelium was rinsed twice with sterile, distilled, charcoal filtered-water. The mycelium was

blended in a 1L Waring blender for 30 sec in 100ml sterile, distilled, charcoal-filtered water, and the fragments were pipetted into sterile Petri dishes. Fragments of mycelium were obtained with sterile forceps and plated onto media containing both benomyl and propiconazole. Approximately 20 fragments were plated onto each plate. The plates were stored at 25C and examined for growth. A percentage of living fragments was determined for each cross.

Molecular studies of chemically marked strains. The two parental strains (M1p, resistant to propiconazole, and CB1b, resistant to benomyl) and five progeny obtained from crosses which had grown out on media containing both benomyl and propiconazole were examined. Mycelium was generated by growing each parent or progeny in PDB.

DNA extraction. Mycelium was filtered to extract most of the liquid and then freeze dried overnight. DNA was extracted using a method developed by Rachdawong (1999). Mycelium was frozen in liquid nitrogen in a mortar, and ground into a powder. The mycelium was placed into 1.5ml centrifuge tubes, and 700µl Lysis buffer was added to each tube (50mM Tris-HCl, 50mM EDTA, 3%SDS, 1% β-mercaptoethanol). After mixing with a pipette tip, the tubes were closed and incubated at 65C for 1h. To each tube, 350 µl saturated phenol and 350 µl chloroform/isoamyl alcohol (24:1) was added and vortexed until homogeneous. Tubes were centrifuged 15min at 14,000 rpm. The top layer was removed and placed in a fresh tube. Only chloroform/isoamyl alcohol (24:1) was added, tubes were inverted and centrifuged for 10 min. The upper layer was removed and reserved in a fresh tube. A 0.03 volume of 3M sodium acetate and 0.5

volume of isopropanol was added to each tube. Tubes were incubated at 4C. Once DNA was noted, tubes were centrifuged 15 min. Supernatant was discarded. DNA was rinsed in 70% ethanol and centrifuged for 2 min. The ethanol was poured off, and the tubes were left open to dry the DNA. Dry DNA was dissolved in 100 µl TE (10mM Tris HCl, 1mM EDTA).

DNA was examined for concentration and purity on a DU-65 Spectrophotometer and by running a gel. A 0.8% agarose gel run in 1X TAE buffer (0.04M Tris, 0.001M EDTA, acetic acid to pH 8) at 100 volts and stained with ethidium bromide (0.5µg/ml) showed that DNA and RNA was present. RNAse A digestion removed the RNA. DNA was then examined at 260 and 280 nm on the spectrophotometer and diluted into stock tubes accordingly.

Polymerase Chain Reactions and RAPD assays. Ready-to-Go RAPD beads (Amersham Pharmacia Biotech Inc., 800 Centennial Avenue, Piscataway NJ 08855) in 0.5 ml centrifuge tubes were used for PCR reactions. Reactions were run separately using Amersham Pharmacia Biotech primers (primer 1, 5'-GGTGCGGGAA-3', primer 2, 5'-GTTTCGCTCC-3', primer 3, 5'-GTAGACCCGT-3', primer 4, 5'-AAGAGCCCGT-3', primer 5'-AACGCGCAAC-3', primer 6, 5'-CCCGTCAGCA-3'). To each reaction was added 5µl primer (total 25pmol primer), 2 µl template DNA (40 ng total), and 19µl sterile distilled water for a total of 25 µl total volume per reaction. The exception to this is in the case of the REP primers (REP 1; 5'-IIIICGICGICATCIGGC-3', REP 2; 5'-ICGICTTATCIGGCCTAC-3') in which 1µl each primer was added (REP 1 and REP 2 at a concentration of 40 pmol each) to each reaction.

PCR reactions were run in an Eppendorf Mastercycler Gradient (Eppendorf Scientific Inc., One Cantiague Road, Westbury NY 11590). The lid was set to 102 C. Amplifications were conducted by programming the thermocycler for one cycle for DNA denaturation at 95 C for 1min; 45 cycles of 95C for 1min (denaturation), 36C for 1 min (annealing), and 72C for 2 min (extension); final extension at 72 C for 7 min with a 4 C soak. Five µl 6X loading dye was either added directly to the cold reaction tubes prior to refrigeration, or reaction tubes were placed directly into the refrigerator. Samples (8µl DNA, 2µl dye, or 10 µl premixed reaction) were examined on a 2% agarose (Fisher Scientific) gel run at 30 volts overnight. REP reactions were run on a 1.5mm thick 6% polyacrylamide 1X TAE gel at 150 volts for 4 hours.

RESULTS

Virulence testing. All *Ggt* isolates tested were pathogenic as determined by the presence of plant damage greater than that indicated in the sterile-PDA-inoculated control plots. Duncan's multiple range test indicated that the relative severity of disease symptoms caused by individual isolates after one month were significantly different at $P \leq 0.05$ (Table 4). The isolates differed in virulence (the ability to damage wheat) yielding a subjective grouping of the isolates into four separate groups (Figure 1).

Growth study. Most *G. graminis* var. isolates tested grew approximately 45-55 mm after 8 d, and several reached the effective growth limit of the plate (approximately 77 mm) after 12-13 d at 25 C. Noted after 8 days, most isolates tested grew approximately 12-15 mm per day (Appendix D, Figures 1-12), note compilation of data

in Table 5. The coloration of cultures after two weeks varied from hyaline/white to black/green, and given a Methuen color rating (Table 3, Kornerup and Wanscher, 1961).

Regression analysis of the average growth rate per day and average total growth per day after eight days (Please see Appendix D, Figures 1-12 for growth data) against shoot weight and root weight (Please see Table 4 and Figure 1) for several *Ggt* isolates was performed. The saprophytic growth rate per day and total growth on PDA in the laboratory indicated a trend that the faster growing isolates caused more damage in the greenhouse bioassay. R values were low (Shoot Weight against Average Total Growth, $R^2 = 0.30$; Root Weight against Average Total Growth, $R^2 = 0.22$; Shoot Weight against Average Growth Rate per Day, $R^2 = 0.23$; Root Weight against Average Growth Rate per Day. $R^2 = 0.14$). Although there was variation among the three tests conducted, identical slopes in all three greenhouse tests indicated a trend that the faster growing isolates caused more damage in the greenhouse bioassay.

Vegetative compatibility. Wildtype isolates paired on PDA exhibited several interactions which were very similar to the description given be Jamil et al. (1984). The barrage zone in control interactions (an isolate against itself) was noted as a less pigmented region at the zone of hyphal interaction (Figure 2). Interactions between different isolates often resulted in a clear zone between the two isolates with adjacent mycelium pigmented in one or both of the isolates. By excising a block of agar and placing a coverslip over the block, hyphae could be noted via light microscopy in the cleared zone. Blocks from a compatible interaction contained many hyaline hyphae with both macrohyphae (slightly pigmented) and microhyphae present. In blocks from a zone

of a putative incompatible interaction, there were fewer hyphae overall, and many of the macrohyphae were highly pigmented. Some microhyphae also appeared to be pigmented. Lysed hyphae were noted in these "incompatible blocks", but lysed hyphae were not confined to the zone of clearing. A barrage zone similar to that of a control barrage was noted between two different isolates only in three cases (four out of 58 pairings, Table 6). All other interactions exhibited a zone of clearing with most interactions exhibiting a darkening of the mycelium adjacent to the cleared zone.

Production of Hyphopodia. Hyphopodia similar to those noted in the literature were produced on a bare polystyrene surface within three days to a week or more (Figures 3-7). Often structures were noted within a week. Most isolates produced simple hyphopodia, which developed as aggregations of short hyphae yielding a "peppered" appearance to the underside of the Petri dish (Figures 3-5). Via brightfield and phase microscopy it was noted that the short hyphae were slightly swollen at the apex. These swellings were approximately 10µm wide. The isolates which produced lobed hyphopodia did not form the "aggregations" as many of the simple-hyphopodia producers, but formed these structures more randomly across the plate. Lobed hyphopodia looked very much like those noted by Epstein et al. (1994). Lobed hyphopodia were slightly darkened and produced several to multiple short to long lobes. Lobed hyphopodia produced by *Ggg* isolate 2033 (see Figure 6) were on average approximately 32 X 25 µm, while the lobed hyphopodia produced by *Ggg* isolate FL-175 (Figure 7) were approximately 28 X 24 µm. The plates which were most successful in the production of these structures were those which retained moisture, whereas dry plates

inhibited the growth of mycelium on the Petri dish surface. In some cases, hyphopodia were produced under the cut edge of the agar. Upon stripping agar off of the plate, hyphopodia remained attached to the plate.

Isolates CK1A (Figures 3 and 5) and Che1 were tested in a molecular study conducted by Rachdawong (1999). According to this study, a genetic probe, which may distinguish between the three varieties of *Ggt*, indicated these two Virginia isolates are variety *graminis* and not variety *tritici*. Isolates of the variety *graminis* traditionally have been thought to produce lobed hyphopodia while *tritici* isolates produce simple hyphopodia. Our results indicate that the production of a particular hyphopodium morphology may not be a good character for the separation of varieties.

PRODUCTION OF PERITHECIA

Production of perithecia in light versus the dark. Sterilized soybean pods inoculated with M1 with half of the plates sealed in aluminum foil indicated that light is required for the production of perithecia. Light, therefore as noted in Asher and Shipton (1981) is required for induction of perithecia. Perithecia developed in approximately two to three weeks, and were counted at four weeks. Perithecia developed on, between, and under the filter paper sheets, and on the soybean pods (Figure 8). Several plates were incubated simultaneously and perithecia were counted for both dark- and light-exposed plates. Approximately 113-117 perithecia were formed in the light exposed plates while only 1-3 perithecia formed in the dark-exposed plates for test one, and 105-136 perithecia were formed in the light while none were produced in the dark for the second test.

Production of perithecia on sterile and nonsterile soybean pods. In the first test, M1 produced approximately 105-136 perithecia on sterile soybean pods (on pods and on surrounding filter paper), while 11-12 large, necked perithecia were produced on nonsterile soybean pods. Perithecia on nonsterile pods were produced close to the point of inoculation on the pod (Figure 10).

In a second test, M1, CB1, and CD1 were inoculated simultaneously onto sterile and nonsterile soybean pods (Figure 9). Approximately 40-200 perithecia were produced on sterile soybean pods, and approximately 10-47 perithecia were produced on nonsterile soybean pods (Figure 10). Average size of perithecia was almost identical except for the size of the base of the perithecium between those formed on sterile versus nonsterile substrates. Perithecia on a sterile substrate for M1 were on average 679 X 432 μm, while those from non-sterile substrate were on average 624 X 273 μm. For CB1 perithecia were 734 X 355 μm and 795 X 289 μm on sterile or nonsterile substrate, respectively, and for CD1, only one perithecium was measured at 1000 X 450 μm and, average nonsterile at 680 X 254 μm. Over the course of the study, perithecia were examined for the production of asci and ascospores (Figures 11-13). Ascospores were similar in size from M1 perithecia formed on both substrates at 74.8 X 3.6 μm and 67.9 X 3.4 μm respectively, 69.7 X 3.7 μm and 64.2 X 3.7 μm respectively for CB1, and 67.6 X 4.4 μm and 65.1 X 3.9 μm respectively for CD1. Many conidia (approximately 5 X 2 μm) were noted for M1 from sterile perithecia, while very few were noted from M1 from non-sterile substrate (Figure 13). No conidia were produced by the other two isolates in this test.

Production of perithecia by single ascospore isolates. Ten single ascospore isolates of M1 (a single 4mm plug) placed onto sterile soybean pods readily produced perithecia and ascospores. After approximately 5 weeks, 23-103 (average 65) well developed perithecia were formed indicating homothallism. By DAPI staining the genetic material, it was evident that three day old generative mycelia were single nucleate. It was evident that ascospores were also single nucleate. This indicates that there must be self pairing of nuclei for the formation of perithecia and ascopospores

Production of perithecia on soybean pods, agar media, and other substrates. Several different substrates were tested for their ability to induce perithecial production using incubator conditions known to induce production. Isolates were tested on soybean pods, Soybean Pod Medium (SPM), Potato Dextrose Agar (PDA), Glucose Asparagine Agar (GAA), Malt Extract Peptone Agar (MPA), sterilized wheat straw, sterile soybean seeds, and sterile filter paper. Perithecia were readily formed on soybean pods and the filter paper surrounding them (approximately 47-200) for isolates M1, CB1, and CD1.

These three isolates were tested on SPM and small dark clumps presumed to be perithecial initials were produced in the agar. After one month M1 produced only several perithecia developed enough to contain ascosopres, but there were numerous “flecks” which did not contain ascospores. CB1 and CD1 produced two and zero necked perithecia, respectively, but did produce “flecks”.

Similarly, PDA did not induce perithecial production very well. Only 0-17 necked perithecia were noted for M1, CB1, and CD1. CB1 produced no perithecia on PDA. No perithecia were noted on any MPA plates, nor were perithecia formed on

sterile filter paper for these three isolates. Sterile soybean seeds (two per plate) induced copious amounts of vegetative growth, but no perithecia were formed.

Ten single spore isolates of M1 were tested on both soybean pods and GAA simultaneously. Many perithecia were formed on both substrates. On soybean pods, 23-103 (average 65) perithecia were formed, while on GAA, 46-221 (average 104) perithecia were formed. To account for the different volumes, soybean pods produced 2.1 perithecia/cm^3 while GAA produced 0.5 perithecia/cm^3. Interestingly, GAA did not perform well in other tests with M1, CB1, and CD1.

M1, CB1, and CD1 were tested on sterile wheat straw (three, 3cm pieces per plate). Very little mycelium was produced, and only after 7-8 weeks were any perithecia noted. Two plates were tested for each isolate. Each isolate had one plate with no perithecia formed, while in the other plate, M1 formed 23, CB1 produced two, and CD1 produced eight. No perithecia were produced on the straw itself.

Production of perithecia and viable ascospores by benomyl resistant strains. Perithecia were produced by benomyl resistant M1, CB1 and CD1 which were morphologically equivilent to the wildtype. The perithecia produced viable ascospores which were resistant to benomyl. As a preliminary test, a suspension of ascospores in sterile distilled water was plated onto PDA or PDA amended with 1.5 µg/ml benomyl. Ascospores grew on PDA alone and on Ben^r X WT and Ben^r X Ben^r for the one isolate tested (M1). No growth was noted for WT X WT ascospores plated onto media containing benomyl.

Single ascospores plated onto PDA and benomyl-amended PDA yielded more information. WT X WT ascospores were not able to grow on the benomyl-amended medium. Crosses in which a benomyl resistant strain was involved resulted in progeny which were capable of growth on benomyl-amended PDA. Crosses in which both strains crossed were resistant to benomyl resulted in almost 100% of the ascospores exhibiting resistance (Table 7).

