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Brassica Mulches and Meal Control Fungal Wheat Pathogens *in vitro* and Take-all Disease in Soil

Thomas Samuel Breeden
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To the Graduate Council:

I am submitting herewith a thesis written by Thomas Samuel Breeden entitled "*Brassica* Mulches and Meal Control Fungal Wheat Pathogens *in vitro* and Take-all Disease in Soil." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Entomology and Plant Pathology.

Bonnie Ownley, Major Professor

We have read this thesis and recommend its acceptance:

Dennis West, Carl Sams, Earnest Bernard

Accepted for the Council:
Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

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A Thesis Presented for the Masters of Science Degree
The University of Tennessee, Knoxville

Thomas Samuel Breeden
December 2005

Dedication

This thesis is dedicated to my parents, Bill and Clare Nell Breeden, and my wife, Angela Breeden. This is as much your work as it is mine. Thanks for encouraging me not to give up and to finish what I started. Finally, I thank God for putting questions and wonder in the mind of Man.

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Abstract

Soilborne pathogens have an economic impact on crops throughout the world. Many survive saprophytically and therefore are difficult to control. Soil fumigation and seed treatments are sometimes effective. However, they are economically impractical for many crops. Crop rotation/alternative crops are often a practical, economical, and successful means of controlling soilborne pathogens. Recent studies have revealed that *Brassica* spp. inhibit a wide array of plant pathogens. Decomposing species of *Brassica* release glucosinolates, which are converted to isothiocyanates (ITC) by the enzyme myrosinase. The ITC compounds inhibit microorganisms. The present study had three objectives. The first was to evaluate the effect of *Brassica* spp. on growth of *Gaeumannomyces graminis* var. *tritici* (*Ggt*), *Fusarium oxysporum, F. solani*, and *F. graminearum*, and to determine if inhibition was fungicidal. The first *in vitro* bioassay was designed as a 3 x 3 factorial in a completely randomized design (CRD) with three isolates of *Ggt* and three mulch treatments, with 3 replicates. The second bioassay was a 6 x 6 factorial with six fungi [three isolates of *Ggt* (A2, WX, and 211.1), *F. solani*, *F. oxysporum*, and *F. graminearum*] and six mulch treatments (*B. juncea* 'Florida Broadleaf' mustard mulch, *B. juncea* mustard seed meal, *B. napus* 'Dwarf Essex Rape', *B. napus* canola, wheat, and no mulch) in a CRD with three replicates. Plant tissue was placed in 490-cm^3 glass jars covered by inverted Petri dishes containing potato dextrose agar (PDA) with a 1-day-old fungal plug. Colony diameters were recorded for eight days. All fungal pathogens tested were inhibited by *B. napus* and wheat mulch, but fungal growth resumed when the mulches were removed. No growth was recorded for fungi

exposed to *B. juncea* mulch and seed meal, where inhibition was fungicidal. The second objective of the present study was to determine the amount of allyl isothiocyanate (AITC) needed to inhibit growth of the take-all pathogen. The tests were designed in a CRD with five rates of AITC or five rates of *B. juncea* seed meal. *Gaeumannomyces graminis* var. *tritici* isolate WX was exposed to AITC, a dominant isothiocyanate compound found in *Brassica* spp. Treatments of pure AITC and AITC from *B. juncea* mustard seed meal (mixed with water) at 0, 0.01, 0.02, 0.03, and 0.04 g were evaluated for the effects on *Ggt* growth. Petri plates with PDA and 1-day-old fungal plugs were inverted over 490- cm^3 glass jars with AITC or seed meal treatments for 5 h. Headspace concentrations of pure AITC and AITC volatized from *B. juncea* seed meal were recorded after 1 and 2 h; mycelial diameter was measured for 9 days to determine suppression or death of the fungus. Mycelial growth was inhibited by all *B. juncea* seed meal treatments. The concentrations of AITC produced by *B. juncea* seed meal that inhibited 50 and 90% of mycelial growth were 0.43 and 0.80 $\mu mol \cdot liter^{-1}$, respectively. Inhibition attributable to pure AITC alone was higher than that achieved by *B. juncea* seed meal. The third objective of the present study was to determine if incorporating *B. juncea* mustard mulch into soil containing *Ggt*-infested wheat crowns would reduce take-all in a subsequent wheat crop, and to evaluate phytotoxicity of *Brassica* mulch to wheat seedlings. The experiment was designed as a factorial in a split plot with two rates of *Ggt* (present or absent), two rates of *B. juncea* 'Florida Broadleaf' mulch (present or absent), and ten replicates. The main plot was *Brassica* mulch; the sub-plot was *Ggt.* There were ten plants per replicate and the study was repeated. For the initial crop, wheat seeds were planted into soil in plastic containers with and without inoculum of *Ggt,* isolates A2 and

WX, and maintained in a growth chamber at 18°C. After 28 days, shoots were excised and wheat roots were left in soil. Soils with healthy and diseased roots were mulched with 'Florida Broadleaf' mustard for five days; mulch was not applied to controls. For the 'final crop' wheat seeds were replanted into soil; take-all severity, shoot height, and percent germination were measured after 28 days. The main effects of mulch and *Ggt*, and the interaction were significant for disease severity in two trials. Treatment of soil containing *Ggt*-infected roots with *Brassica* mulch significantly reduced take-all in the subsequent wheat planting in two tests. In one test, height of seedlings was reduced in soil with *Brassica* mulch and seedling germination was reduced with *Brassica* mulch regardless of the presence of *Ggt*.

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Part I

Literature Review: Effect of *Brassica* Mulches on Soilborne Pathogens of Wheat

Take-all of Wheat

Gaeumannomyces graminis var. *tritici* (*Ggt*) is a soilborne pathogen that causes take-all of wheat. The term "take-all" was first used in Australia where the disease was recognized as early as 1852 (Rothrock, 2001). In the U.S., take-all disease is best known as a root and crown rot. It is the most significant wheat disease worldwide, and U.S. yield losses exceed $1 billion/year (USDA, 1994). Generally, it occurs sporadically in wheat fields planted continuously to wheat or in fields infested with perennial grass weeds. The damage to wheat is related to the extent of root and basal stem colonization by *Ggt*. Wheat plants can withstand mild infections with only minimal yield loss. Mild infections do not result in obvious disease symptoms. However, when symptoms become obvious, yield losses can be high (Cook and Veseth, 1991).

The most conspicuous field symptom of take-all is the occurrence of stunted, yellow plants in circular patches during early stages of growth. Occasionally, dead plants can be found scattered throughout the stand. Take-all can be identified by the dark brown to black rotted roots or stolons. Initially, roots have small black lesions that expand and later coalesce. Heavily rotted roots are very brittle and much of the root system remains in the soil when plants are pulled out of the soil. Under a microscope, dark brown "runner hyphae" and mycelial mats on roots or stolon surfaces are easily seen and are helpful in diagnosing this root disease (Huber and McCay-Buis, 1993).

Yield loss is attributed to the premature death of plants soon after heading and before grain filling. Prematurely killed plants produce bleached, empty heads known as whiteheads. Whiteheads are conspicuous in contrast to the normal green color of the

crop. Whiteheads often have no grain, but may contain a few shriveled kernels. During wet weather, the dead plants become covered with saprophytic fungi, which mask the bleached whiteheads by turning them blackish in color. In seasons of abnormally wet weather, *Ggt* can extend up the stems 2.5 to 5-cm (Cook and Veseth, 1991).

Although the fungus can produce ascospores, they are unimportant in the spread of the disease (Hornby and Cook, 1990). The take-all fungus exists within soil, in old roots and tiller bases of the previous wheat crop and uses these plant remains as a food base for survival and as a bridge to support its growth onto new roots of the next crop. Wheat plants become infected when their roots contact infested residues or living plants harboring the fungus. The fungus spreads from residues to the root surface and from one root to the next by growth of "runner hyphae" through the soil (Cook and Veseth, 1991).

Take-all is usually more severe in sandy soils with high pH. Severity in these soils is often because nutrients involved in host defense, such as manganese (Mn) are electrochemically unavailable to wheat (Huber and McCay-Buis, 1993). Generally, the earlier infection takes place, the more disease and the greater the yield loss at harvest. Thus, infections of young plants soon after planting result in the most severe yield losses. Moisture retaining, poorly drained soils, or abnormally wet weather, especially in the second half of the growing season, favor development of the disease because high moisture is needed for mycelial growth (Cook and Veseth, 1991). Maintaining adequate levels of nitrogen, phosphorus, and potassium for crop growth reduces take-all (Huber and McCay-Buis, 1993).

Control of Take-all

Fumigation and Seed Treatment

Fumigation is an option in many crops that are affected by soilborne pathogens. However, given the low economic value of wheat in the southeastern U.S. , fumigation is not an option. Fungicide seed treatments are effective in managing take-all (Duffy, 2000; Dawson, 2001). However, they are often economically impractical because take-all is sporadic in a field. In addition, there are no commercially available cultivars of wheat with a high degree of resistance. Therefore, alternative control methods are used.