Production of perithecia by other *Gaeumannomyces graminis* isolates. Several other isolates were grown on soybean pods. Other than M1, CB1, and CD1, isolates CS1 and CE2 produced the most perithecia. ATCC isolate 28230 (Ggt), 12761 (Ggg) and 15419 (Gga) did not produce perithecia on soybean pods. Further testing will need to be conducted to determine the significance of these data.

Chemical resistant mutants. Putative benomyl- or propiconazole resistant (Ben^r and Prp^r respectively) isolates for isolates M1 and CB1 grew on a higher concentration of chemical than did the wildtype (WT) of the same isolate. Propiconazole resistant isolates were resistant to propiconazole to approximately 18 µg/ml propiconazole. All WT isolates were killed at very low concentrations of benomyl.

Paired chemical resistant strains always exhibited a dark mycelial band as did the WT pairings between two different isolates. Pairings between the same isolate/strain (WT X Ben^r, WT X Prp^r, and Ben^r X Prp^r) resulted in the same apparently compatible zone of interaction as did the strictly WT X WT pairings of the same isolate.

Through the pairing studies and plating of plugs onto agar amended with both benomyl and propiconazole, it was evident that the use of plugs gave results which were

difficult to interpret. Mycelium was capable of growth on the plug which may have diluted the concentration of chemical. Blending of mycelium yielded much clearer results. Using this technique, a low level of anastomosis between these two putatively incompatible strains was noted (Table 8).

It was noted that in each case in which growth occurred on lower propiconazole concentration dual (benomyl and propiconazole) agar, the percentage of Ben^r fragments living on the dual medium was high. Benomyl resistant strains were capable of growing on high levels of propiconazole.

RAPD analysis. Primers obtained from Amersham Pharmacia Biotech (Primers 1-6) all yielded amplified *Ggt* DNA. Polymorphisms were noted between the two parent strains (M1p and CB1b) for primers 3 and 6 (Fig. 14). The combination of REP 1 and 2 yielded amplified DNA and several polymorphisms between the two parents (Fig 15).

DISCUSSION

Virulence testing. The virulence tests showed that the *Ggt* isolates used were all pathogenic on wheat, but the relative virulence among isolates after one month varied (note Tables 4 and 5). This is not at all unexpected as isolates originated from a variety of locations throughout Virginia. By using this technique, it was also possible to discriminate among the *Ggg* and *Gga* isolates, and many, but not all of the *Ggt* isolates. In the subjective grouping of isolates based on relative plant damage, three of the *Ggt* isolates tested were placed into the same category as both the *Ggg* and *Gga* isolates. The implications of the results of this study are that Virginia isolates of *Ggt* are variable in their virulence. Asher, (1980), Great Britain, and Dewan and Sivasithamparam, (1989)

Australia, obtained similar information, indicating that isolates tested in their respective regions were variable. Dewan and Sivasithamparam noted differences among individual isolates based on shoot weight, root weight, and a root necrosis rating. In Asher's tests it was noted, however, that the mean virulence (based on dry shoot weight) of populations from different cropping sequences was not significant.

In work conducted by Rachdawong (1999), a probe was developed with the ability to distinguish between the three *Gaeumannomyces graminis* varieties. Isolates used in this study were assayed with her genetic probe. CK1A and CE1 were determined to be variety *graminis*, not variety *tritici*. This information in combination with the low virulence level of these two isolates tested indicates this may be the case. These two isolates produced simple hyphopodia however, indicating that the formation of simple hyphopodia may not be an appropriate indicator of variety.

Growth studies. Growth studies conducted indicate that the growth rate of the isolates tested from Virginia, Montana, and Britain were different than those obtained by Asher (1980). Asher tested many isolates at 22.5 C in 90 mm Petri dishes using a 4 mm fungal disc on PDA containing streptomycin. Growth rates were approximately 70-77 mm after 7d. Isolates tested in this laboratory grew approximately 50 mm after 8d at 25C on 20 ml PDA (100 X 15 mm Petri dish) growing from an 8 mm fungal plug taken from a plate containing 20 ml PDA. It would appear that our isolates grew more slowly, but Asher does not state the volume of agar in each plate, nor the volume of agar contained in the plug used. Asher noted variation in the growth rate of individual isolates, but the mean growth rates of populations were not significantly different.

Regression analysis. Regression of the growth data against the virulence data indicated that there was a trend which indicated that the faster growing isolates on PDA in the laboratory caused more damage on wheat in the greenhouse. There was variation among the three greenhouse tests, but the trend was the same over the three tests. Therefore, under these conditions it would not be useful to predict virulence for a given growth rate from these data. However, it is appropriate to suggest that saprophytic growth under these conditions may have contributed in an isolate's ability to penetrate, infect, and colonize a living wheat plant within the first month after planting. Dr. S. D. Garrett (1970) has indicated however that *Gaeumannomyces graminis* is not a good saprophyte (competitive saprophytic ability). Even in the controlled conditions of the greenhouse, R-values relating growth rate to virulence, were low. It should be expected therefore that under field conditions, little should be inferred from these data relating saprophytic growth rate to virulence.

Vegetative compatibility. Pairing studies resulted in interactions very similar to those noted by Jamil et al. (1984) in which vegetative compatibility (VC) groupings were determined. Almost all paired isolates caused an interaction resulting in clearing and in most cases adjacent mycelial darkening between the two isolates tested unless they were paired with a clone of the same isolate. Only in four cases out of 58 pairings where there was not a pairing between the same isolate was there a potentially compatible reaction (Table 6). These results suggest that different *Ggt* individuals within a field and within Virginia do not readily anastomose and share nuclei. It has been suggested by Jamil et

al.'s study, however, that due to similarities in double-stranded RNA from *Ggt* isolates that anastomosis may occur at a low level regardless of VC grouping.

Our tests, the first such tests conducted in the State of Virginia with *Ggt*, indicate that in all but three pairings among different isolates there was an incompatible reaction as previously described by Jamil et al (1984). Jamil et al. (1984) noted that a definite zone of interaction was produced as a result of putatively incompatible crosses. According to Leslie's review, this would indicate that since there are many VCGs, the population may be sexually reproducing. Studies examining the number of VCGs among multiple individuals within fields would be beneficial in studying this further.

Asher and Shipton (1981) noted that in previous studies, heterokaryon formation among isolates was rarely achieved. One study reported that only six of 45 pairs were apparently compatible while selfed pairs were compatible. A different study indicated that the frequency of heterokaryon formation was extremely low and noted that heterokaryon formation was even low between an auxotrophic mutant and a WT strain of itself. Our study indicated similarly that in only four pairings out of 58 different pairings (exluded self-pairings) was there an apparently compatible interaction.

Chemical resistant mutants. This is the first report that indicates benomyl resistance in *Ggt* may confer resistance to propiconazole as well as to other chemicals tested as putative chemical markers (nystatin, cyloheximide, and azoxystrobin).

In experiments testing for anastomosis, 100% of the wildtype mycelial fragments could not grow on media containing both benomyl and propiconazole. The percentage of living mycelial fragments from crosses in which benomyl resistant strains were crossed

with themselves was very high. At a propiconazole concentration of 15 μg/ml, 78% of the M1b X self fragments could grow, 20% of the CB1b X self, and 5% of the CD1b X self fragments. In all cases, 100% of the M1p X self-fragments were killed by the presence of benomyl.

Other chemical markers were not tested as extensively (see Tables 2-4 appendix C), but Ben^r *Ggt* isolates used in this study had become nystatin resistant up to approximately 4.5 μg/ml which was above the highest resistance of the putative nystatin resistant mutants. On agar amended only with nystatin, Ben^r isolates did grow better in two out of three isolates, but there was growth in all isolates and all three conditions (Ben^r, Ny^r, and WT). On agar containing both nystatin and benomyl, only benomyl isolates grew while all other isolates (Ny^r, and WT) were killed. The use of benomyl, nystatin, azoxystrobin, and cycloheximide were chosen due to their very different modes of action.

Benomyl is a mitotic inhibitor. The breakdown product of benomyl, methyl benzimidazole carbamate (MBC) binds to microtubular protein disrupting chromosome migration. Fungal resistance to benomyl is believed to be conferred by single base changes in the β-tubulin gene, thus altering the amino acids which make up β-tubulin (Yarden and Katan, 1993).

Nystatin, a polyene antibiotic, requires the presence sterol in the membrane for toxicity. It is hypothesized that pores are created in membranes by multiple polyene molecules forming hydrogen bonds with sterol molecules in the membrane. Additional hydroxyl groups of the polyene molecules face toward each other forming a pore, and the

amino sugar found on each molecule is pushed inward (inside the cell) or outward (toward the cell wall) away from the membrane. Cells are killed by the subsequent leakage of cellular constituents (Holz, 1979; Pratt and Fekety, 1986). Nystatin resistant fungi have a marked decrease in their ergosterol content (Vanden Bossche, 1997). Beezer et al. (1986), however, suggests that resistance may involve changes in cell wall components, specifically the amino-acid content and fatty acid chain length.

Cycloheximide, specific for cytosolic ribosomes, is a protein synthesis inhibitor (Oleinick, 1975). Protein production is inhibited by preventing the amino acids bound to RNA into protein (Sisler and Siegel, 1967). Resistance is conferred by one to many genes (Sisler and Siegel, 1967).

It is apparent that benomyl resistance confers resistance to other non-related antimicrobials. Goldway et al. (1995) suggested that the BEN super(r) gene of *Candida albicans* (Robin) Berkhout confers resistance in *Saccharomyces cerevisiae* Meyen ex E. C. Hansen to six structurally and functionally non-related drugs. Disruption of this gene leads to susceptibility of many of the previously ineffective chemicals. The basis for this resistance is unknown, but it is thought that impermeability in the fungal cell membrane may be responsible (Jia et al., 1993; Yaacov et al., 1994). The Ben super(r) gene is responsible for resistance in *C. albicans* to benomyl, but also to cycloheximide, benzitriazoles, 4-nitroquinoline-N-oxide, and sulfmeturon methyl (Yaacov et al., 1994).

Our tests indicate benomyl resistance did not confer cycloheximide resistance in the three *Ggt* isolates tested. This suggests that the Ben^r isolates created here are not exhibiting an impermeability to cycloheximide similar to other reports. The literature

does not mention benomyl resistance conferring resistance to nystatin as was the case in *Ggt*.

Conclusions Regarding Anastomosis and Chemically Marked Strains. Pairing isolates on PDA resulted in many putatively incompatible reactions resulting in a darkened barrage zone. This included the chemically marked strains, CB1b and M1p. We saw a low level of anastomosis however by crossing two chemically marked strains and forcing them to grow on a medium containing both chemicals. This indicated that even putatively incompatible strains could anastomose and share nuclei. This must mean therefore that under natural field conditions there is a possibility that on wheat or other host roots, in field debris such as wheat stubble, or potentially even in soybean debris, anastomosis may take place. Anastomosis creates a heterokaryon, which allows for greater diversity in the population of this organism. It is not specifically known what role conidia and ascospores have in the life cycle of this fungus, but with the production of perithecia on wheat or soybean debris, both ascospores and conidia would be produced and capable of dissemination. To confirm anastomosis among incompatible strains, similar testing will need to be continued. Using nitrate nonutilizing (Nit) mutants may be an alternative marker, which could enable the tracking of nuclei in *Ggt*.

Polymerase Chain Reactions and RAPD assays. Enough genetic differences existed between isolates in this study to see polymorphisms between the two parents studied. It is evident that RAPDs will be a valuable tool in fingerprinting specific *Ggt* isolates and studying populations. This is the first report of the use of bacterial Repetitive Extragenic Palindrome sequence (REP) primers for the fingerprinting of this

genus. DNA from *Ggt* generated approximately 26 major and minor bands with REP primers, which exhibited valuable polymorphisms between the two isolates tested. Traditionally REP primers have been used in the analysis of bacterial DNA from a variety of sources including those bacteria that cause plant disease (Versaloic et al. 1991; Lupski and Weinstock, 1992; Kerr, 1994; Louws et al. 1995 (a & b); Hurek et al. 1997; Townsend et al. 1997). Some fungal genomes have been examined with REP primers however (Edel et al. 1995; George et al. 1997). In George et al.'s study (1997), REP primers were effectively able to show differences in multiple *Magnaporthe grisea* (T. T. Hebert) Yaegashi & Udegawa isolates, including polymorphisms between those capable of infecting rice (*Oryzae sativa* L.) and those that infect other hosts. Edel et al.'s study (1995) showed that using REP primers was simpler and more rapid than using RFLPs of total DNA, and was able to discriminate well among isolates of *Fusarium oxysporum* Schlect. emend. Snyd. & Hans. We will need to test more primers for the amplification of polymorphisms. Primers showing differences between both parents and the progeny will be most effective in determining if anastomosis actually took place, and if both nuclei were in the same thallus.

Perithecial production and affect of double-croppage into soybeans. Traditionally, perithecia formed by *Ggt* could be found occasionally on diseased wheat debris. In the laboratory, no true single method has been established for perithecial production. Previous laboratory techniques involved complex media and procedures or the often unsuccessful surface disinfestation of wheat seed. This group determined that sterile soybean pods inoculated with *Ggt* and incubated under fluorescent light induced

the formation of perithecia. This method was extremely easy and reliable, and allowed the production of numerous perithecia. Crosses on soybean pods involving the use of a genetic marker were quickly and reliably achieved.

In tests in our laboratory, many isolates produced perithecia very well on sterile and nonsterile soybean pods. This may have epidemiological and ecological significance since nearly 100% of small grain fields are no-tillage-double-cropped to soybeans. Dr. Barry Cunfer (Plant pathologist, University of Georgia) stated in Southeast Farm Press that in the Southeast, take-all has "become a problem, possibly due to the double-croppage of soybeans" (Hollis, 1998). Double-croppage with soybeans is not a true rotation, and his work suggests that soybeans maintain the fungus well (Hollis, 1998). The closely related variety *Gaeumannomyces graminis* var. *graminis* has been isolated from soybean pods (Roy et. al. 1982). Virginia growers have increased acreage of intensively managed wheat, made common the practice of double-cropping with soybeans, and have noted an increased presence of take-all in Virginia's intensively managed wheat fields. Further work will need to be conducted to evaluate the importance of the epidemiological and ecological significance of this information. It should be noted that perithecia were produced even on sterile wheat straw as well as on some artificial media, although in much lower numbers. The implication is that sexual reproduction and the subsequent formation of perithecia and ascospores with new combinations of genetic material could naturally occur on wheat or soybean debris in the field.

The goal of this work was to evaluate *Gaeumannomyces graminis* isolates, many of which were collected in Virginia, genotypically and phenotypically. Based on pathogenicity and virulence testing, variation existed among isolates based on their relative ability to damage wheat. Several isolates which had less of an ability to damage wheat relative to the other isolates tested, produced simple hyphopodia. Based on work conducted by Rachdawong (1999), these isolates were *Ggg* based on a PCR-based discrimination test. Due to this, the use of hyphopodium morphology as a discriminating factor must be questioned. Many vegetative compatibility groups were determined. Based on a review by Leslie (1993), this may mean that the population is sexually reproducing. This also means that many genes must be in control of incompatibility in *Ggt* reducing the possibility of the transmittal of viruses. Based on chemical markers in two putatively incompatible *Ggt* strains, anastomosis took place at low levels. More testing among greater numbers of *Ggt* isolates will need to take place to determine variability across and within geographic boundaries. Soybean pods were discovered to be an excellent substrate for the production of perithecia. Not only will this technique be valuable as a common laboratory method for the induction of fruiting bodies, but the fact that soybean pods are such a good substrate leads to ecological and agricultural concerns. Approximately 100% of Virginia's wheat crops are double-cropped into soybeans. It is evident through this work that soybean pods could be an ideal substrate for the induction of *Ggt* fruiting bodies in the field. The challenge will be to determine if the presence of soybean debris leads to the increased variability among populations of *Gaeumannomyces graminis*.

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Table 1. Selected chemical and physical properties of a Kempsville loam (fine-loamy, siliceous, thermic Typic Hapludult) soil utilized in greenhouse bioassays.

Parameter	Analyses	Method†
Textural Classification	Sandy Loam	
Sand %	59.7‡	
Silt %	24.7	
Clay %	15.7	
Organic matter %	1.4	29-3.5.2
CEC (meq/100g)	3.9	(sum of cations)
pH	5.7	12-2.6, 12-3.4.4
NO_3 (µg/g)	9.0	33-3.2.1, 33-8.3
P (µg/g)	49.0 (Bray 1)	24-5.1
K (µg/g)	91.0	13-3.5.2
Mn(µg/g)	25.7	19-3.4

†All methods are listed in Methods of Soil Analyses Part 2. 1982. Chemical and Microbiological Properties 2nd Edition. American Society of Agronomy and Soil Science Society of America.

‡ Average of three separately analyzed samples

Table 2. *Gaeumannomyces graminis* variety isolates used in this study with Isolate origin, isolation information, and morphology of hyphopodia.*

Lab code	Origin	Year isolated or Reference	Hyphopodia
CB1	Bedford, VA	94	Simple
CD1	Dinwiddie, VA	94	Simple
CE1	Essex, VA	95	Simple
CE2	Essex, VA	96	Simple
CH1	Hanover, VA	95	Simple
Che1	Henrico, VA	95	Simple
CK1A	King & Queen, VA	95	Simple
CK1B	King & Queen, VA	95	Simple
CO1	Orange, VA	96	…
CS1	Southhampton, VA	95	Simple
CVab	Virginia Beach, VA	97	None formed
M1	Montana, USA	94	Simple
TGg	ATCC 12761	78†	Simple
TGgt	ATCC 28230	74‡	None formed
TOga	ATCC 15419	No reference available	Simple
Warsaw Richmond, VA		96	Simple
Ggt 3056			None formed
Ggg FL-175	Florida		Lobed
Ggg 2033			Lobed

*Isolates obtained from ATCC (12301 Parklawn Dr., Rockville, MD, 20852), Dr. D. Mathre (Montana State University, Boseman MT), or isolated from Virginia wheat plants with the aid of the selective medium SMGgt-3

†See Frick and Lister, 1978.

‡See Deacon, 1974.

Table 3. Appearance of the underside of 2-week-old *Gaeumannomyces graminis* var. isolates in culture.

Lab code	Bottom coloration	Comments
CB1	Met. 2B 2; 2F 2*	Some overall darkening
CD1	Met. 2B 2; 2F 2	Overall dark coloration
CE1	Met. 2B 3; 2F 3	Some overall coloration
CE2	Met. 2B 2; 2E 3	Darker overall coloration
CH1	Met. 1A 2	No darkening
Che1	Met. 2B 3; 2E 3	Slight browning around plug
CK1A	Met. 1A2; 1B 2	Slight gray-browning
CK1B	Met. 2B 2; 2E 3	Dark overall
CO1	Met. 2B 2; 2F 2	Some overall darkening
CS1	Met. 2B 2; 2F 3	Some overall coloration
CVab	Met. 2C 3; 2F 2	Some overall coloration
M1	Met. 2B 3; 2F 2	Striated graying
TGg	Met. 2D 3; 2F 2	Light mycelium with dark striations
TOga	Met. 4B 2; 4F 4	Even browning
War	Met. 2B 3; 2F 3	Some overall coloration

*Color was noted from the bottom of 2 week old cultures, 20 ml Sigma PDA where the first Met. (Methuen color chart, Kornerup and Wanscher, 1961) color represents the lighter mycelium and the second Met. color represents the dark pigmented mycelium which simultaneously occurred in plates.

Table 4. Relative plant damage in *Gaeumannomyces graminis* var-inoculated greenhouse-grown plants.*

	Test #											
	1	2	3	1	2	3	1	2	3	1	2	3
Isolate	Root necrosis rating (0-5)**			Root weight (mg)			Shoot weight (mg)			Shoot/Root		
M1	4.6 a	3.3 c	4.1 ab	26.7 cd	61.3 b	63.2 cd	41.7 cd	50.6 b	69.6 cde	1.9 ab	0.9 b	1.4 abc
CB1	4.4 a	...	4.1 ab	28.9 cd	...	46.4 d	45.3 cd	...	60.2 a	1.6 ab	...	1.5 ab
CD1	4.3 ab	5.0 a	4.1 ab	33.4 cd	18.6 c	72.5 bcd	42.7 cd	26.5 c	78.9 bcd	2.1 a	1.5 ab	1.3 bc
CE1	2.6 c	0.6 d	0.4 cd	53.2 bc	110.7 a	106.6 ab	58.9 abc	78.3 a	98.6 ab	1.3 abc	0.9 b	1.0 bc
CE2	3.7 ab	4.4 ab	3.4 b	55.7 bc	59.8 b	89.9 abc	57.7 a-d	53.8 b	88.0 abc	1.2 bc	1.1 ab	1.2 bc
CH1	…	0.6 d	...	...	107.9 a	...	...	75.0 a	...	...	0.7 b	…
Che1	3.4 b	...	...	70.9 ab	...	...	67.9 ab	...	...	1.0 c	...	…
CK1A	1.3 d	...	...	78.9 ab	...	...	73.9 a	...	...	1.1 bc	...	…
CK1B	4.3 ab	4.4 ab	4.1 ab	55.6 bc	41.7 bc	56.2 cd	51.4 bcd	43.1 bc	70.0 cde	1.4 abc	1.9 a	1.4 abc
CO1	3.9 ab	...	4.7 a	35.5 cd	...	37.7 d	43.9 cd	...	50.6 e	1.9 ab	...	1.8 a
CS1	4.3 ab	4.3 ab	3.9 ab	20.0 d	32.3 bc	51.5 d	40.8 d	36.1 bc	75.5 b-e	2.1 a	1.3 ab	1.5 ab
Toga	0.1 e	0.4 d	0.9 c	71.9 ab	96.7 a	109.2 ab	73.2 a	75.6 a	105.5 a	1.0 bc	8.0 b	1.0 bc
Tgg	0.7 de	...	0.4 cd	72.3 ab	...	109.5 ab	73.0 a	...	100.9 ab	1.1 bc	...	1.8 bc
War	...	4.0 bc	...	...	46.0 bc	...	...	45.0 bc	...	...	1.2 ab	...
PDA Check	0.0 e	0.0 d	0.0 d	86.1 a	118.8 a	114.2 a	72.5 a	85.1 a	99.9 ab	0.9 c	0.7 b	0.9 c

* Determined after one month growth in a greenhouse by each *Gaeumannomyces* sp. isolate inoculated as two, eight mm plugs at seed level at planting. Means followed by the same letter do not significantly differ ($p \leq 0.05$)

**Rating: 0 =no root lesions, 1 = 1 to several root lesions, 2 = extensive root necrosis, 3 = lesions on roots as well as any darkening of crown, 4 = extensive darkening of crown, 5 = plant killed or nearly dead with extensive necrosis, … = not tested..

Table 5. Compilation of *Gaeumannomyces graminis* var. isolates relative virulence level, growth rate, and total growth.

Lab code	Approximate Virulence Level*	Approximate** Growth Rate/d	Total Growth (mm) ***
CB1	3	12	49
CD1	4	16	50
CE1	1	11	23
CE2	2	12	42
CHe1	1	6	8
CK1A	1	16	44
CK1B	2	11	36
CO1	3	14	44
CS1	4	17	54
M1	2	16	48
TGg	1	12	17
TOga	1	12	46
War	3	…	…

*0=non-pathogenic, 1= slightly virulent, 2=virulent, 3=very virulent, 4= extremely virulent; a subjective rating based on the virulence data presented in Table 4 and compiled in Figure 1.
**After 8 days, in mm.
***After 13 days, in mm.

Table 6. Interaction between wildtype *Gaeumannomyces graminis* var. *tritici* isolates paired on PDA.*

	M1	CB1	CD1	CE1	CE2	CH1	CK1A	CK1B	CO1	CS1	CVab	War
M1	C	R	R	R	R	R	R	R	R	R	R	R
CB1		C	R	R	R	R	R	R	R	C	R	R
CD1			C	R	R	R	R	C	R	R	R	R
CE1				C	R	R	R	R	…	R	R	…
CE2					C	R	C	R	R	R	R	…
CH1						C	R	C	…	R	R	…
CK1A							C	R	R	R	R	…
CK1B								C	R	R	R	…
CO1									C	R	R	R
CS1										C	R	R
CVab											C	…
War												C

*Four plugs consisting of two isolates placed opposite each other (approximately 4-5 cm) creating a test barrage (between plugs of different isolates) and a control barrage (between plugs of the same isolate) on the same plate grown at 25 C in the dark, and examined for any interaction between pairings at the barrage zones. All except CH1, CVab, and War tested twice.

**C = apparent compatibility, R = apparent reaction.

Table 7. Percent growth of single spored ascospores resulting from crosses between wildtype or benomyl resistant strains of *Ggt* on soybean pods, plated onto PDA or benomyl-amended PDA.

Isolate	Cross	Percent* ascospore living at benomyl concentration (µg/ml) 0	1.5	2.5
M1	WT X WT	100	0	0
	Ben^r X WT	100	53	47
	Ben^r X Ben^r	100	87	93
CB1	WT X WT	93	0	0
	Ben^r X WT	100	0	0
	Ben^r X Ben^r	100	93	100
CD1	WT X WT	100	0	…+
	Ben^r X WT	100	87	73
	Ben^r X Ben^r	93	100	100

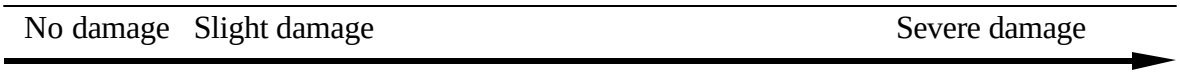
*Fifteen ascospores plated for each cross and benomyl concentration. At least 8-10 perithecia were chosen across the plate and ascospores combined to represent each cross.

+Plates contaminated.

Table 8. Percent growth of fragments of wildtype (WT) isolates and crossed chemical resistant strains of *Gaeumannomyces graminis* var *tritici* on benomyl and propiconazole amended agar.

	Percentage of living mycelial fragments at 18 µg/ml propiconazole and 1.2 µg/ml benomyl	
	TEST 1	TEST 2
Cross		
M1p* X CB1b	10	2
M1p X self	0	0
CB1b X self	0	0
WT M1 X self	0	0
WT CB1 X self	0	0
WT M1 X WT CB1	0	0

*p=propiconazole resistant, b = benomyl resistant, WT = wildtype



PDA Check	Toga	CE2	CB1	CS1
	Tgg	CK1B	CO1	CD1
	CK1A	M1	Warsaw	
	CE1			
	CHe1			

Figure 1. Subjective grouping of *Gaeumannomyces graminis* varieties based on their ability to cause damage to soft red winter wheat plants after one month in the greenhouse. Bioassay based on the dated presented in Table 4.

A

B

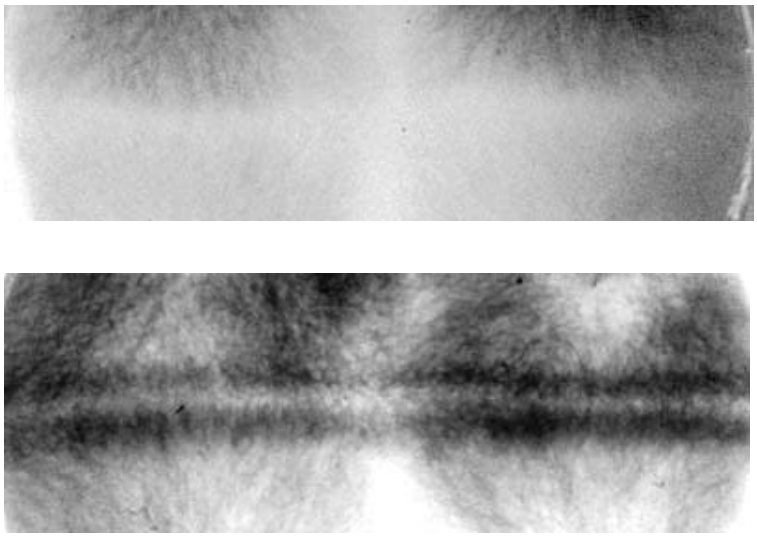


Figure 2. Interaction between putatively compatible (A) and incompatible (B) strains of *Ggt* paired on Potato Dextrose Agar.

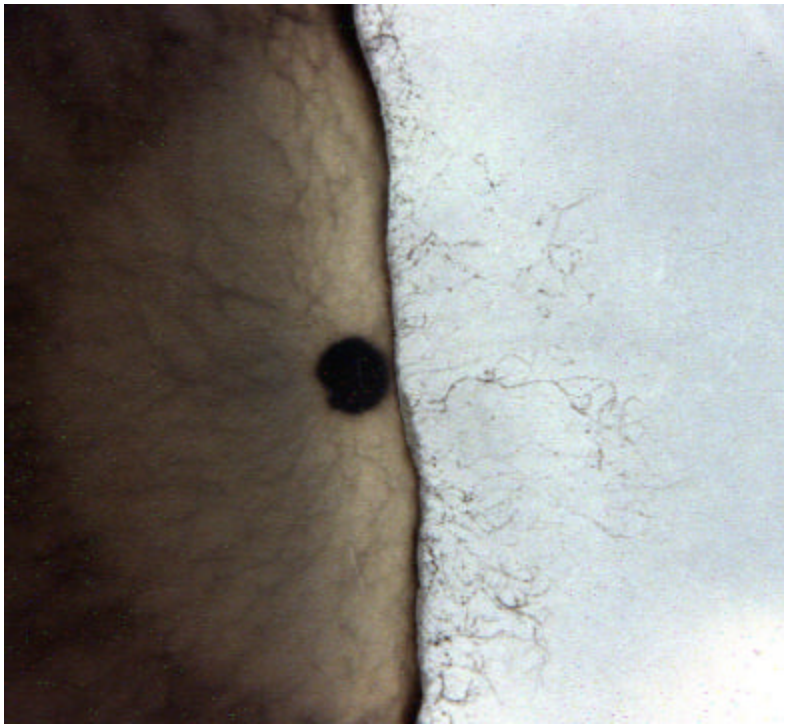


Figure 3. Hyphopodia **"**peppered" bare polystyrene Petri dish bottom from mycelial growth of isolate CK1A. Note the dark central 4 mm dia. mycelial plug located on the upper surface of the dilute V8 agar.