Irrigation

Rush et al. (1999) have suggested that irrigation management practices should be studied further. Specifically, they propose that irrigating at variable rates using Global Position Systems (GPS) will contribute to less severe outbreaks of take-all. Take-all disease is severe on highly irrigated cropping systems. Using GPS as a tool for irrigation decisions may be promising, but no substantial results have been concluded. In areas where wheat is not irrigated, such as the southeastern U.S., this control option has no practical value.

Nitrogen

Nutrition management is another approach to control take-all. In root segments located below lesions longer than 1-cm, nitrogen was reduced by half compared to healthy roots and root segments above lesions (Schoeny, 2003). This reduction in nitrogen probably resulted from the invasion and breakdown of phloem by the fungal hyphae. Although still capable of nitrate uptake through their functioning proximal

segments, in the field it is necessary to provide enough nitrogen in the upper profile of the soil where efficient root segments are located.

In addition, stage of growth of the wheat plant at the time of fertilizer application plays a role in disease. Take-all losses were more severe when nitrogen was applied at late boot stage instead of pre-boot stage (Howard et al., 2002a; Howard et al., 2002b). Form of nitrogen is also important in disease management. Ammonium nitrate and ammonium sulfate fertilizer were more efficient for suppression of take-all than urea, urea-ammonium nitrate, and urea-ammonium nitrate + calcium nitrate (Howard et al., 2002a).

Manganese

Manganese plays a vital role in take-all development. Manganese interacts with nitrogen metabolism and is intimately involved in respiration, photosynthesis, hormone metabolism, and the synthesis of secondary metabolites associated with the defense of plants against pathogens (Huber and McCay-Buis, 1993). However, *Ggt* oxidizes Mn^{2+} to Mn^{+4}. Manganese is available to the plant only in the reduced state of Mn^{2+} but Mn was detected around roots infected with *Ggt* in the oxidized, Mn^{+4}, a state that is unavailable for plant uptake (Schulze, 1995). Virulence of *Ggt* isolates, and number and total length of lesions, were positively correlated with the Mn-oxidation capacity of *Ggt* isolates (Pedler, 1996). A manganese application rate of 2.25 kg ha^{-1} was effective in suppressing take-all of bentgrass, caused by the fungus *Gaeumannomyces graminis* f. sp. *avenae* (Heckman, 2003). Over time, previous applications of Mn fertilizer were less effective in suppressing take-all patch than were the most recent applications of Mn.

Alternative Approaches

Crop rotation/alternative crops are often a practical, economical, and successful means of controlling soilborne pathogens. Also, management of inoculum levels, and the soil environment have been studied as ways to control take-all. Crop rotation/alternative crops is a method of cultural control, while managing inoculum levels is predominantly done by physical control.

Soybeans, other legumes, and corn are popular crops for rotations with wheat. In the southeastern U.S., often wheat is doubled-cropped with soybean. However, an increase in take-all disease following double cropping with soybeans has been reported (Cook, 1981). Soybean and sunflower root exudates significantly increase the pathogenicity of *Ggt* in comparison with maize and tobacco root exudates (Mass, 1990). Furthermore, naturally occurring microbial antagonists to *Ggt* are greater in maize and tobacco soils than in soybean or sunflower soils (Mass, 1990).

Crop rotation with other small grains, except oat, does not break the take-all disease cycle. Wheat following triticale, barley, or rye has similar disease severity and percent whiteheads, whereas wheat following oat has significantly less take-all symptoms (Rothrock, 1991). Although oat is not susceptible to *Ggt*, it is susceptible to *G. graminis* var. *avenae*.

Soil tillage is an example of physical control that can reduce inoculum levels of *Ggt*. However, this approach may conflict with soil conservation methods in the southeastern U.S. Finally, managing the soil environment uses both biological and cultural methods to control pathogen growth. In summary, no single method has emerged to control take-all of wheat. An integrated approach is the most feasible way to

manage take-all at low to moderate levels.

Crown Rot of Wheat

Crown rot in wheat is caused by *Fusarium graminearum* and a variety of other *Fusarium* spp. *Fusarium* spp. can invade a wide range of crops. They are efficient saprophytes that survive and multiply on many crop residues.

On wheat, *F. graminearum* survives as chlamydospores in the soil or stubble. Chlamydospores formed in conidia or mycelium can exist in the soil for months. These germinate and produce hyphae, which infect mainly through crown roots and wounds sustained during crown root emergence (Cook and Veseth, 1991).

Although moisture is essential for infection, crown rot of wheat tends to be most severe in drought conditions. Infected crowns and roots are brown and rotted, and a reddish brown discoloration of the stem may be present. Leaf sheaths look normal until peeled away to reveal a discolored culm. If the plant is water stressed during the final stage of plant development, premature ripening and white heads may develop. Later, pink or red mycelium can be observed if the stem is split (Inglis and Cook, 1986).

Control of Crown Rot

Some lines of wheat have resistance to *F. graminearum* based on depth at which the crown is formed (Wildermuth, 2001). However, crop rotation is the most effective means of disease control. Break crops such as legumes can reduce the amount of inoculum in the soil. Break crops (*Brassica* spp. and chickpeas) significantly reduced the severity of crown rot in both a susceptible (37 - 47% reduction) and tolerant wheat crop (21 - 51% reduction) compared to growing wheat after wheat or wheat after barley.

Brassica spp. were generally more effective than chickpea in reducing the severity of crown rot. Denser canopies created by the *Brassica* crops and increased populations of *Trichoderma* spp. (isolated from wheat following *Brassica*) made *Brassica* spp. a more effective break crop (Simpfendorfer, 2005).

Where the disease incidence is high, alternative crops should be considered in the rotation. Alternative crops should be grown for at least two years if there is a moderate level of crown rot and at least three years if there is a high level of disease (Simpfendorfer, 2005). During the growth of alternative crops, grasses should be controlled at an early growth stage so they do not maintain the fungus during the rotation (Simpfendorfer, 2005).

Crown rot disease is more severe when nitrogen fertilizer is applied at high rates (Wildermuth, 2002). In these cases the full benefit of the fertilizer will not be realized until the disease is reduced. Crown rot is also more severe when plants are deficient in zinc (Wildermuth, 2002).

Brassica Mulches

Brassica mulches (*Brassica juncea* cv. Indian Mustard and *B. napus* cv. Dwarf Essex Rape) have been reported to inhibit *Ggt in vitro* (Angus, 1994). Mulches of *Brassica* spp. such as *B. juncea* and *B. napus* produce glucosinolates (GS) (Table I-1; All tables and figures are located in Appendix I). These are secondary metabolites found in intact plant tissues. When tissue is damaged the enzyme myrosinase (Fig. I-1) (normally physically separated by myrosin cells from GS) catalyzes the conversion of GS (Fig.I-2)

to isothiocyanates (ITC) (Fig. I-3) (Andréasson et al., 2001; Chew, 1988), which have activity against plant pathogens (Charron and Sams, 1999). The ITCs are highly biocidal to a diverse range of organisms including nematodes, bacteria, fungi, insects, and germinating seeds (Brown and Morra, 1997; Borek, 1998).

Although the total soil fungal population is strongly increased after *Brassica* mulch treatments, *Pythium* spp. were suppressed due to the GS degradation products released during plant chopping. The fungitoxic effect of these degradation products overcame the stimulant effect of organic matter amendment (Lazzeri and Manici, 2001). Intact 'Dwarf Essex' meal, with its high concentration of glucosinolates and intact myrosinase, completely suppressed *Aphanomyces* root rot of pea (*Pisum sativum*) and water extracts of meal completely inhibited mycelial growth even at 50% dilution (Smolinska, 1997).

The release of ITCs is dependent on many factors. When tissue is frozen, thawed, and dried, cellular disruption is maximized, resulting in high ITC release. Freeze-dried tissue can increase ITC release efficiencies 14 to 26% because of the cellular disruption that occurs compared to no cellular disruption (Morra and Kirkegaard, 2002). However, the molar amount of ITC produced by hydrolysis is lower than the amount of corresponding GS present in the plant tissues. The conversion of GS to ITC is limited by soil moisture, soil retention, incomplete hydrolysis, and the formation of non-ITC hydrolysis products by reactions with proteins and amino acids. (Warton, 2001).

Brassica spp. can increase the overall health of wheat plants when incorporated into a cropping system. Wheat grown after *Brassica* spp. had greater dry matter than wheat grown after wheat (Kirkegaard, 1994). Furthermore, in soil infested with *Ggt*,

wheat after *Brassica* break-crops yielded a 29% increase in shoot biomass at anthesis compared to wheat after wheat (Kirkegaard, 1994).

The GS side chain is very important to biological activity. Small changes in side chain structure can have significant effects. For example, while methylthioalkly GS produces volatile and pungent isothiocyanates, methylsulphinylalkyl GS (the next product in the biochemical pathway) produces non-volatile ITCs with relatively mild flavors, such as those found in broccoli. Removal of the methylsulphinyl group and the addition of a double bond results again in a volatile ITC. Finally, addition of a hydroxyl group to 3-butenyl and 4-pentenyl GS results in the spontaneous cyclization of the unstable ITC and the production of a non-volatile product (Mithen, 2001).