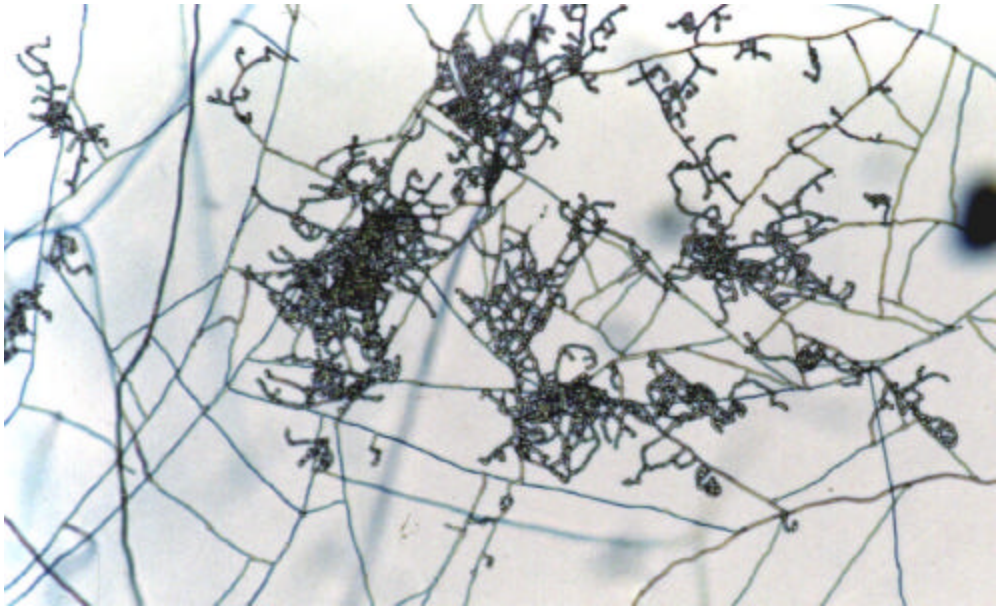


Figure 4. Aggregations of short hyphae produced by isolate CE2, typical of the formation of simple hyphopodia. (100X).

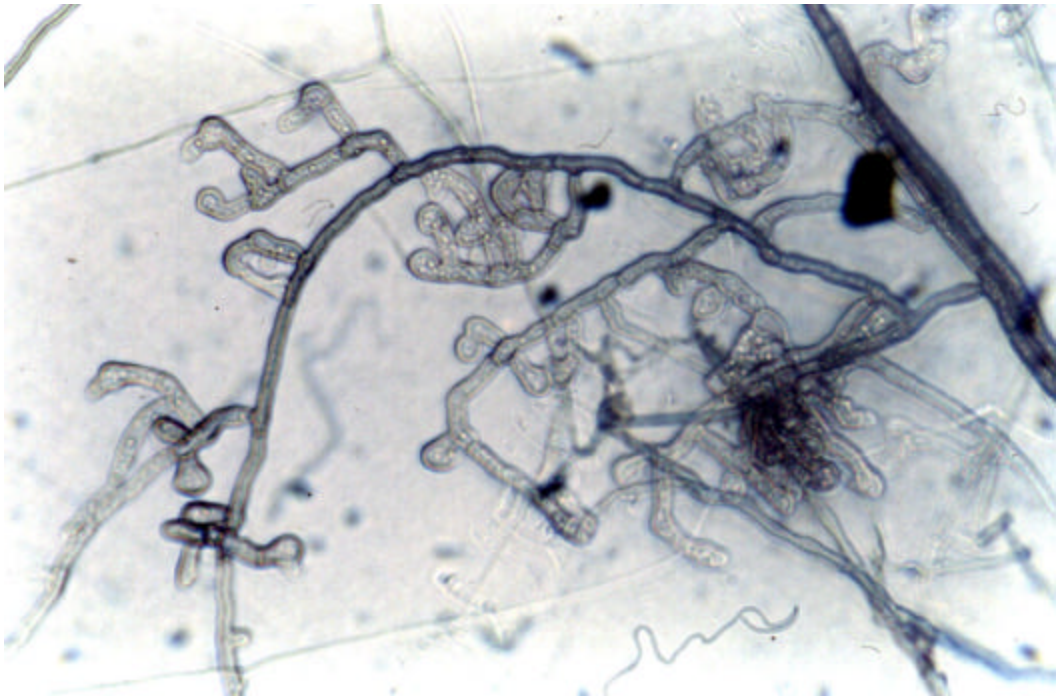


Figure 5. Aggregations of short hyphae produced by isolate CK1A, typical of the formation of simple hyphopodia. (400X). Hyphopodia are approximately 10 µm in width.

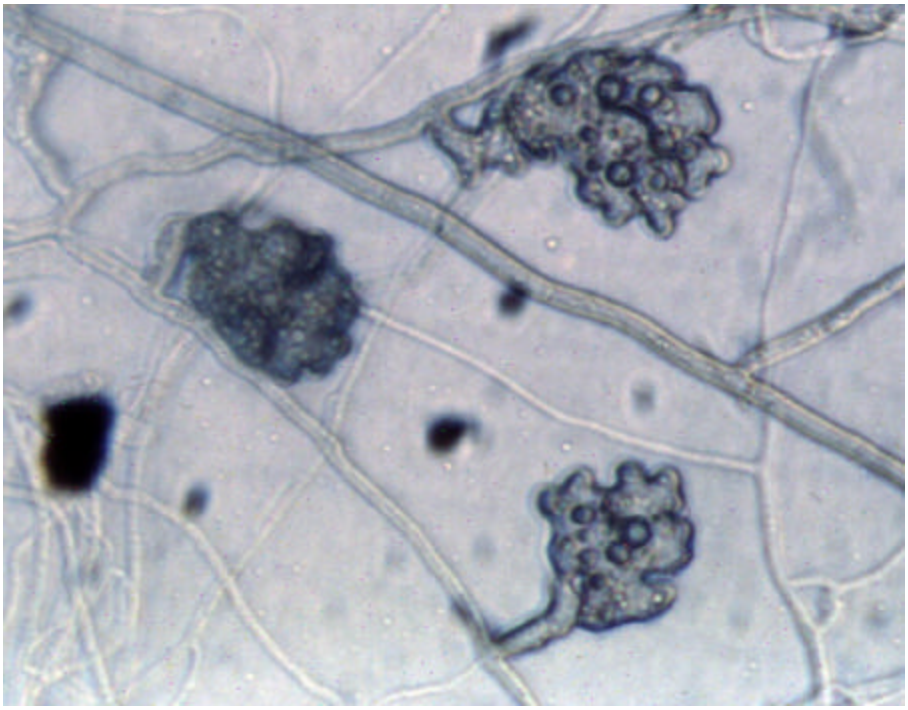


Figure 6. Lobed hyphopodia produced by *Ggg* isolate 2033. (400X).

Hyphopodia are approximately 32 X 26 µm.

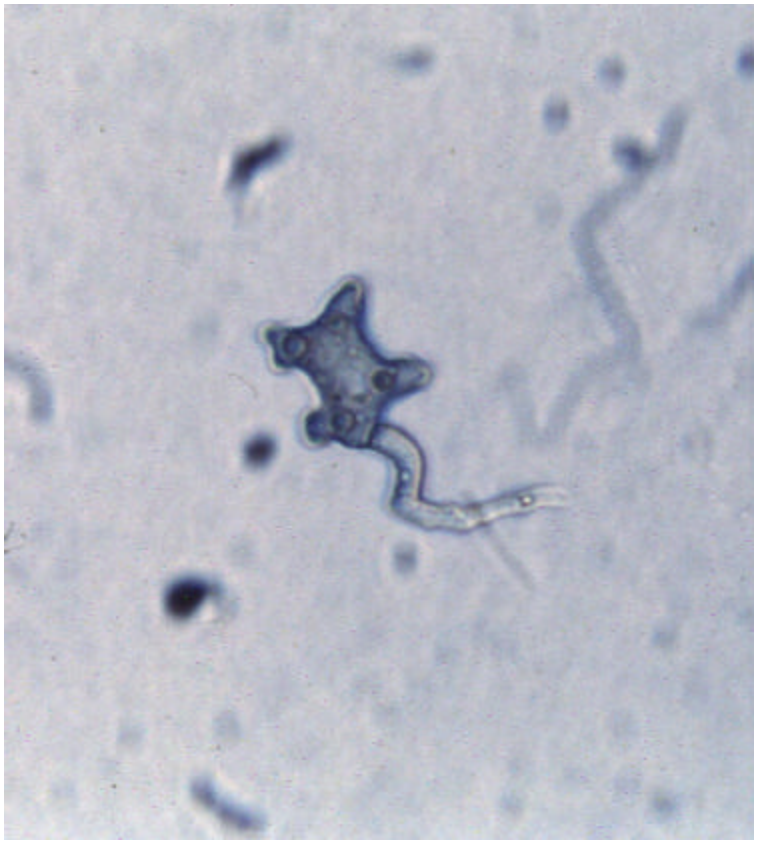


Figure 7. Lobed hyphopodium produced by *Ggg* isolate FL-175. (400X). Hyphopodium is approximately 28 X 24 µm.

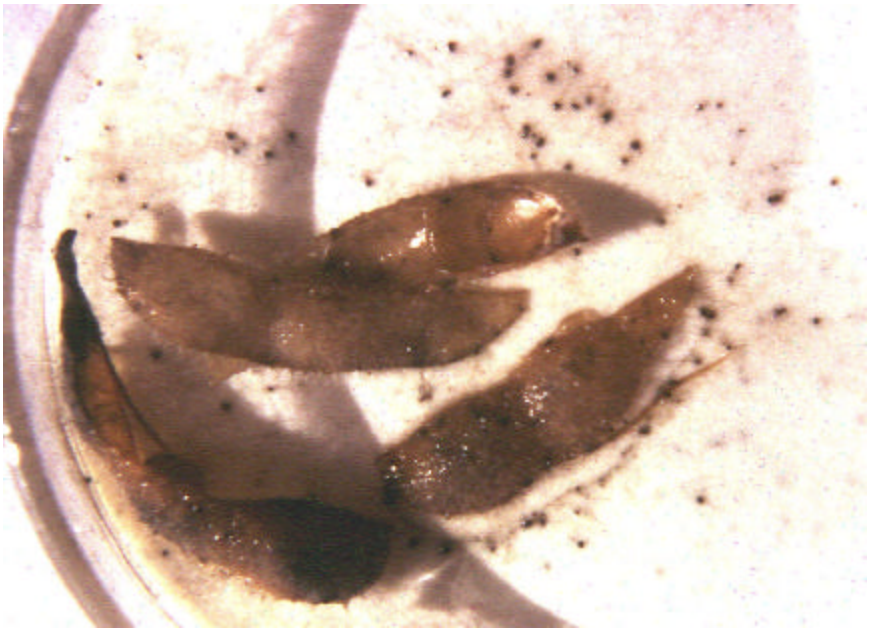


Figure 8. Production of perithecia on sterile soybean pods and filter paper. Soybean pods are approximately 3.0-4.0 cm in length.

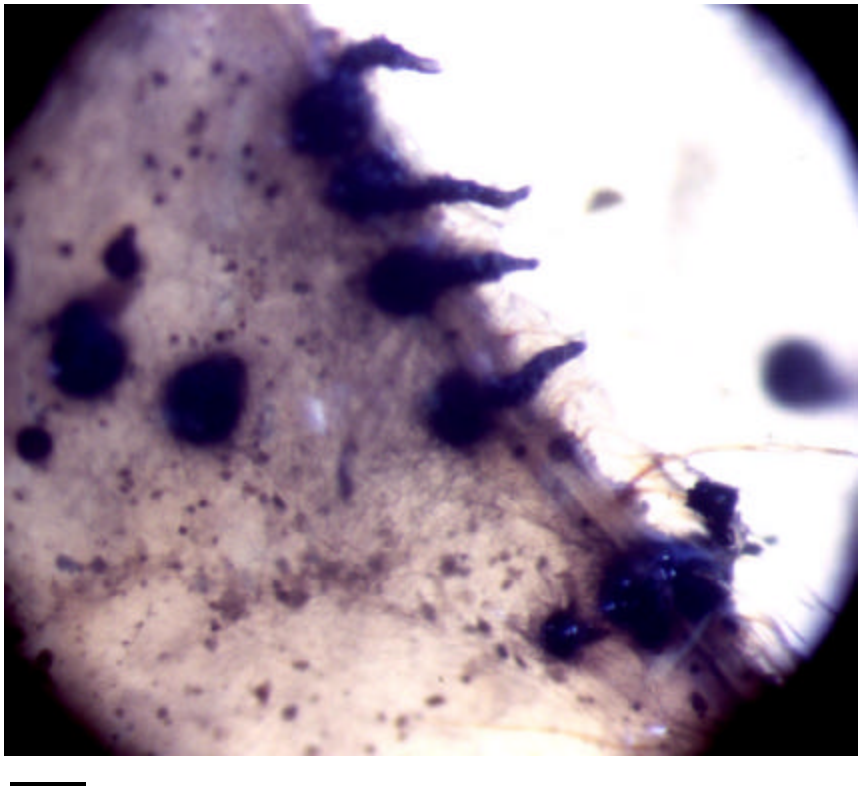


Figure 9. Necked perithecia formed on sterile soybean pods by *Ggt* isolate CB1. Bar = 350 μm.