More study on the effectiveness of *Brassica* spp. for suppression of soilborne pathogens is needed because *Brassica* spp. benefit the soil environment and can increase overall health of crops.

Thesis Objectives

The objectives of this research are;

1. To evaluate inhibition of *Ggt* isolates and *Fusarium* spp. by *Brassica* spp. *in vitro*.

2. To determine the amount of pure allyl isothiocyanate (AITC) and AITC from *B. juncea* mustard seed meal needed for inhibition of *Ggt*.

3. To determine the ability of *B. juncea* 'Florida Broadleaf' mulch incorporated into soil, to suppress take-all disease from natural inoculum (infected roots and crowns) in a controlled growth chamber environment.

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Appendix I

Table I-1. Compounds found in *B. napus* and *B. juncea* (Brown and Morra, 1997; Charron and Sams, 1999).

B. napus	*B. juncea*
Allyl [CH_2CHCH_2]	Allyl [CH_2CHCH_2]
3-Butenyl [$CH_2CHCH_2CH_2$]	3-Butenyl
Hexyl [$CH_3(CH_2)_5$]	3-Indolylmethyl
5-Hexenyl[$CH_2CH(CH_2)_4$]	3-Methylthiopropyl
R-2-hydroxy-3-butenyl [$CH_2CH(OH)CHCH_2$]	4-Methoxyindolyl-3-methyl
S-2-hydroxy-3-butenyl [$CH_2CH(OH)CHCH_2$]	Phenylethyl
4-Hydroxy-3-indolylmethyl (4-$OHC_8H_5NCH_2$)	2-Phenylethyl [$CH_6CH_5(CH_2)_2$]
2-Hydroxy-4-pentenyl [$CH_2CHOH_2(OH)CHCH_2$]	
4-Hydroxybenzyl [4-$OHC_6H_5CH_2$]	
3-Indolylmethyl ($C_8H_6NCH_2$)	
5-Methylhexyl [$CH_3(CH_3)CH(CH_2)_4$]	
4-Methylpentyl [$CH_3(CH_3)CH(CH_2)_3$]	
2-Methylpropyl [$CH_3(CH_3)CHCH_2$]	
4-Methylsulfinyl-3-butenyl [$CH_3SOCHCH(CH_2)_2$]	
4-Methylsulfunylbutyl [$CH_3SO(CH_2)_4$]	
3-Methylsulfinylpropyl [$CH_3SO(CH_2)_3$]	
4-Methylthiobutyl [$CH_3S(CH_2)_4$]	
3-Methylthiopropyl [$CH_3S(CH_2)_3$]	
1-Methoxy-3-indolylmethyl [1-$(CH_3O)C_8H_5NCH_2$]	
1-Methoxy-3-indolylmethyl [4-$(CH_3O)C_8H_5NCH_2$	
4-Pentenyl [$CH_2CH(CH_2)_3$]	
2-Phenylethyl [$CH_6H_5(CH_2)_2$]	

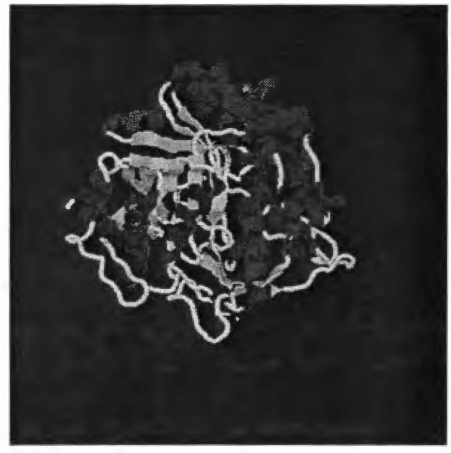

Fig. I-1. Myrosinase (Berman, 2000)

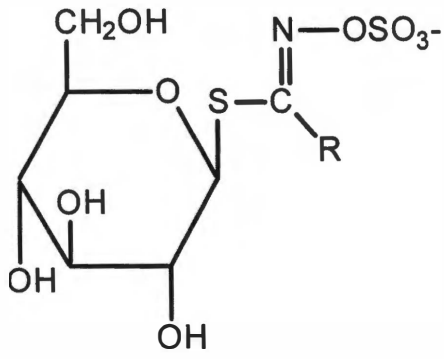

Fig. I-2. Glucosinolate

Fig. I-3. Isothiocyanate

Part II

Effect of *Brassica* Mulches on Growth of Soilborne Wheat Pathogens *in vitro*

Abstract

Fusarium oxysporum, *F. solani*, *F. graminearum*, and *Gaeumannomyces graminis* var. *tritici* (*Ggt*), are soilborne plant pathogens of wheat. Decomposing *Brassica* spp. release glucosinolates, which inhibit microorganisms. The objectives of this study were 1) to evaluate the effect of *Brassica* spp. on growth of *Ggt* (A2, WX, and 211.1) and *Fusarium* and 2) to determine if inhibition was fungicidal. The first *in vitro* bioassay was designed as a 3 x 3 factorial in a completely randomized design (CRD) with three isolates of *Ggt* and three mulch treatments, with 3 replicates. The second bioassay was a 6 x 6 factorial with six fungi [three isolates of *Ggt* (A2, WX, and 211.1), *F. solani*, *F. oxysporum*, and *F. graminearum*] and six mulch treatments (*B. juncea* 'Florida Broadleaf' mustard mulch, *B. juncea* mustard seed meal, *B. napus* 'Dwarf Essex Rape', *B. napus* canola, wheat, and no mulch) in a CRD with three replicates. Plant tissue was placed in 490-cm^3 jars covered by inverted Petri dishes containing potato dextrose agar (PDA) with a fungal plug. Colony diameters were recorded for eight days. All fungal pathogens tested were inhibited by *B. napus* and wheat mulch. Fungal plugs were removed from mulch treatments and were observed for growth (six to seven days). In the second bioassay no growth occurred for fungi exposed to *B. juncea* mulch and meal, where inhibition was fungicidal. In the first bioassay growth of *Ggt* isolate WX was observed at five days with *B. juncea* 'Florida Broadleaf' mulch. In both assays growth of pathogens resumed after removal of mulches that did not contain *B. juncea*.

Introduction

Pathogens

The fungal pathogens *Gaeumannomyces graminis* var. *tritici* (*Ggt*) and *Fusarium graminearum* have a major impact on wheat production in the southeastern U.S. *Gaeumannomyces graminis* var. *tritici* causes take-all of wheat and barley crops worldwide (Rothrock, 2001). *Fusarium graminearum* causes foot and crown rot in wheat (Cook and Veseth, 1991). *Fusarium oxysporum* and *F. solani* cause seedling and root rots in wheat (Farr et al., n.d.; Shivas, 1989). These wheat pathogens have ascomycetous sexual stages and exist within soil, in the old roots or tiller bases of previous wheat crops. The wheat residue serves as a source of inoculum and supports growth of these pathogens onto new roots or crowns of the next crop (Cook and Veseth, 1991).

Often take-all is found in moist soils, or in dry wheat production areas under heavy irrigation. Infection by *Ggt* occurs through young seminal roots (Cook and Veseth, 1991). Foot and crown rot pathogens attack wheat plants by growing from crop residue into the crown roots of the plant. The fungus continues to grow into the crown tissue, rotting the crown and destroying the plant tissues responsible for moving water from the roots to the above ground parts of the plant. Plants must be water-stressed for the disease to progress. Under adequate moisture conditions, the plants may be infected, but the disease will not spread into the crown (Draper, 2000).

Fumigation is an option in many crops that are affected by soilborne pathogens. However, given the low economic value of wheat crops in the southeast, fumigation is not an option. Seed treatments with fungicides are effective in managing take-all (Duffy,

2000; Dawson, 2001). However, they are often economically impractical because take-all is sporadic in a field. No cultivars of wheat are commercially available with a high degree of resistance against take-all. Some lines of wheat have shown resistance to *F. graminearum* based on depth at which the crown is formed (Wildermuth, 2001). Currently, cultural practices, such as crop rotation and planting dates, are the most economical ways to control these diseases.

***Brassica* spp.**

Mulches of *Brassica* spp. such as *Brassica juncea* and *B. napus* produce glucosinolates (GS), which are secondary metabolites found in intact plant tissues. When tissue is damaged the enzyme myrosinase (normally physically separated from GS) catalyzes the conversion of GS to isothiocyanates (ITC) (Chew, 1988), which have activity against plant pathogens (Charron and Sams, 1999). This allelopathic activity has stimulated research using *Brassica* species as "break crops" to reduce disease loss (Angus, 1994).

The release of ITC is dependent on many factors. When tissue is frozen, thawed, and dried, cellular disruption is maximized, resulting in high ITC release. Freeze-dried tissue can increase ITC release efficiencies from 14 to 26% because of the cellular disruption that occurs compared to no cellular disruption (Morra and Kirkegaard, 2002). However, it is well known that the molar amount of ITC produced by hydrolysis is lower than the amount of corresponding GS present in the plant tissues. Several limiting factors are present. Soil moisture, soil retention, incomplete hydrolysis, and the formation of non-ITC hydrolysis products by reacting with proteins and amino acids are possible

limiting factors (Warton, 2001).

The objective of this study was to determine if mulches of *B. juncea* cv. Florida Broadleaf, *B. napus* cv. Dwarf Essex Rape, *B. juncea* mustard seed meal, and *B. napus* canola affect mycelial growth of three isolates of *Ggt* and three species of *Fusarium in vitro*.

Materials and Methods

Bioassay

All pathogens (Table II-1; All tables and figures are located in Appendix II) were isolated from diseased wheat roots. *Fusarium* spp. were isolated from diseased wheat roots at the University of Tennessee Highland Rim Research and Education Center, Springfield, TN. *Gaeumannomyces graminis* var. *tritici* isolates A2, 211.1, and WX were found on diseased wheat roots in Tennessee (our laboratory), and Georgia and South Carolina, respectively (Mazzola et al., 1995). Isolates were cultured on potato dextrose agar (PDA) for 7 days at 28 to 32°C. Mycelial plugs were transferred to fresh PDA and grown overnight before use in the bioassay.

Brassica plants were grown as a winter annual crop at the East Tennessee Research and Education Center, Blount Farm, Knoxville, TN. No insecticides, fungicides, or herbicides were applied to foliage after planting. Small, young leaves, which normally have the highest GS content, were harvested by hand and transferred quickly to the laboratory to prevent loss of volatile compounds. *Brassica juncea* mustard seed meal was from unprocessed, seed used for mustard as a condiment. Leaves from

Brassica spp. were macerated separately in a food processor for 20 s. Tissue (10 g) from each *Brassica* treatment was transferred to sterile 490-cm^3 glass jars. For mustard seed meal, 1 g of meal and 9 ml of water were added to jars. The water was added to activate the release of ITC.

After 10 g of fresh, macerated tissue or meal was added to sterile jars, the bottom of 100-mm-diam. Petri dishes containing 1-day-old plugs of *Ggt* or *Fusarium* spp. growing on PDA were inverted on top of the jars and the joint was sealed with Parafilm to reduce loss of volatile compounds. The bioassay was incubated for 6 to 8 days at room temperature and growth of mycelia, based on colony diameter, was recorded daily after plating. Because *Ggt* is a relatively slow growing fungus, no measurement was taken the first day. After 6 to 8 days, plugs were removed from the mycelia exposed to mulch or meal treatments and plated on fresh PDA. Colonies were allowed to grow for six to seven days with no mulch or meal treatment. Mycelial growth (colony diameters) was measured to determine if inhibition was fungicidal.

Experimental design and statistical analysis

Bioassay 1 was designed as a 3 x 3 factorial with three isolates of *Ggt* and three mulch treatments (Table II-2) in a completely randomized design (CRD) with three replicates. Bioassay 2 was designed as a 6 x 6 factorial with six fungal pathogens, six mulch treatments (Table II-3), and three replicates in a CRD. In Bioassay 1, the control was not exposed to mulch (untreated). In Bioassay 2, controls were the untreated and wheat mulch. Data were analyzed with the Mixed procedure of PC-SAS, (Version 8.2, SAS Institute, Cary, NC). Significant effects were further analyzed with Fisher's-protected least significant difference test at $P = 0.05$.

Results

For Bioassay 1 and Bioassay 2, at each measurement date, the effects of fungi, mulch treatment, and the interaction were significant ($P < 0.0001$). Across all *Ggt* isolates (Bioassay 1) and *Fusarium* spp. (Bioassay 2), *B. juncea* mustard seed meal and *B. juncea* 'Florida Broadleaf' mustard mulch were the most effective mulches for mycelial inhibition (Figs. II-1 and II-2). *Brassica* napus canola type, *B. napus* 'Dwarf Essex Rape', and wheat inhibited growth at intermediate levels compared to the mustard treatments.

The patterns of growth inhibition by mulch treatments were similar for all fungi (Figs. II-1 to II-5 [Bioassay 1] and Tables II-4 to II-9 [Bioassay 2]). *Fusarium* spp. were less sensitive to mulch treatments than *Ggt* isolates. The *Ggt* isolate A2 (Table II-4) was less susceptible to *B. napus* 'Dwarf Essex Rape' than isolates 211.1 and WX. Growth of all *Ggt* isolates was strongly inhibited by *B. juncea* 'Florida Broadleaf' mulch and *B. juncea* mustard seed meal.

After removal of mulch treatments, fungi subjected to *B. napus* 'Dwarf Essex Rape', wheat, and *B. napus* canola recovered and resumed growth (Tables II-10 to II-12). With one exception, *Ggt* isolate WX, fungi exposed to *B. juncea* 'Florida Broadleaf' mulch and *B. juncea* mustard meal had no growth when treatments were removed (Table II-10). Thus, in general, fungi treated with *B. juncea* 'Florida Broadleaf' mustard mulch and mustard seed meal in Bioassay 1 and 2 were killed as a result of exposure to these mulch treatments.

Discussion

In these studies, mulches and meal of *B. juncea* significantly inhibited growth of all three *Ggt* isolates and three species of *Fusarium*; *F. oxysporum*, *F. graminearum*, and *F. solani*. *Brassica juncea* 'Florida Broadleaf' was more inhibitory than mulch from *B. napus* canola, wheat, or *B. napus* 'Dwarf Essex Rape'. Hence, *B. juncea* 'Florida Broadleaf' has a greater potential to be used as a break-crop to reduce inoculum of these pathogens.

Brassica juncea 'Florida Broadleaf' mulch and *B. juncea* mustard seed meal generally proved to be fungicidal because, after removal of the mulch, only one isolate of *Ggt* exposed to 'Florida Broadleaf' mulch recovered. In all other replicates no growth was observed after 7 days. When *Ggt* isolates and *Fusarium* spp. were removed from exposure to *B. napus* 'Dwarf Essex Rape', *B. napus* canola, and wheat mulch, all isolates recovered and grew. The results of these experiments demonstrate the potential of using *Brassica* spp. as an alternative method for reducing inoculum of soilborne pathogens.

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Appendix II

Table II-1. Fungi tested in bioassay experiments

Ggt Isolates	*Fusarium* spp.
Ggt WX	*F. oxysporum*
Ggt A2	*F. graminearum*
Ggt 211.1	*F. solani*

Table II-2. Experimental parameters for Bioassay 1

Mulch Treatments	Fungal Isolates
Untreated	*Ggt* WX
B. napus	*Ggt* A2
B. juncea	*Ggt* 211.1

Table II-3. Experimental parameters for Bioassay 2

Mulch Treatments	Fungal Isolates
Untreated	*Ggt* WX
B. napus 'Dwarf Essex Rape'	*Ggt* A2
B. napus Canola	*Ggt* 211.1
Wheat	*F. oxysporum*
B. juncea 'Florida Broadleaf'	*F. graminearum*
B. juncea Mustard Meal	*F. solani*