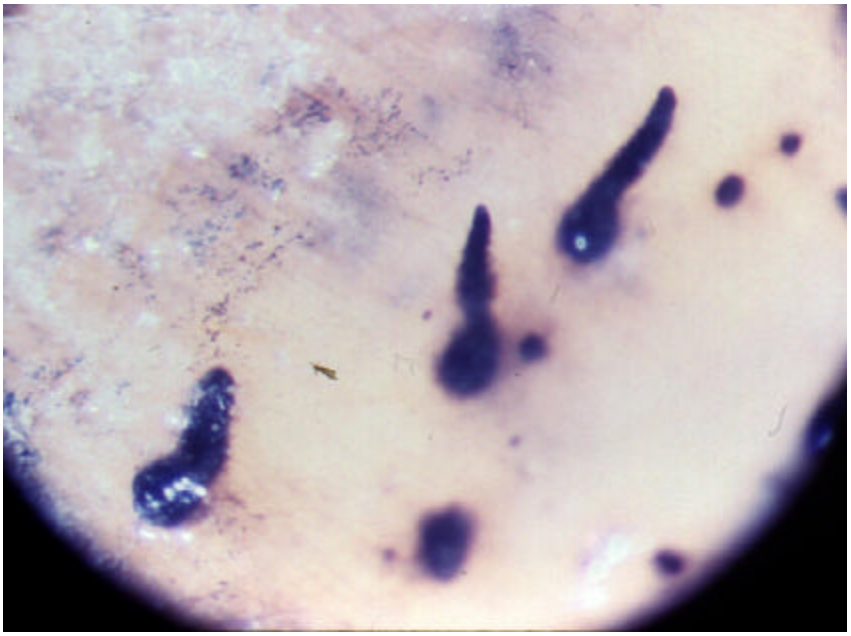


Figure 10. Necked perithecia produced on a non-sterile soybean pod by *Ggt* isolate CD1

Bar = 250 µm.

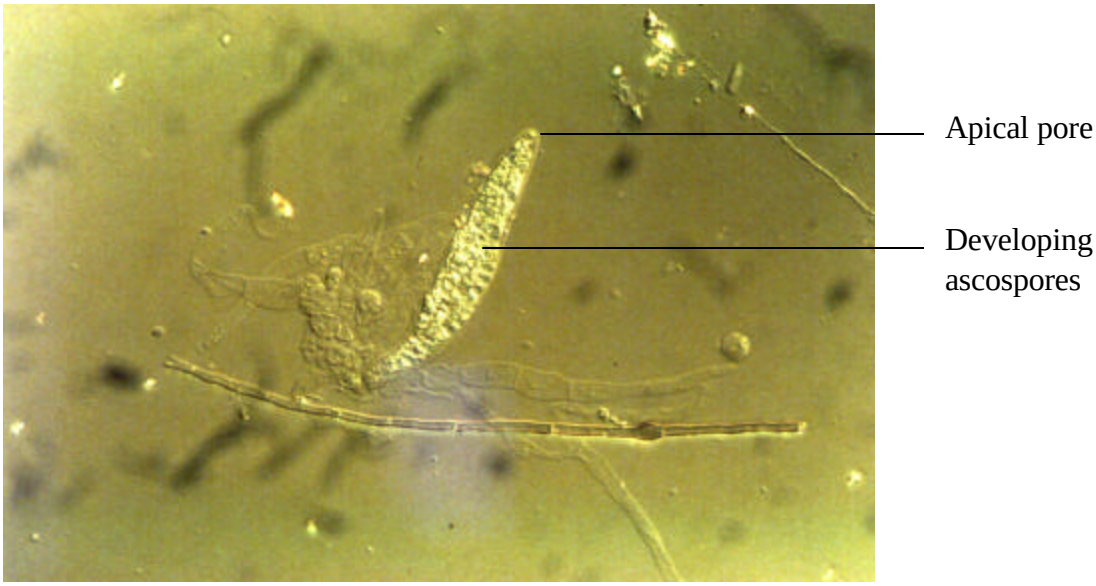


Figure 11. Young developing ascus with refractive apical pore typical of *Gaeumannomyces graminis*.

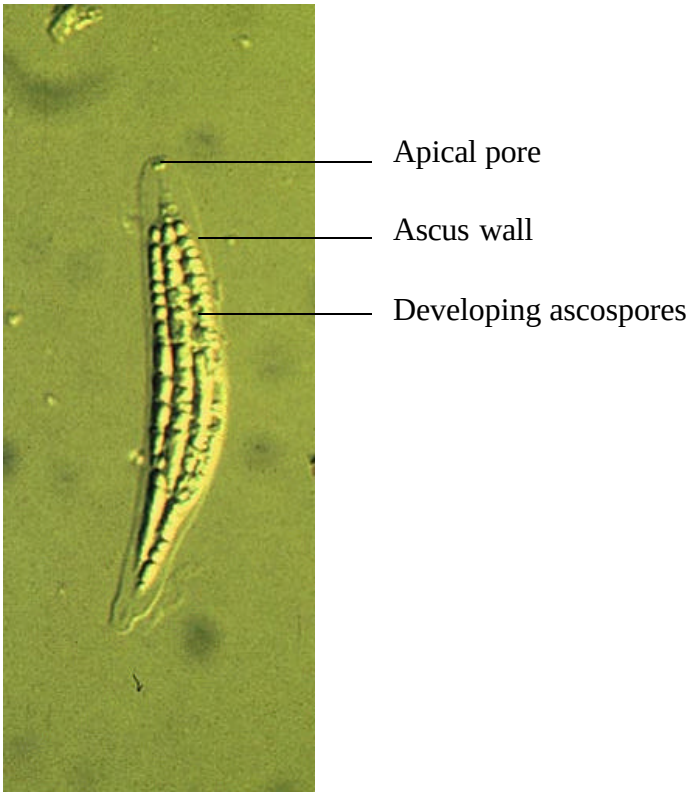


Figure 12. Ascus with ascospores and refractive apical pore typical of *Gaeumannomyces graminis*. Typically eight, 60-110 µm slightly curved ascospores are formed.

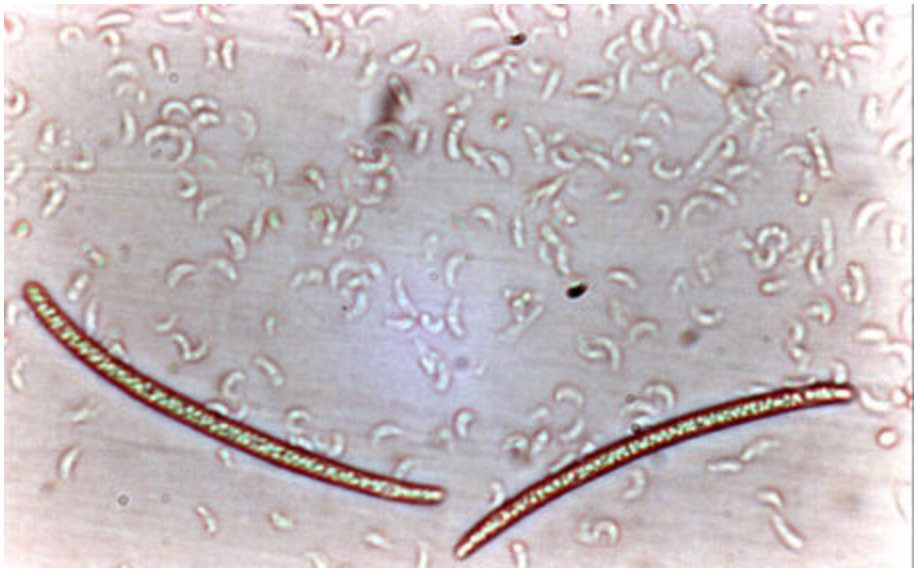


Figure 13. Typical long (60-110 X 3-5 µm) slightly curved ascospores, and small curved conidia (approximately 5 X 2 µm) of *Gaeumannomyces graminis* which are capable of forming on phialides, budding from ascospores, or are found in the matrix exuded from the osteole of perithecia.

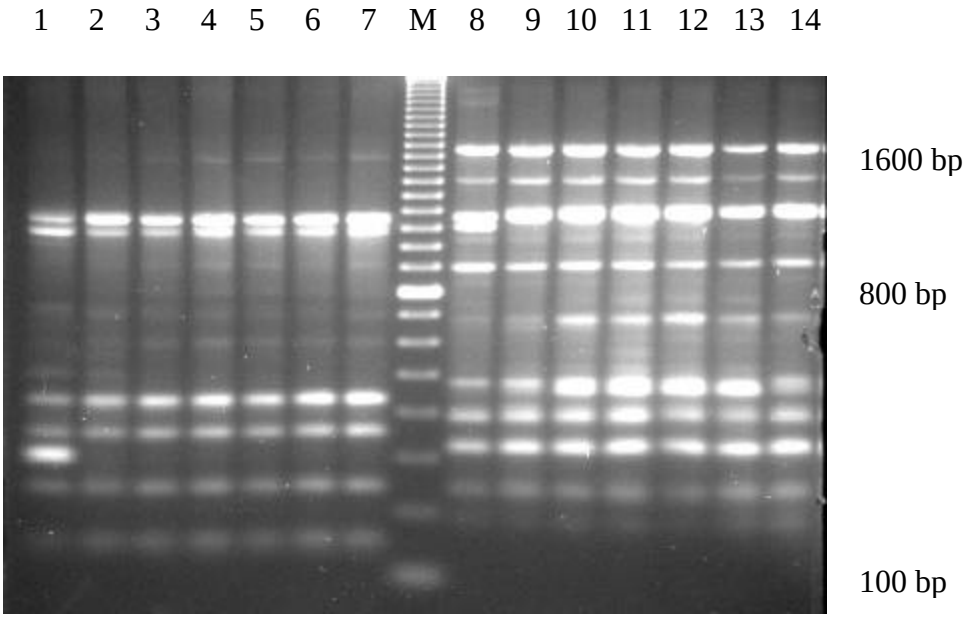


Figure 14. Randomly amplified polymorphic DNA (RAPD) banding patterns generated with Amersham Pharmacia Biotech primers 3 (5'-GTAGACCCGT-3') and 6 (5'-CCCGTCAGCA-3') from two chemically marked *Gaeumannomyces graminis* var *tritici* parents and their five progeny. The 100 base pair molecular marker is in lane M where the DNA in the bright middle band is 800 base pairs. From left to right, lanes 1-7 are M1p, CB1b, progeny 1, progeny 2, progeny 3, progeny 4, and progeny 5 with primer 3. Lanes 8-14 are M1p, CB1b, progeny 1, progeny 2, progeny 3, progeny 4, and progeny 5 with primer 6.

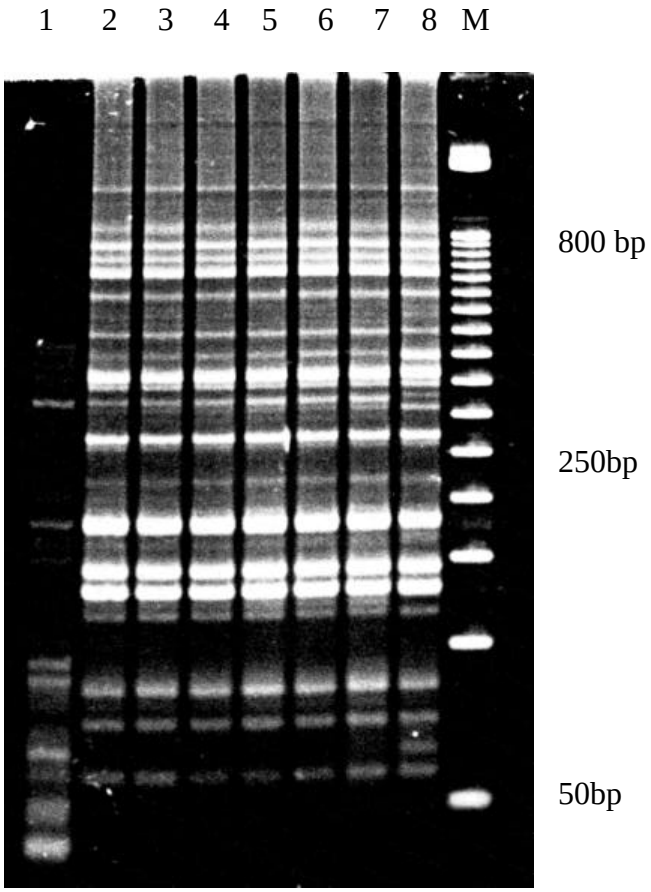


Figure 15. Randomly amplified polymorphic DNA (RAPD) banding patterns generated with Repetitive Extragenic Palindrome (REP) primers (REP 1; 5'-IIIICGICGICATCIGGC-3', REP 2; 5'-ICGICTTATCIGGCCTAC-3') in combination from two chemically marked *Gaeumannomyces graminis* var *tritici* parents and their five progeny. The 50 base pair molecular marker is in lane M. From left to right, lane 1 is no template (REP 1 and REP 2 primers only), lanes 2-8 are progeny 5, progeny 4, progeny 3, progeny 2, progeny 1, CB1b, and M1p.

APPENDIX A

Media:

1. PDA (potato dextrose agar)

Autoclave (121 C) for 15-20 min 24 g Sigma® PDB (potato dextrose broth) and 15 g Sigma® agar in 1 L distilled water (Sigma, P.O. Box 14508, St. Louis, MO, 63178, USA).

2. Selective medium (Sm-Ggt3)

Sigma PDA is prepared as above

To one L cooled medium (approximately 50 C), add:

10 mg ai dichloran (12.5 mg Botran 75 WP®, Gustafson Inc., Dallas, TX 75266)

10 mg ai metalaxyl (40 µl Subdue® 2 E, Novartis, Greensboro, NC 27419)

25 mg ai Hoe 703 (experimental and no longer available chemical from Hoecht)

100 mg streptomycin sulfate

500 mg L-DOPA

3. WA (water agar)

Autoclave (121 C) for 15-20 min 15 g Sigma agar in 1 L distilled water.

4. Benomyl, nystatin, cycloheximide, azoxystrobin, and propiconazole-amended agars

Sigma PDA is prepared as above with the chemical added to a volumetric flask and brought up to volume with ethanol, methanol, dimethyl sulfoxide (DMSO), or sterile distilled water as appropriate for the particular chemical. DMSO was the better of the two solvents for benomyl and nystatin at 10µg / 50 ml, while ethanol was appropriate for 5 µg/ml benomyl. Methanol was used to dissolve the azoxystrobin, and the sterile distilled water was used with the propiconazole which was from the commercial product Banner®. Fresh chemical stock solution was added to cooled (approximately 50 C) agar.