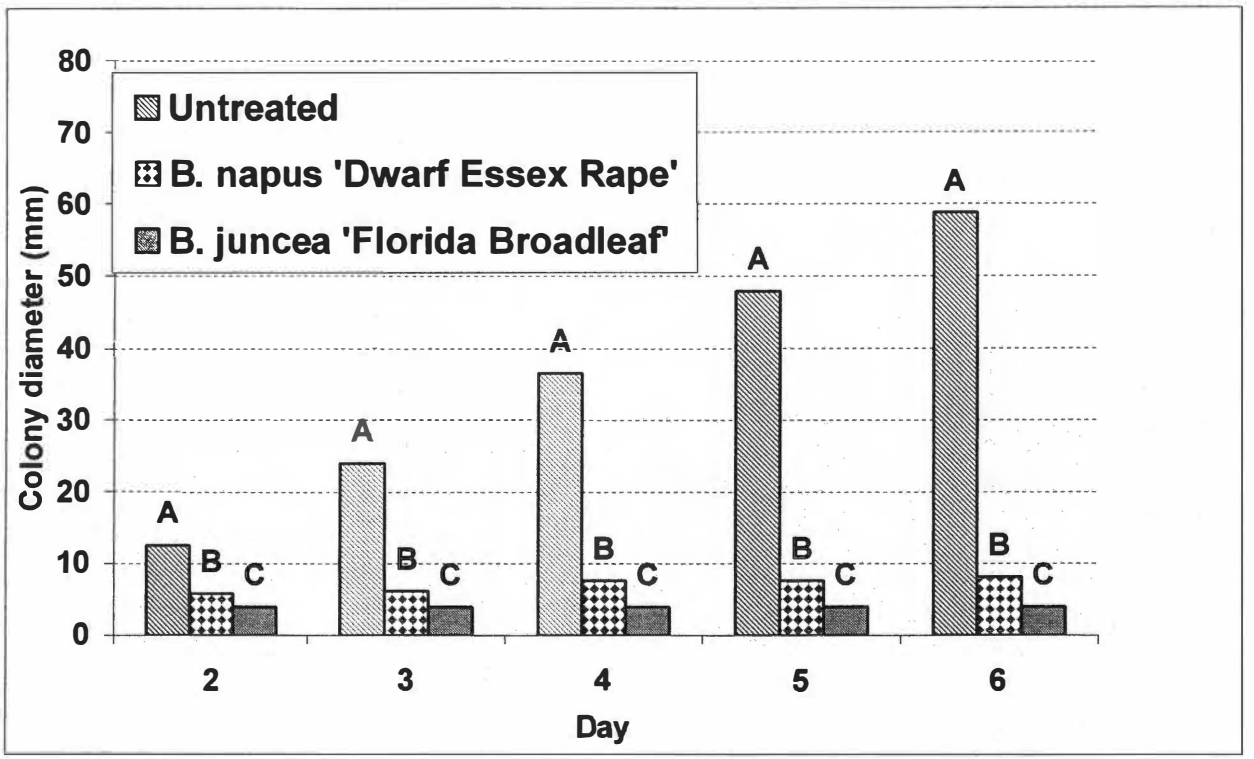

Fig. II-1. Main effect of *Brassica* mulches on growth of *Gaeumannomyces graminis* var. *tritici* isolates A2, 211.1 and WX in Bioassay 1. Data for all fungal isolates were combined. Within each day, bars with the same letters are not significantly different according to a Fisher's-protected least significant difference test at $P = 0.05$

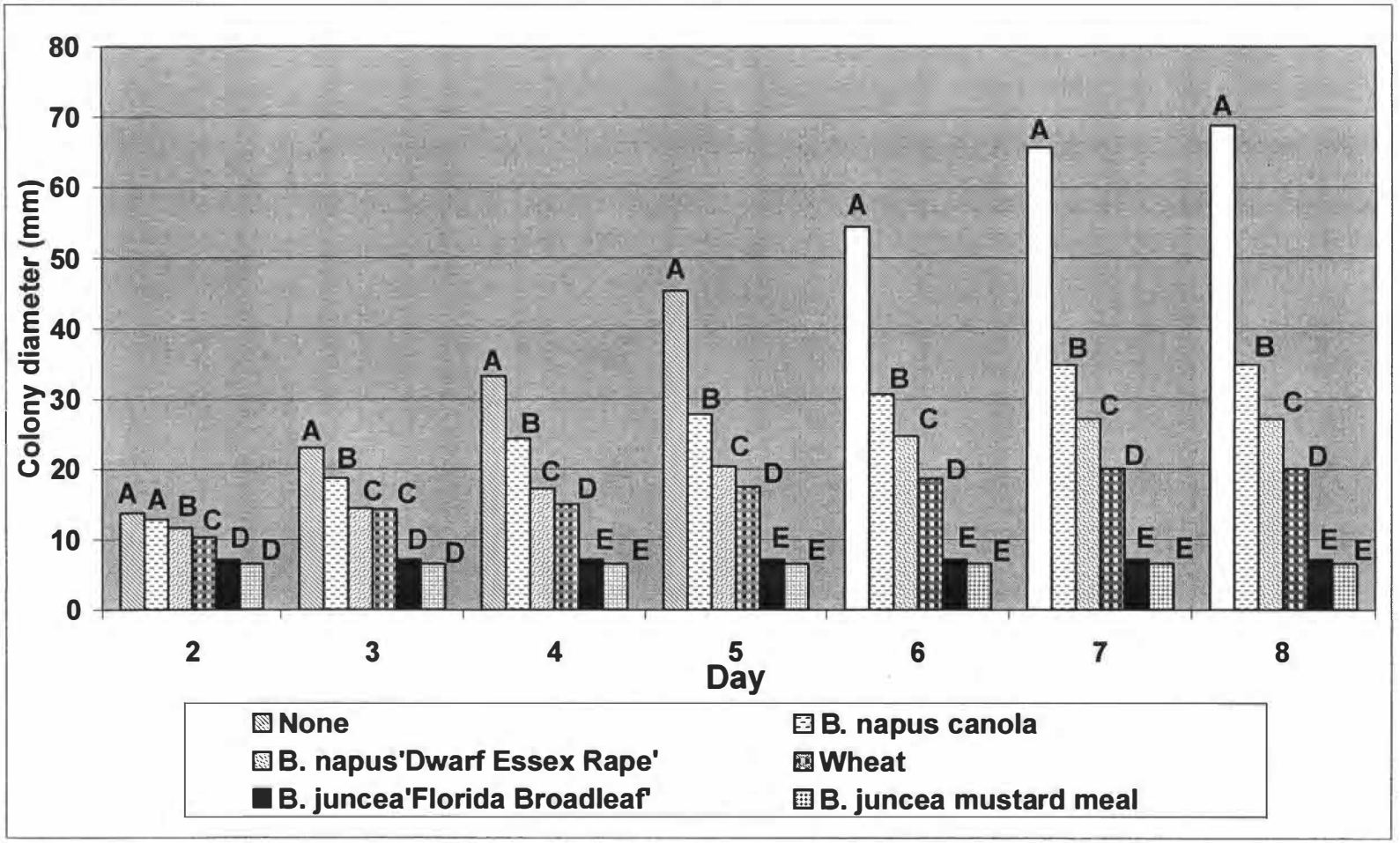

Fig. II-2. Main effect of mulch treatments on mycelia growth of *Gaeumannomyces graminis* var. *tritici* isolates A2, 211.1, WX, and *Fusarium oxysporum*, *F. graminearum*, and *F. solani* in Bioassay 2. Growth measurements are based on mean colony diameter of all fungal isolates. Within each day, bars with the same letters are not significantly different according to a Fisher's-protected least significant difference test at $P = 0.05$

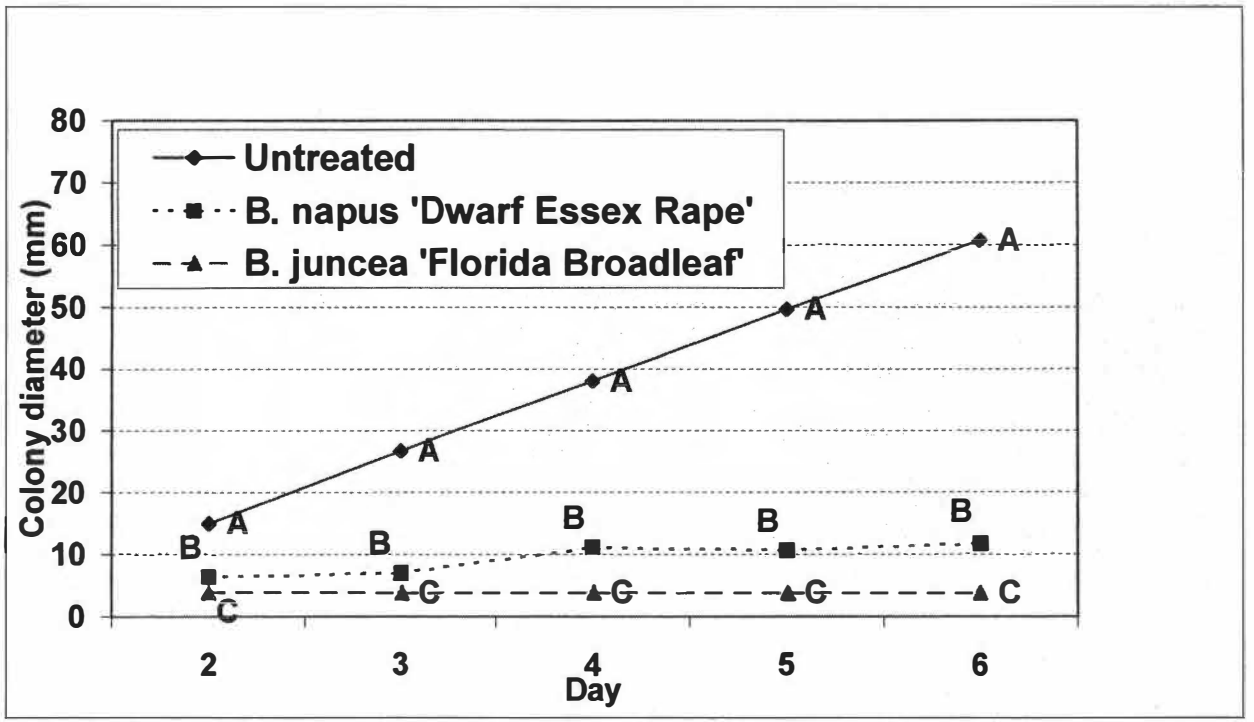

Fig. II-3. Effect of mulch treatments on growth of *Gaeumannomyces graminis* var. *tritici* isolate A2 in Bioassay 1. Within each day, data points with the same letter are not significantly different according to a Fisher's-protected least significant difference test at $P = 0.05$

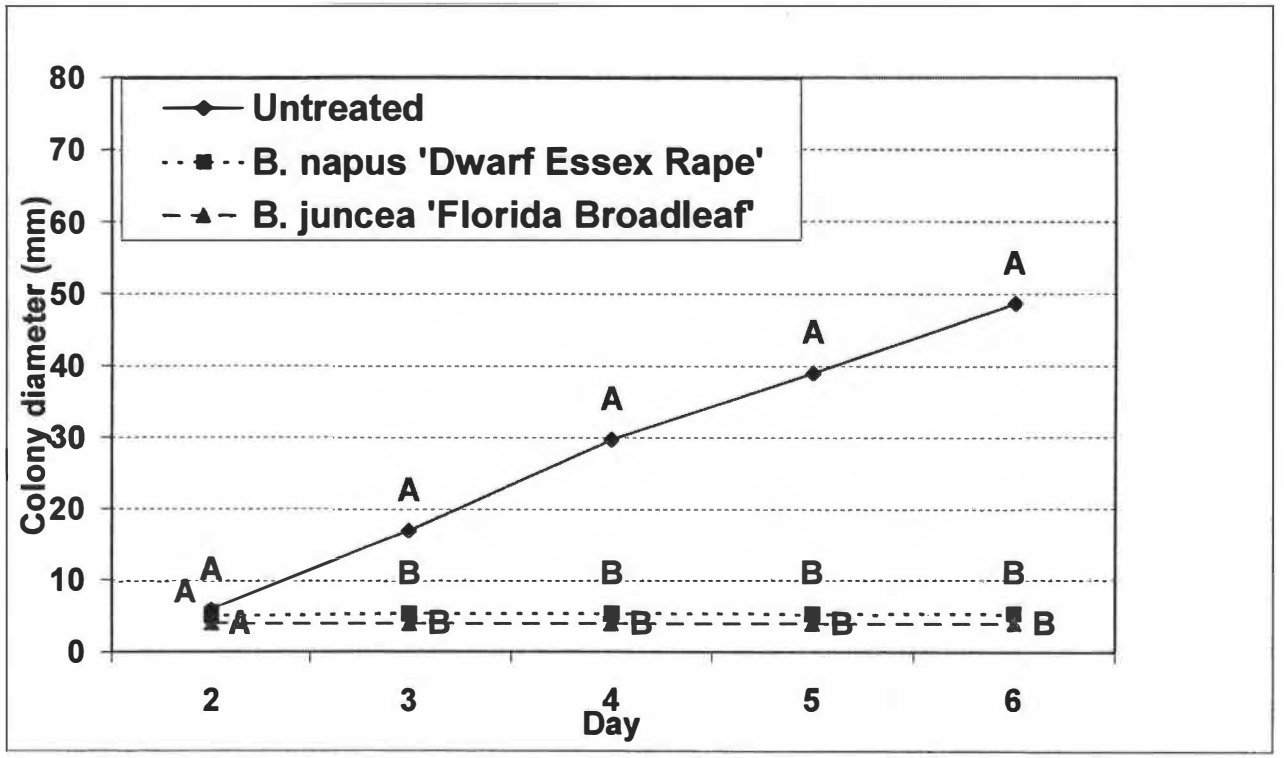

Fig. II-4. Effect of mulch treatments on growth of *Gaeumannomyces graminis* var. *tritici* isolate WX in Bioassay 1. Within each day, data points with the same letter are not significantly different according to a Fisher's-protected least significant difference test at $P = 0.05$

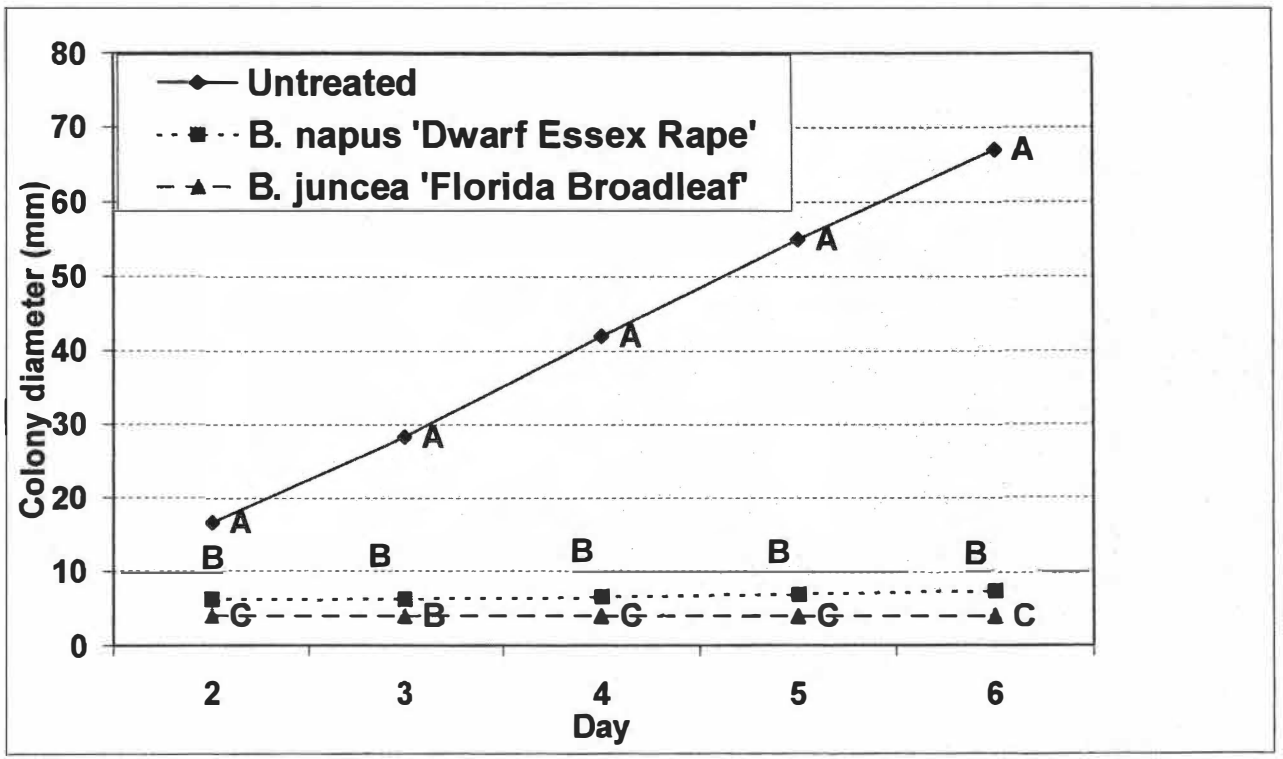

Fig. II-5. Effect of mulch treatments on growth of *Gaeumannomyces graminis* var. *tritici* isolate 211.1 in Bioassay 1. Within each day, data points with the same letter are not significantly different according to a Fisher's-protected least significant difference test at $P = 0.05$

Table II-4. Effect of mulch on mycelial growth of *Ggt* isolate A2 for day 2, 4, and 6

	Day[z]		
Treatment	2	4	6
Untreated	16.00 A	33.00 A	53.33 A
B. napus Canola	9.66 B	16.00 B	21.00 B
B. napus 'Dwarf Essex Rape'	8.00 B	8.00 C	8.00 C
Wheat	6.66 C	7.33 CD	7.33 CD
B. juncea 'Florida Broadleaf'	7.66 B	7.66 CD	7.66 CD
B. juncea Mustard meal	4.66 C	4.66 D	4.66 D

[z]Within each day, numbers with the same letters are not significantly different according to a Fisher's-protected least significant difference test at $P = 0.05$, Bioassay 2.

Table II-5. Effect of mulch on mycelial growth of *Ggt* isolate 211.1 for day 2, 4, and 6

	Day[z]		
Treatment	2	4	6
Untreated	6.33 A	22.00 A	45.33 A
B. napus Canola	6.00 A	15.33 B	17.66 B
B. napus 'Dwarf Essex Rape'	5.66 A	6.00 CD	6.00 CD
Wheat	7.16 A	7.83 C	7.83 C
B. juncea 'Florida Broadleaf'	4.66 A	4.66 D	4.66 D
B. juncea Mustard meal	4.66 A	4.66 D	4.66 D

[z]Within each day, numbers with the same letters are not significantly different according to a Fisher's-protected least significant difference test at $P = 0.05$, Bioassay 2.

Table II-6. Effect of mulch on mycelial growth of *Ggt* isolate WX for day 2, 4, and 6

	Day[z]		
Treatment	2	4	6
Untreated	3.83 A	23.33 A	38.33 A
B. napus Canola	5.00 A	11.00 B	13.33 B
B. napus 'Dwarf Essex Rape'	4.33 A	4.33 C	4.33 C
Wheat	4.00 A	4.00 C	4.00 C
B. juncea 'Florida Broadleaf'	3.66 A	3.66 C	3.66 C
B. juncea Mustard meal	3.66 A	3.66 C	3.66 C

[z]Within each day, numbers with the same letters are not significantly different according to a Fisher's-protected least significant difference test at $P = 0.05$, Bioassay 2.