Mineral solutions:

One of the following added to 1 L distilled water, 40 ml of which is added to cones to yield a 30 µg / ml solution per cone:

530.7 mg $(NH_4)_2SO_4$

820.0 mg $CaNO_3$

321.4 mg NH_4NO_3 (or 428.6 mg for 30ml solution added to cone for 30 µg / ml)

546.8 mg $CaSO_4$ added to $CaNO_3$ and NH_4NO_3 solutions to balance SO_4^-

Isolation of *Ggt* fungus:

Fresh wheat samples from the field were washed under running tap water to remove soil from the roots. Roots were cut into several mm pieces and “cookies” were made from necrotic crown and culm tissue with a sharp razor blade. Wheat tissue was

placed into 10 % Clorox® (0.5% hypochlorite) for approximately 1 min with agitation, then transferred to sterile distilled water, rinsed, and blotted on sterile paper towels. Several pieces of wheat tissue were placed onto Sm-Ggt3 medium with sterile forceps. After several days at 25 C, plates were examined for the presence of fungal growth, specifically growth accompanied by a darkening of the medium. Potential isolates were transferred to Sigma PDA for further observation.

Hyphal tipping of *Ggt* isolates:

Several plugs of *Ggt* mycelium were placed onto sterile millet seed (Setaria italica L.), and allowed to colonize the seed at 25 C until all of the seed appeared to be coated with the fungus. Colonized millet seed was sprinkled onto water agar and incubated at 25 C for several days. An alternative procedure was to lace very small peices of fungal tissue directly onto water agar, and mince the peices into very small peices, spreading them across the agar surface. In either case, with the development of hyphae, hyphal tips were removed with the aid of a dissecting microscope and a curved sterile metal pick. Hypal tips were transferred to PDA for growth at 25 C.

APPENDIX B

Table 1. *Bacillus* isolate BII evaluated for the suppression of take-all caused by *Gaeumannomyces graminis* var. *tritici* which had been mixed into the soil as one month old millet seed inoculum (10 g/ Kg soil) with plant health measured by dry shoot weight, root weight, and a root necrosis rating after being grown in a greenhouse for one month.

	Test #							
	1*	2**	1	2	1	2	1	2
Treatment	Root Rating (0-5)***		Root Weight (mg)		Shoot Weight (mg)		Shoot/Root	
B- Ggt- TSB****	0.3 b	...	74.6 a	...	81.2 a	...	1.2 a	...
B+Ggt -TSB	0.1 b	...	89.4 a	...	86.2 a	...	1.0 a	...
B- Ggt +TSB	4.6 a	...	28.6 b	...	33.3 b	...	1.5 a	...
B+Ggt +TSB	4.7 a	...	24.9 b	...	29.4 b	...	1.5 a	...
B- Ggt - PDB	0.3 b	0.3 b	79.6 a	7.6 a	81.9 a	67.3 a	1.0 a	1.8 a
B+Ggt - PDB	...	0.3 b	...	41.2 a	...	79.7 a	...	2.0 a
B- Ggt +PDB	...	5.0 a	...	10.2 b	...	30.0 b	...	2.4 a
B+Ggt +PDB	4.6 a	4.1 a	31.8 b	32.4 a	36.9 b	45.2 b	1.2 a	1.8 a

Means in columns followed by the same letter do not significantly differ ($P \leq 0.05$)

* Seed dressing, 4.1 X 10^4 CFU

** Drench, 1.5 X 10^7 bacteria / seed

*** Rating: 0 =no root lesions, 1 = 1 to several root lesions, 2 = extensive root necrosis, 3 = lesions on roots as well as any darkening of crown, 4 = extensive darkening of crown, 5 = plant killed or nearly dead with extensive necrosis.

****B+/- = with or without bacterium, Ggt +/- = with or without Ggt, TSB =TryptoseSoyBroth,PDB=PotatoDextroseBroth

Table 2. *Bacillus* Isolate 202-10B evaluated for the suppression of take-all caused by *Gaeumannomyces graminis* var. *tritici* which had been mixed into the soil as one month old millet seed inoculum (10 g/ Kg soil) with plant health measured by dry shoot weight, root weight, the shoot to root ratio, and a root necrosis rating after being grown in a greenhouse for one month

	Test #															
	1*	2**	3***	4****	1	2	3	4	1	2	3	4	1	2	3	4
Treatment	Root Necrosis Rating (0-5)[V]				Root Weight (mg)				Shoot Weight (mg)				Shoot/Root			
Ggt +	5.0 a	4.7 a	4.6 a	4.7 a	10.2 b	29.4 b	17.7 b	8.8 b	30.0 c	56.6 b	40.7 b	25.8 b	2.4 a	2.0 a	4.1 a	3.1 a
Ggt -	0.3 b	0.3 b	0.3 b	0.1 b	37.6 a	56.3 a	54.6 a	29.2 a	67.3 ab	83.4 a	86.9 a	63.9 a	1.8 a	1.5 b	1.6 b	2.4 a
Ggt + B+	4.1 a	4.3 a	4.8 a	4.7 a	30.9 a	37.4 b	31.9 b	9.4 b	52.4 b	52.4 b	46.7 b	27.3 b	1.8 a	1.5 b	1.9 b	3.7 a
Ggt - B -	0.3 b	0.2 b	0.4 b	0.4 b	41.4 a	56.8 a	46.9 a	29.2 a	74.0 a	91.5 a	80.6 a	66.8 a	1.8 a	1.7 b	1.6 b	2.4 a

Means in columns followed by the same letter do not significantly differ ($P \leq 0.05$)
B+/- = with or without bacterium, Ggt +/- = with or without Ggt

* Drench, 1.5 X 10^7 bacteria / seed

** Drench, 4.4 X 10^9 bacteria / seed

*** Drench, 1.1 X 10^9 bacteria / seed

**** Drench, 6.0 X 10^8 bacteria / seed

[V]Rating: 0 =no root lesions, 1 = 1 to several root lesions, 2 = extensive root necrosis, 3 = lesions on roots as well as any darkening of crown, 4 = extensive darkening of crown, 5 = plant killed or nearly dead with extensive necrosis.

Table 3. Fluorescent pseudomonad 407-7 evaluated for the suppression of take-all caused by *Gaeumannomyces graminis* var. *tritici* which had been mixed into the soil as one month old millet seed inoculum (10 g/ Kg soil) with plant health measured by dry shoot weight, root weight, the shoot to root ratio, and a root necrosis rating after being grown in a greenhouse for one month

	Test #															
	1 *	2**	3***	4****	1	2	3	4	1	2	3	4	1	2	3	4
Treatment	Root Necrosis Ratingt (0-5)[v]				Root Weight (mg)				Shoot Weight (mg)				Shoot/Root			
Ggt +	5.0 a	4.7 a	4.6 a	4.7 a	10.2 b	29.4 b	17.7 b	8.8 b	30.0 b	56.6 b	40.7 b	25.8 b	2.4 a	2.0 a	4.1 ab	3.1 ab
Ggt -	0.3 b	0.1 c	0.3 b	0.0 b	37.6 a	57.1 a	54.6 a	30.8 a	67.3 a	84.4 a	86.9 a	66.1 a	1.8 a	1.5 b	1.6 b	2.0 b
Ggt + B+	4.8 a	4.0 b	5.0 a	4.7 a	17.2 b	52.9 a	7.9 b	9.1 b	34.1 b	66.1 b	30.6 c	28.7 b	2.2 a	1.4 b	5.6 a	4.0 a
Ggt - B -	0.3 b	0.3 c	0.7 b	0.4 b	41.0 a	49.3 a	52.6 a	30.3 a	74.6 a	87.3 a	79.8 a	67.2 a	1.9 a	1.8 ab	1.6 b	2.4 ab

Means in columns followed by the same letter do not significantly differ ($P \leq 0.05$)
B+/- = with or without bacterium, Ggt +/- = with or without Ggt

* Drench, 1.5×10^5 bacteria / seed

** Drench, 1.0×10^{10} bacteria / seed

*** Drench, 2.1×10^9 bacteria / seed

**** Drench, 2.8×10^8 bacteria / seed

[V]Rating: 0 =no root lesions, 1 = 1 to several root lesions, 2 = extensive root necrosis, 3 = lesions on roots as well as any darkening of crown, 4 = extensive darkening of crown, 5 = plant killed or nearly dead with extensive necrosis.

APPENDIX C

Table 1. General observations on the production of perithecia *in vitro* where an 8mm plug of Ggt mycelium was placed into a test tube containing a wheat seedling germinating in 0.25 strength PDA.

Isolate	Production of Perithecia	Ascospore Length
CB1	Yes	83.6 µm (77.1-97.7)
CD1	Yes	81.5 µm (64.3-95.1)
CE1	Abundantly	85.3 µm (69.4-95.1)
CH1	Yes	…
Che1	One noted	…
CK1A	Yes	…
CK1B	Abundantly	…
CS1	One noted	…
M1	Yes	79.4 µm (72.0-87.4)
Tgg	None	…
TOGa	Toga	…

Table 2. Interaction between wildtype (WT) isolates and chemical resistant strains of *Gaeumannomyces graminis* var *tritici*. paired on PDA.*

	TEST 1[B]	TEST 2[B]	TEST 3[D]	TEST 4		TEST 5		TEST 6	
Cross or isolate	# plugs & reaction[E]	#plugs & reaction[E]	Reaction	Reaction[F] (set a)[A]	(set b)[B]	Reaction[F] (set a)[B]	(set b)[C]	Reaction[F] (set a)[B]	(set b)[C]
CB1WT *	1-	...	...	...	...	...	...	...	...
CD1WT	1-	...	...	NG	NG	...	...	...	...
CB1Ny^r	1-	...	...	...	...	...	...	...	...
CB1Ben^r	1-	...	...	...	...	...	...	...	...
CD1Ny^r	1-	...	...	...	...	...	...	...	...
CD1Ben^r	1-	...	...	...	...	...	...	...	...
CB1WT X CB1WT	...	...	...	HT	HT	NG	NG	NG	NG
CB1WT X CB1Ny^r	...	...	...	HT	HT	NG	NG	NG	NG
CB1WT X CB1Ben^r	...	...	...	G	G	G	HT	G	HT
CD1WT X CD1WT	...	...	...	...	...	NG	NG	NG	NG
CD1WT X CD1Ny^r	...	...	...	NG	NG	NG	NG	NG	NG
CD1WT X CD1Ben^r	...	...	...	G	G	G	HT	G	HT

CB1Ny^{r} X CB1Ben^{r}	5-	10-	G***	G	G	G	HT	G	HT
CD1Ny^{r} X CD1Ben^{r}	2-	9-	G	G	G	G	HT	G	HT
CD1Ny^{r} X CB1Ben^{r}	5+	5+	G	G	G	G	G	G	HT
CD1Ben^{r} X CB1Ny^{r}	4-**	12-	G	G	G	G	HT	G	HT
M1WT X M1WT	...	...	...	...	...	NG	NG	NG	NG
M1WT X M1Ny^{r}	...	...	...	...	...	NG	NG	NG	NG
M1WT X M1Ben^{r}	...	...	...	...	...	G	HT	G	HT
M1Ny^{r} X M1Ben^{r}	...	...	...	...	...	G	HT	G	HT
M1Ny^{r} X CB1Ben^{r}	...	...	...	...	...	G	HT	G	HT
M1Ben^{r} X CB1Ny^{r}	...	...	...	...	...	G	HT	G	HT
M1Ny^{r} X CD1Ben^{r}	...	...	...	...	...	G	HT	G	NG
M1Ben^{r} X CD1Ny^{r}	...	...	...	...	...	G	HT	G	HT

* Plugs consisted of two isolates placed opposite each other creating a test barrage and a control barrage on the same plate were grown at 25 C in the dark, and were examined for anastomosis between pairings at the barrage zones by placing barrage plugs on a dual nystatin(ny) / benomyl(ben) medium, or covering entire colonies with the dual medium and examining for growth.

^{A} Four mm plugs plated onto dual medium consisting of 1.2µgbenomyl&2.5µg nystatin / ml agar.

^{B} Four mm plugs plated onto dual medium consisting of 1.2µgbenomyl&3.5µg nystatin / ml agar.

^{c} Four mm plugs plated onto dual medium consisting of 1.5µgbenomyl&4.5µg nystatin / ml agar.

^{D}Fifteen, 4mm plugs from each isolate alternated on a 150 X 25 mm petri dish with 80 ml PDA.

[E] + or - refers to growth or no growth respectively, Test 1 plated after 5 d, Test 2 plated after 15 d
[F] Five plugs per plate after 7-10 d, G = growth, NG = no growth, HT = hyphal tips < 1mm
[*] WT = wildtype, Ben^r = benomyl, Ny^r = nystatin
[**] One plug grew a minute amount of mycelium
[***] Growth in test 3 was only after approximately 6 weeks

Table 3. *Ggt* isolates CB1, CD1, and M1 [Wildtype (WT), nystatin resistant (Ny^r), and benomyl resistant (Ben^r)] grown on PDA containing 0.5 µg / ml or 0.7 µg / ml cycloheximide for approximately 5 - 7 d.

Isolate	Resistance	Reaction					
		Test 1*	Test 2*	Test 3		Test 4	
				A*	B**	A*	B**
CB1	WT	NG	G	G	G	G	HT
	Ny^r	G	G	G	G	G	G
	Ben^r	HT	G	G	G	G	G
CD1	WT	NG	G	HT	HT	NG	HT
	Ny^r	NG	G	HT	HT	HT	NG
	Ben^r	G	G	HT	NG	NG	NG
M1	WT	NG	HT	G	G	…	HT
	Ny^r	NG	HT	G	G	G	G
	Ben^r	G	HT	HT	HT	NG	NG

* 0.5 µg / ml
**0.7 µg / ml
G = growth, NG = no growth, HT = hyphal tips < 1mm

Table 4. *Gaeumannomyces graminis* var isolates CD1 and M1 [nystatin resistant (Ny^r), and benomyl resistant (Ben^r)] grown on PDA amended with 1.2 µg benomyl / ml and 0.7 µg / ml cycloheximide for approximately 13 d.