Table II-7. Effect of mulch on mycelia growth of *Fusarium oxysporum* at 2, 4, and 6 days after treatment

	Day[z]		
Treatment	2	4	6
Untreated	24.00 A	43.66 A	67.00 A
B. napus Canola	24.33 A	36.66 B	43.33 B
B. napus 'Dwarf Essex Rape'	22.33 A	29.00 C	35.00 C
Wheat	19.66 B	31.66 C	43.66 B
B. juncea 'Florida Broadleaf'	13.66 C	13.66 D	13.66 D
B. juncea Mustard meal	13.00 C	13.00 D	13.00 D

[z]Within each day, numbers with the same letters are not significantly different according to a Fisher's-protected least significant difference test at $P = 0.05$, Bioassay 2.

Table II-8. Effect of mulch on mycelia growth of *Fusarium graminearum* at 2, 4, and 6 days after treatment

	Day[z]		
Treatment	2	4	6
Untreated	15.33 A	42.33 A	67.00 A
B. napus Canola	16.66 A	36.00 B	46.66 B
B. napus 'Dwarf Essex Rape'	14.33 B	20.66 D	29.00 C
Wheat	10.66 C	26.00 C	43.66 B
B. juncea 'Florida Broadleaf'	4.83 D	4.83 E	4.83 D
B. juncea Mustard meal	5.33 D	5.33 E	5.33 D

[z]Within each day, numbers with the same letters are not significantly different according to a Fisher's-protected least significant difference test at $P = 0.05$, Bioassay 2.

Table II-9. Effect of mulch on mycelia growth of *Fusarium solani* at 2, 4, and 6 days after treatment

	Day[z]		
Treatment	2	4	6
Untreated	17.00 A	35.33 A	55.33 A
B. napus Canola	15.66 AB	31.00 B	42.33 B
B. napus 'Dwarf Essex Rape'	15.33 AB	22.00 C	29.33 C
Wheat	14.00 B	26.33 D	41.66 B
B. juncea 'Florida Broadleaf'	8.33 C	8.33 E	8.33 D
B. juncea Mustard meal	8.00 C	8.00 E	8.00 D

[z]Within each day, numbers with the same letters are not significantly different according to a Fisher's-protected least significant difference test at $P = 0.05$, Bioassay 2.

Table II-10. Effect of the interaction of mulch and *Ggt* isolate on mycelial growth at 3, 5, and 7 days after removal of the mulch treatment, Bioassay 1

		Day after mulch removed[z]		
Mulch Treatment	*Ggt* Isolate	3	5	7
B. napus 'Dwarf Essex Rape'	A2	23.5 A	28.5 A	29.5 A
	211.1	10.7 B	15.3 AB	18.3 AB
	WX	5.3 B	10.8 B	14.0 AB
B. juncea 'Florida Broadleaf'	A2	3.8 B	3.8 B	3.8 B
	211.1	4.0 B	4.0 B	4.0 B
	WX	4.0 B	4.0 B	13.5 B

[z]Within each day, numbers with the same letters are not significantly different according to a Fisher's-protected least significant difference test at $P = 0.05$, Bioassay 2.

Table II-11. Effect of the interaction of mulch and *Ggt* isolate on mycelial growth at 2, 4, and 6 days after removal of the mulch treatment, Bioassay 2

		Days after mulch removed[Y]		
Mulch Treatment	*Ggt* Isolate	2	4	6
B. napus Canola	A2	41.66 A	59.00 A	80.00 A
	211.1	33.66 AB	55.33 A	76.33 AB
	WX	26.00 BC	44.33 AB	61.00 BC
B. napus 'Dwarf Essex Rape'	A2	14.33 D	31.00 BC	46.66 C
	211.1	4.66 E	8.66 E	15.66 D
	WX	4.33 E	11.00 DE	15.66 D
Wheat	A2	19.66 C	27.00 BC	25.33 D
	211.1	11.00 DE	*[Z]	*
	WX	22.33 C	18.66 CD	20.00 DE
B. juncea 'Florida Broadleaf'	A2	7.66 E	7.66 E	7.66 DE
	211.1	4.66 E	4.66 E	4.66 E
	WX	3.66 E	3.66 E	3.66 E
B. juncea Mustard meal	A2	4.66 E	4.66 E	4.66 E
	211.1	4.66 E	4.66 E	4.66 E
	WX	3.66 E	3.66 E	3.66 E

[Y]Within each day, numbers with the same letters are not significantly different according to a Fisher's-protected least significant difference test at $P = 0.05$, Bioassay 2

[Z]* Contaminated with bacteria

Table II-12. Effect of the interaction of mulch and *Fusarium* species on mycelial growth at 2, 4, and 6 days after removal of the mulch treatment, Bioassay 2

		Day after mulch removed[z]		
Mulch Treatment	*Fusarium* spp	2	4	6
B. napus Canola	*oxysporum*	44.66 CD	80.00 A	80.00 A
	solani	68.33 B	53.33 C	80.00 A
	graminearum	80.00 A	80.00 A	80.00 A
B. napus 'Dwarf Essex Rape'	*oxysporum*	46.00 CD	60.33 BC	75.66 A
	solani	48.33 CD	66.66 ABC	78.00 A
	graminearum	52.33 C	76.00 AB	80.00 A
Wheat	*oxysporum*	64.66 B	73.33 AB	75.33 A
	solani	80.00 A	80.00 A	80.00 A
	graminearum	80.00 A	80.00 A	80.00 A
B. juncea 'Florida Broadleaf'	*oxysporum*	13.66 E	13.66 D	13.66 B
	solani	8.33 E	8.33 D	8.33 B
	graminearum	7.66 E	7.66 D	7.66 B
B. juncea Mustard seed meal	*oxysporum*	13.00 E	13.00 D	13.00 B
	solani	8.00 E	8.00 D	8.00 B
	graminearum	5.33 E	5.33 D	5.33 B

[z]Within each day, numbers with the same letters are not significantly different according to a Fisher's-protected least significant difference test at $P = 0.05$, Bioassay 2.

Part III

Effect of Allyl Isothiocyanate from *Brassica juncea* on Growth of *Gaeumannomyces graminis* var. *tritici*

Abstract

Gaeumannomyces graminis var. *tritici* (*Ggt*) is a soilborne pathogen that affects wheat. Recently, *Brassica* spp. have been reported to suppress soilborne wheat pathogens *in vitro*. The objective of this experiment was to determine the amount of allyl isothiocyanate (AITC) released from *B. juncea* needed to inhibit growth of *Ggt*. *Gaeumannomyces graminis* var. *tritici* was exposed to AITC, a dominant isothiocyanate compound found in *Brassica* spp. Treatments of pure AITC and AITC from *B. juncea* seed meal (mixed with water) at 0, 0.01, 0.02, 0.03, and 0.04 g were evaluated for effects on *Ggt* mycelial growth. Petri plates with potato dextrose agar and fungal plugs were inverted over 490-cm^3 glass jars containing AITC treatments for 5 h. Headspace concentrations of pure AITC and AITC from *B. juncea* were recorded and colony diameter was measured for 9 days to determine suppression or death of *Ggt*. Mycelia growth was inhibited by all *B. juncea* treatments. The concentrations of AITC produced by *B. juncea* that inhibited 50 and 90% mycelial growth of *Ggt* were 0.43 and 0.80 μmol• $liter^{-1}$, respectively. The average amount of AITC for each treatment is reported. Inhibition attributable to pure AITC alone was greater than that achieved by *B. juncea*.

Introduction

Gaeumannomyces graminis var. *tritici* is the most important soilborne pathogen affecting wheat worldwide (USDA, 1994). It is prevalent in the Southeastern United States due to high rainfall. This pathogen causes take-all of wheat and is most severe in areas with high precipitation or irrigation and poor drainage. Various controls have been

applied to *Ggt* but most are ineffective or not economical.

The damage to wheat is related to the extent of root and basal stem colonization by *Ggt*. Wheat plants can withstand mild infections with only minimal yield loss. Mild infections do not result in obvious symptoms of the disease. However, when symptoms become obvious, yield losses can be high. Yield loss is attributed to the premature death of plants soon after heading and before grain filling.

Wheat is most vulnerable to take-all during the seedling stage. For this reason seed treatments with fungicides have been applied with some success (Duffy, 2000; Dawson, 2001). However, take-all is sporadic in a field and seed treatments have not proven to be economical. Crop rotation and double cropping are common practices in the southeast to effectively utilize the growing season and to reduce inoculum of pathogens in soil. However, a rotation of wheat and legumes tends to aggravate take-all leading to an increase of the disease (Cook, 1981).

Double cropping of wheat with green mulches that have allelopathic potential is being considered as an alternative approach to control take-all. Glucosinolates (GS), allelopathic compounds, are found in plants of the Brassicaceae family (Chew, 1988).