Cross or isolate	Reaction					
	Test 1		Test 2		Test 3	
	5d	13d	5 d	12d†	7d**†	13d**†
$M1Ben^r$	NG	G	G	G	G	G
$CD1Ben^r$	NG	G	G	G	G	G
$M1Ny^r$	NG	NG	NG	NG	NG	NG
$CD1Ny^r$	NG	NG	NG	NG	NG	NG
$CD1Ben^r$ X $CD1Ny^r$	HT	G	HT	G	HT	G
$M1Ben^r$ X $M1Ny^r$	G	G	G	G	G	G
$CD1Ny^r$ X $M1Ben^r$	G	G	HT	G	G	G
$CD1Ben^r$ X $M1Ny^r$	HT	G	HT	G	G	G

*G = growth, NG = no growth, HT = hyphal tips < 1mm
†Same result at 1.1 µg / ml cycloheximide / 1.2 µg benomyl
**Plated onto 100 X 15 mm plates

APPENDIX D

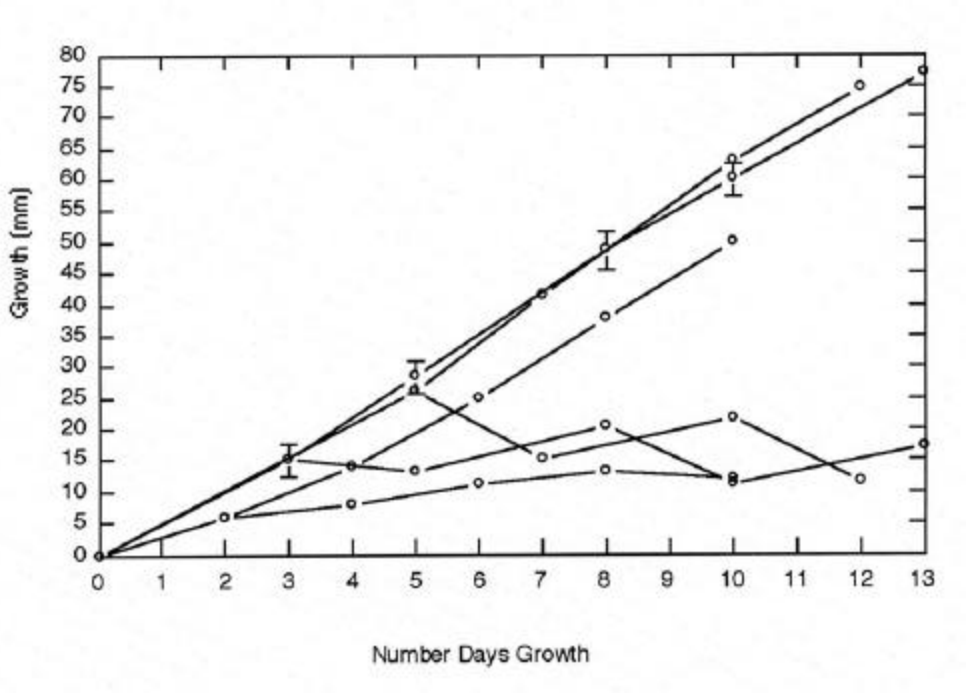


Figure 1. Linear and average growth of *Ggt* isolate M1. An 8mm plug was placed at the edge of a 100 X 25 mm Petri dish containing 20 ml Sigma PDA. Cultures were grown at 25 C. One to three repetitions were measured, and error bars represent standard deviation.

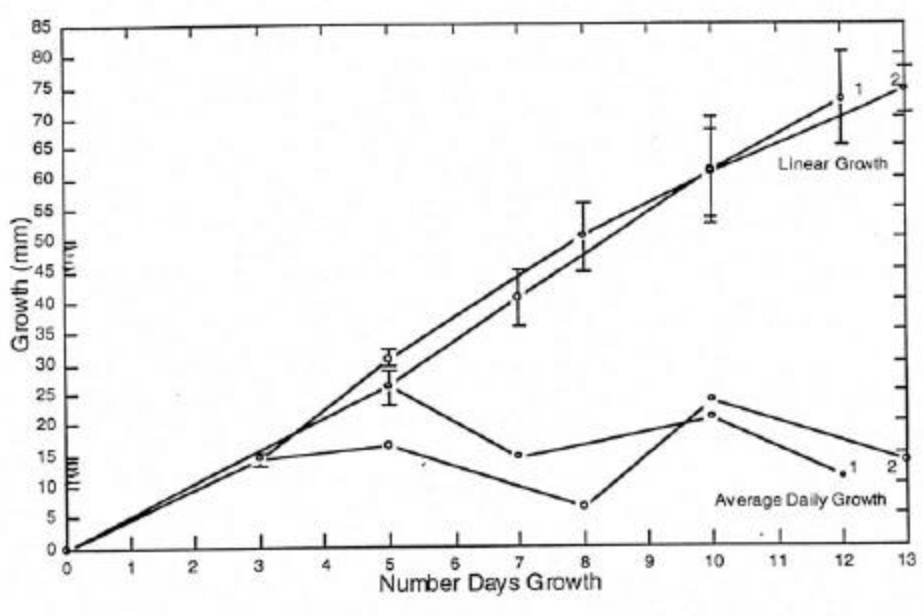


Figure 2. Linear and average growth of *Ggt* isolate CB1. An 8mm plug was placed at the edge of a 100 X 25 mm Petri dish containing 20 ml Sigma PDA. Cultures were grown at 25 C. One to three repeitions were measured, and error bars represent standard deviation.

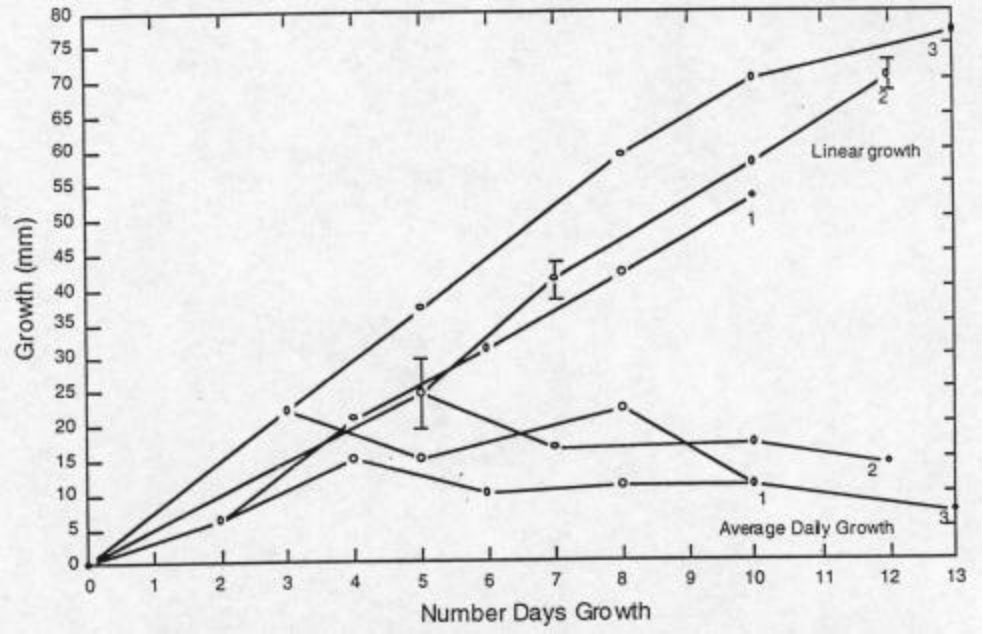


Figure 3. Linear and average growth of *Ggt* isolate CD1. An 8mm plug was placed at the edge of a 100 X 25 mm Petri dish containing 20 ml Sigma PDA. Cultures were grown at 25 C. One to three repetitions were measured, and error bars represent standard deviation.

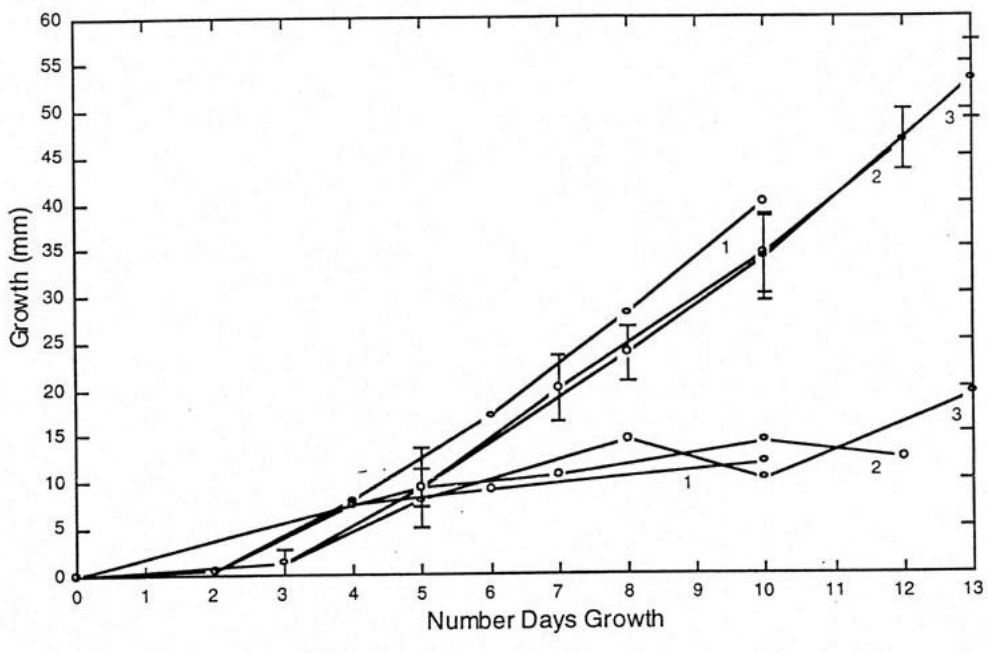


Figure 4. Linear and average growth of *Ggt* isolate CE1. An 8mm plug was placed at the edge of a 100 X 25 mm Petri dish containing 20 ml Sigma PDA. Cultures were grown at 25 C. One to three repetitions were measured, and error bars represent standard deviation.

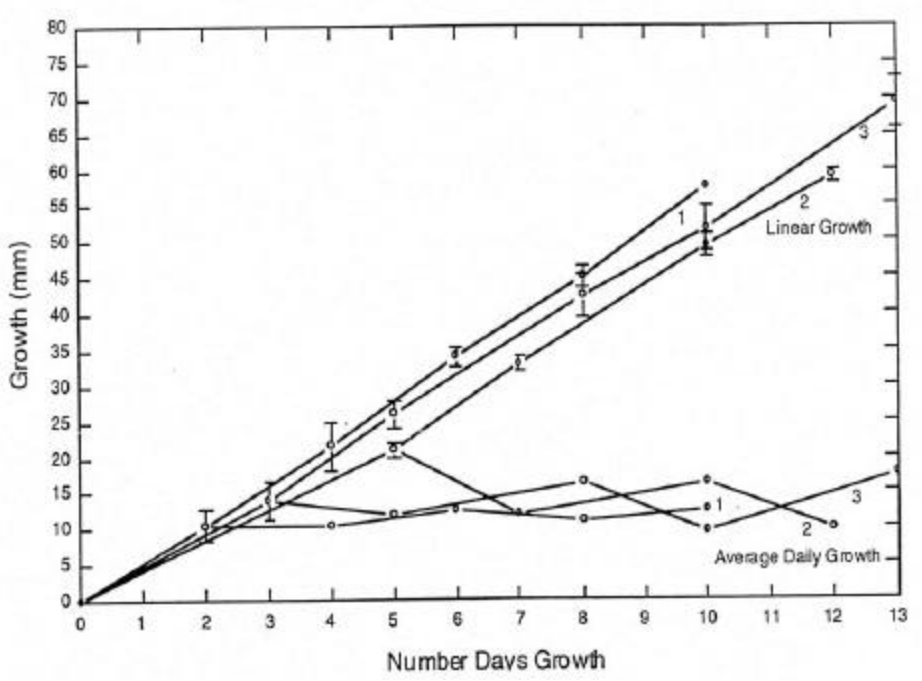


Figure 5. Linear and average growth of *Ggt* isolate CE2. An 8mm plug was placed at the edge of a 100 X 25 mm Petri dish containing 20 ml Sigma PDA. Cultures were grown at 25 C. One to three repetitions were measured, and error bars represent standard deviation.

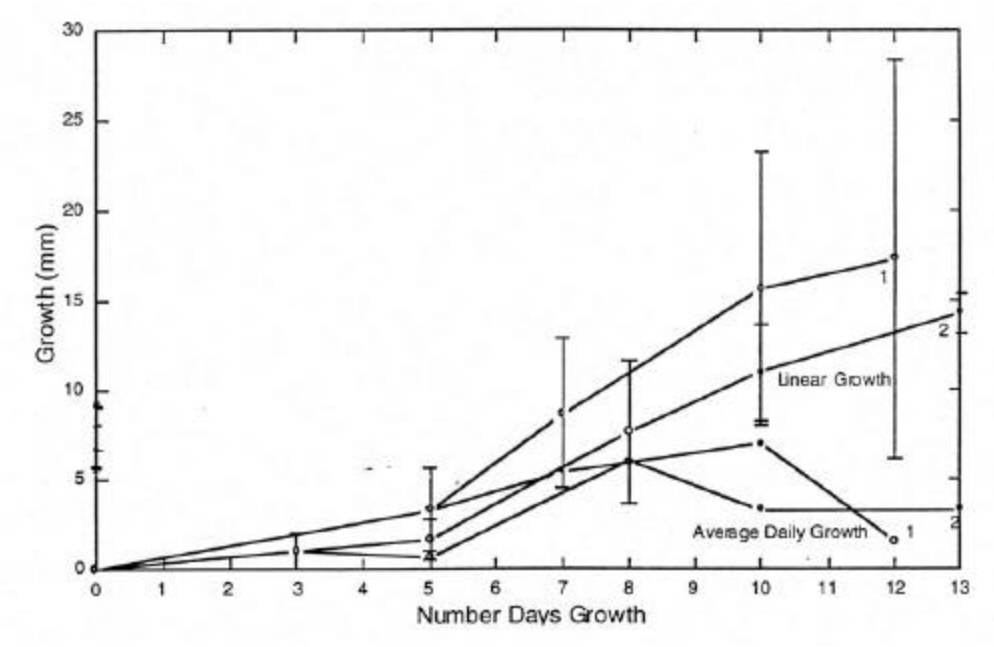


Figure 6. Linear and average growth of *Ggt* isolate CHe1. An 8mm plug was placed at the edge of a 100 X 25 mm Petri dish containing 20 ml Sigma PDA. Cultures were grown at 25 C. One to three repetitions were measured, and error bars represent standard deviation.

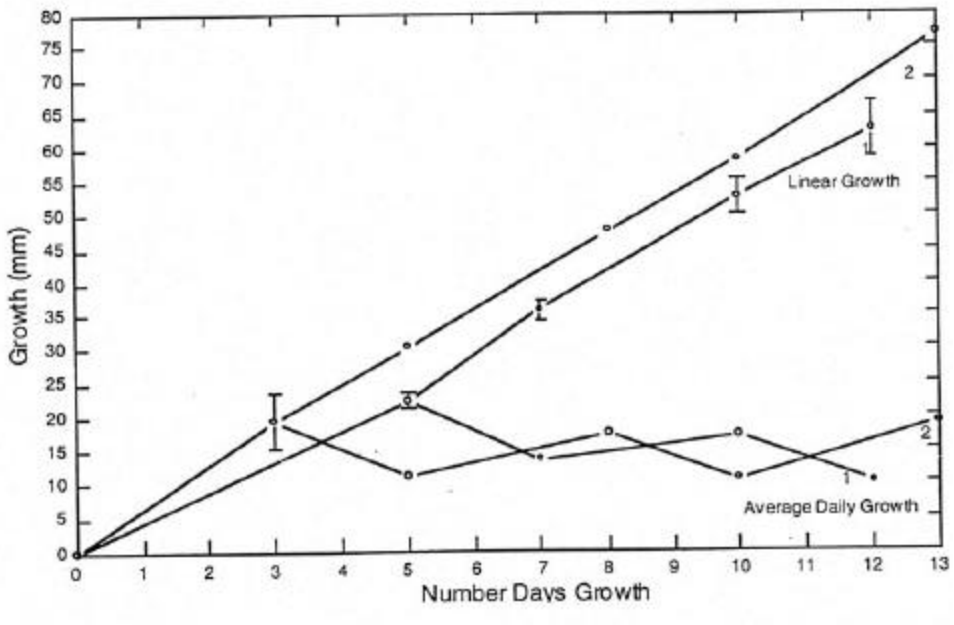


Figure 7. Linear and average growth of *Ggt* isolate CK1A. An 8mm plug was placed at the edge of a 100 X 25 mm Petri dish containing 20 ml Sigma PDA. Cultures were grown at 25 C. One to three repetitions were measured, and error bars represent standard deviation.

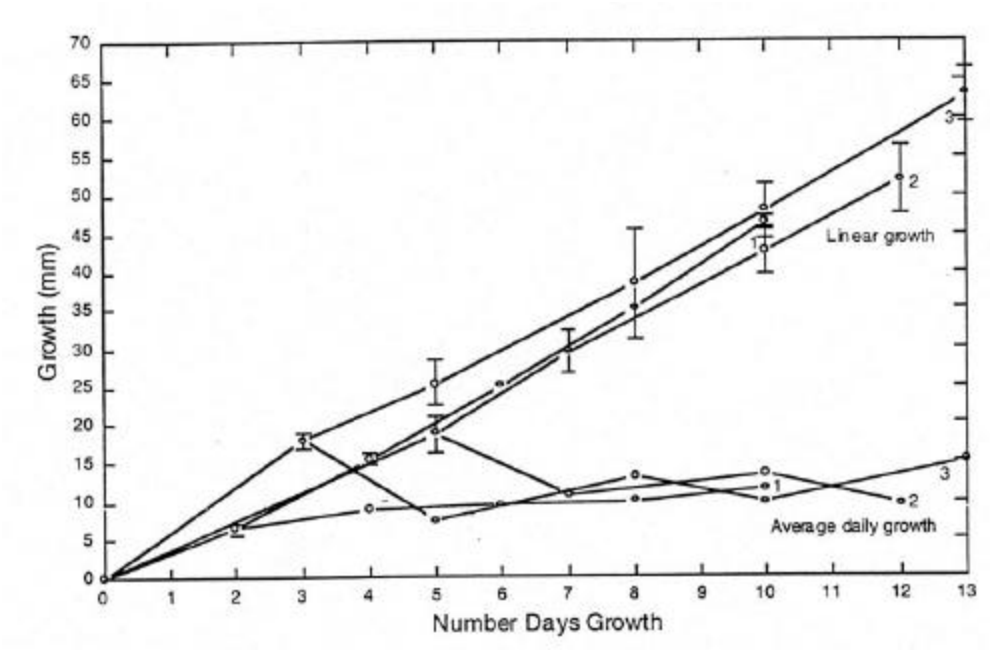


Figure 8. Linear and average growth of *Ggt* isolate CK1B. An 8mm plug was placed at the edge of a 100 X 25 mm Petri dish containing 20 ml Sigma PDA. Cultures were grown at 25 C. One to three repetitions were measured, and error bars represent standard deviation.

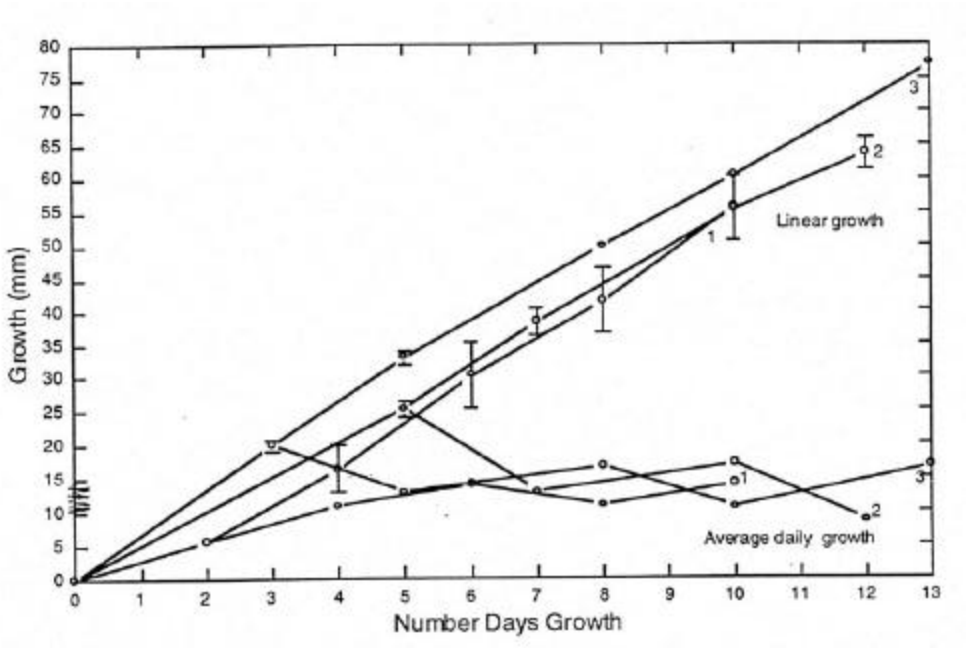


Figure 9. Linear and average growth of *Ggt* isolate CO1. An 8mm plug was placed at the edge of a 100 X 25 mm Petri dish containing 20 ml Sigma PDA. Cultures were grown at 25 C. One to three repetitions were measured, and error bars represent standard deviation.

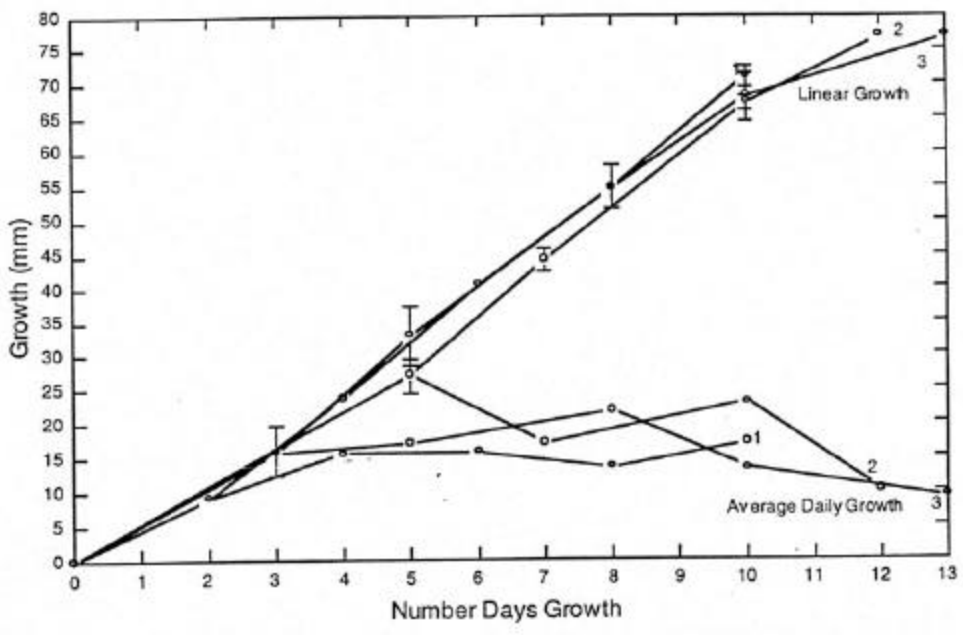


Figure 10. Linear and average growth of *Ggt* isolate CS1. An 8mm plug was placed at the edge of a 100 X 25 mm Petri dish containing 20 ml Sigma PDA. Cultures were grown at 25 C. One to three repetitions were measured, and error bars represent standard deviation.

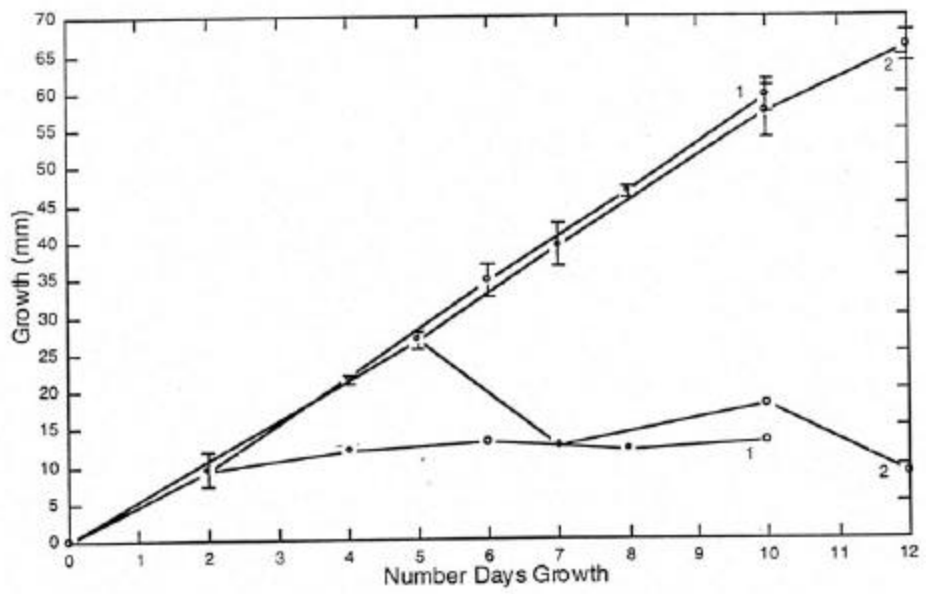


Figure 11. Linear and average growth of *Gga* isolate 15419. An 8mm plug was placed at the edge of a 100 X 25 mm Petri dish containing 20 ml Sigma PDA. Cultures were grown at 25 C. One to three repetitions were measured, and error bars represent standard deviation.

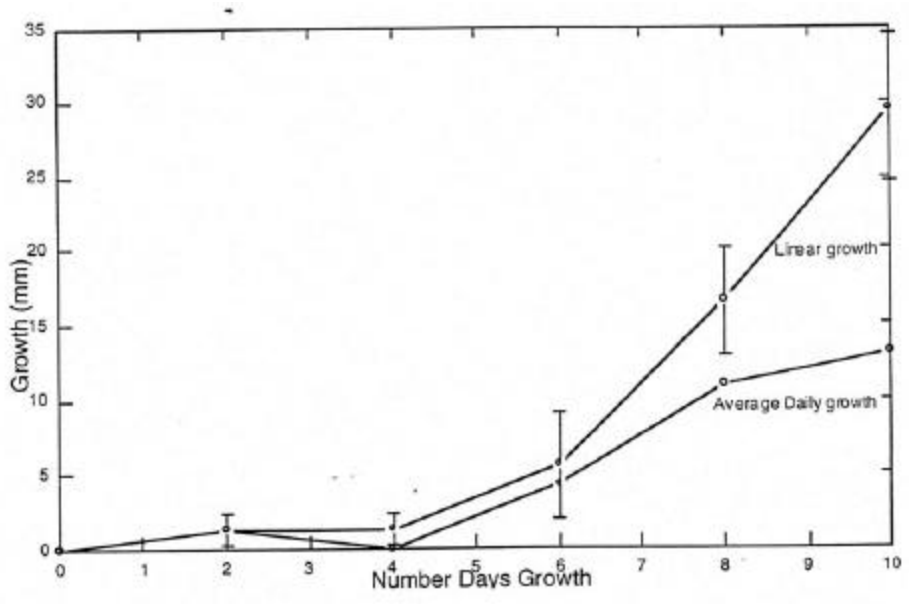


Figure 12. Linear and average growth of *Ggg* isolate 12761. An 8mm plug was placed at the edge of a 100 X 25 mm Petri dish containing 20 ml Sigma PDA. Cultures were grown at 25 C. One to three repetitions were measured, and error bars represent standard deviation.

Literature

1. Juhnke, M. E., Mathre, D. E., and Sands, D. C. 1984. Selective medium for *Gaeumannomyces graminis var. tritici.* Plant Disease. 68:233-236.

APPENDIX E

Methods of soil analysis used by A & L Eastern Agricultural Laboratories, Inc. to analyze the soil used in all greenhouse bioassays. All methods from Methods of Soil Analysis, Part 2. Chemical and Microbiological Properties, Second Edition. 1982. A. L. Page, Editor. American Society of Agronomy, Inc., and Soil Science Society of America, Inc.:

PARAMETER	SOIL ANALYSIS METHOD	PAGE
Organic Matter	Walkley-Black procedure	570
PH and Buffer pH	Glass Electrode-Calomel Electrode pH Meter method and SMP Single-Buffer method	206, 215
Available Phosphorus	Bray 1, P soluble in in Dilute Acid-Fluoride	416
Exchangeable Potassium	NCR-13 Exchangeable Potassium procedure	235
Exchangeable calcium and Magnesium	NCR-13 Exchangeable Potassium procedure including 14-4	235, 258
Exchangeable Sodium	NCR-13 Exchangeable Potassium procedure	235
Hydrogen (acidity)	SMP Single Buffer method	215
Nitrate-N	Extraction of Exchangeable Ammonium Nitrate and Nitrite and the Copperized Cadmium Reduction method	649, 679
Ammonium-N	Extraction of Exchangeable Ammonium Nitrate and Nitrite and the Indophenol Blue method	649, 674

Sulfate-S	Acetate-Extractable Sulfur	532
Extractable Zinc, Manganese		
Iron, Copper	Dilute Hydrochloric Acid Extraction	333
Boron	Hot Water Extract	443
Soluble Salts	Saturation Extract and Electrical Conductivity	168, 172
Chloride	Automatic Titration Method for Chloride	460
Total Nitrogen	Regular Kjeldahl method	610
Total Phosphorus	Digestion method	406

VITA

Brooks Crozier was born in Latrobe, Pennsylvania in 1968. He lives in Salem Virginia with his wife, Jennifer, and his two children, Ellen and Jamie. He received his BS in biology at Roanoke College, concentrating on plant and fungal biology under the late Dr. Philip C. Lee Jr., and received his Masters degree from VPI & SU under the direction of Dr. R. Jay Stipes. He remained at VPI & SU to study under Dr. E. L. Stromberg. He has been teaching part time at Roanoke College, teaching Mycology, Phycology, Plant Anatomy and Physiology, Microbiology, and of course Biology 101. He was promoted last year to Part-Time Senior Lecturer, and will remain there as a lecturer.

James Brooks Crozier