The concentration of GS varies throughout the leaves, stems, roots, and seeds (Mithen, 2001). In general, GS are allocated in proportion to tissue importance relative to reproduction, with seeds containing the greatest quantity (Wallace and Eigenbrode, 2001). When tissue is damaged, GS react with the enzyme myrosinase, in the presence of water, to form isothiocyanates (ITC). The ITC compounds function as natural fumigants and have proven to inhibit a wide array of plant pathogens (Charron and Sams, 1999). The GS-myrosinase interaction has been considered a plant defense system. However,

little work has been done to discover which specific ITC are directly involved with inhibition of pathogens.

Allyl isothiocyanate (AITC) is a dominant ITC found in *Brassica juncea* 'Florida Broadleaf'. This cultivar is grown for food consumption or animal forage and inhibits mycelial growth of *Ggt* and *Fusarium in vitro* (Breeden et al., 2003).

In this study pure AITC and mustard seed meal from *B. juncea* were tested to determine the amount of AITC needed to inhibit growth of *Ggt*.

Materials and Methods

Treatment application

Fungal plugs of *Gaeumannomyces graminis* var. *tritici* were taken from actively growing mycelia of isolate WX, placed on 100-mm Petri dishes with 20 ml of potato dextrose agar (1/4 strength), and incubated for 24 h at 22°C. Mycelial growth was observed on fungal plugs after 24 h. Isolate WX was isolated from roots of wheat with take-all disease (Mazzola et al., 1995).

Petri dishes were inverted on 490-cm^3 glass jars that had been flamed with 70% ethanol. Pure AITC (95%, Sigma Aldrich, St. Louis, MO) was diluted with hexane (HPLC grade, Sigma Aldrich, St. Louis, MO) to obtain desired treatment concentrations of 0.0, 0.72, 1.56, 2.5, 3.125, and 4.76 μmol • $liter^{-1}$. An airtight 10-μl syringe (Agilent Technologies, Palo Alto, CA) was used to inject pure AITC into the bottom of jars. *Brassica juncea* seed meal treatments (0.0, 0.01, 0.01, 0.02, 0.03, 0.04 g) were placed in the bottom of jars and deionized water (50, 50, 100, 100, 150, and 200 μl respectively)

was added to hydrolyze the GS and activate the release of ITC from *B. juncea* (Table III-1; All tables and figures are located in Appendix III). Jars were sealed with Parafilm and incubated at 23°C for 1 h before AITC was measured.

The AITC concentrations were determined at 1, 2, and 3 h using an airtight 50-µl syringe (Agilent Technologies, Palo Alto, CA). Headspace samples were taken from a hole (0.635-cm-diam.) that was covered with two layers of adhesive tape on the side of the glass jar.

Gas Chromatograph

Headspace samples were injected into a Hewlett Packard 5890 gas chromatograph equipped with a Hewlett Packard 5972 mass selective detector to desorb for 1 min. The column was an Alltech EC Wax 30 x 0.25 x 0.25 (Alltech Associates, Inc., Deerfield, IL). The inlet and outlet temperatures were 200 and 280°C, respectively. The oven parameters were programmed at 60°C for 1 min, and then increased by 5°C per min to a maximum of 150°C. Detector response was quantified based on the equation for the AITC standard curve.

After 5 h of exposure to pure AITC and *B. juncea* mulch in sealed jars, Petri dishes were removed, resealed with Parafilm and a new Petri lid, and *Ggt* was allowed to grow for 9 days. Fungicidal properties of pure AITC and *B. juncea* seed meal treatments were determined by measuring the colony diameter of fungal plugs.

Data Collection

Rates of pure AITC and the resulting counts, taken at one and two hours, were used to generate a regression equation (Fig III-1). This equation was used to calculate

concentrations of *B. juncea* seed meal that inhibited 50 and 90% (IC50 and IC90) of *Ggt* mycelial growth (Fig. III-2 and Fig. III-3).

Design and Analysis

This experiment utilized a completely randomized design with three replicates and was repeated. Treatments were five rates of pure AITC or five rates of *B. juncea* seed meal (Tables III-1 and III-2). Two treatments of *B. juncea* with 0.01 g were used; however one treatment (denoted with an asterisk) had 100 µl of water added in order to obtain a lower amount of AITC. Linear regression with PC-SAS software (Version 9.1, SAS Institute, Cary, NC) was utilized to produce the equations for quantifying AITC production and for describing the relationship between AITC release and *B. juncea.*

Results

The linear regression equation, developed using known concentrations of AITC, was $y = 905.99x + 134.61$ (Fig. III-1). The R^2 of this equation was 0.85. Mycelia of *Ggt* were sensitive to volatiles produced by *B. juncea* seed meal. Treatments containing 0.03 g or more resulted in death of *Ggt.* Inhibition of *Ggt* mycelia at 50% (IC50) and 90% (IC90) were, 0.43 and 0.80 $\mu mol \cdot liter^{-1}$, respectively, by *B. juncea* were calculated using the linear regression equation (Fig. III-2).

Concentrations of GS activity can vary between samples of *B. juncea*, therefore IC50 and IC90 values of *B. juncea* meal (g) were not determined. Instead, AITC from GS activity in *B. juncea* treatments were measured and related to inhibition of *Ggt.* The *Ggt* isolate WX was sensitive to pure AITC (Fig. III-3). The IC50 and IC90 values of

pure AITC were 0.22 and 0.49 μmol•liter^{-1} respectively. These values were calculated using the equation developed from a standard curve (Figure III-1).

The average concentration of AITC evolved from each treatment of *B. juncea* ranged from 0.39 μmol•liter^{-1} for 0.01 g meal (diluted with 100 μl water) to 1.77 μmol•liter^{-1} for 0.04 g meal (diluted with 200 μl water) (Table III-1). The AITC (μmol•liter^{-1}) evolved from pure AITC ranged from 0.06 (0.72 μM treatment) to 0.92 (4.76 μM treatment) (Table III-2). Absorption of volatiles by experimental equipment occurred in all treatments of pure AITC and AITC from *B. juncea* meal.

Discussion

In a study on inhibition of *Pythium ultimum* and *Rhizoctonia solani* by *B. juncea*, more than 90% of the volatiles recorded were AITC. This suggested that AITC was a significant factor in suppression of the pathogens by volatiles emitted from macerated mustard leaves (Charron and Sams, 1999).

In experiments with *Sclerotium rolfsii*, mycelia and sclerotia were sensitive to volatiles produced by freeze-dried *B. juncea* Indian mustard. However, predicted concentrations of Indian mustard necessary for inhibition of *S. rolfsii* mycelial growth were four times the concentrations actually observed, if AITC alone was responsible for the inhibition. This suggested that other chemicals released by the Indian mustard contributed to its toxicity. Harvey concluded that AITC and other compounds acted synergistically to provide greater inhibition than either compound alone (Harvey et al., 2002).

Results of the current study differed from the aforementioned. The LC50 for pure

AITC was 0.22 μmol^{-1} [332.71 Gas Chromatograph Count (GCC)] and the LC90 was 0.49 μmol^{-1} (578.86 GCC). The LC50 for AITC mustard seed meal from *B. juncea* was 0.43 μmol^{-1} (524.61 GCC) and the lC90 was 0.80 μmol^{-1} (858.25 GCC). The equation y = 905.99x + 134.61 was used to calculate projected values. The results suggest that AITC is the primary ITC responsible for inhibition of *Ggt*.

In this study AITC from *Brassica juncea* inhibited *Ggt* mycelia. It has been reported that only 1% or less of the predicted ITC concentration from GS were measured in soil amended with tissues of high GS content (Morra and Kirkegaard, 2002).

Mustard seed meal from *B. napus* cv. Dwarf Essex stimulated microbial organisms antagonistic to *Rhizoctonia solani* (Mazzola et al., 2001). Total bacterial populations in soil increased from log 7 colony-forming units (CFU) g^{-1} to greater than log 8 CFU g^{-1} 4 wk after application of 'Dwarf Essex' seed meal. Furthermore, fluorescent pseudomonad and actinomycete populations increased significantly in response to application of 'Dwarf Essex' seed meal. Nematode populations were reduced also in one of two soils tested (Mazzola et al., 2001). More work is needed to determine the biofumigation properties of AITC from *Brassica* sp. and whether biofumigation will be effective in reducing primary inoculum of *Ggt*.

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Appendix III

Table III-1. Amount of allyl isothiocyanate (AITC) (μmol•liter^{-1}) measured from each treatment of *B. juncea* (g) after 1 h

Brassica juncea (g)[a]	AITC (μmol•liter^{-1})
0.01	0.41
0.01	0.39
0.02	0.86
0.03	1.62
0.04	1.77

[a] diluted with 50, 100, 100, 150, and 200 μl deionized water, respectively.

Table III-2. Amount of pure allyl isothiocyanate (AITC) (μmol•liter^{-1}) recorded from each treatment of AITC (μM) diluted with 95% HPLC hexane after 1h

AITC (μM) treatment	AITC (μmol•liter^{-1})
0.72	0.06
1.56	0.23
2.50	0.42
3.125	0.83
4.76	0.92

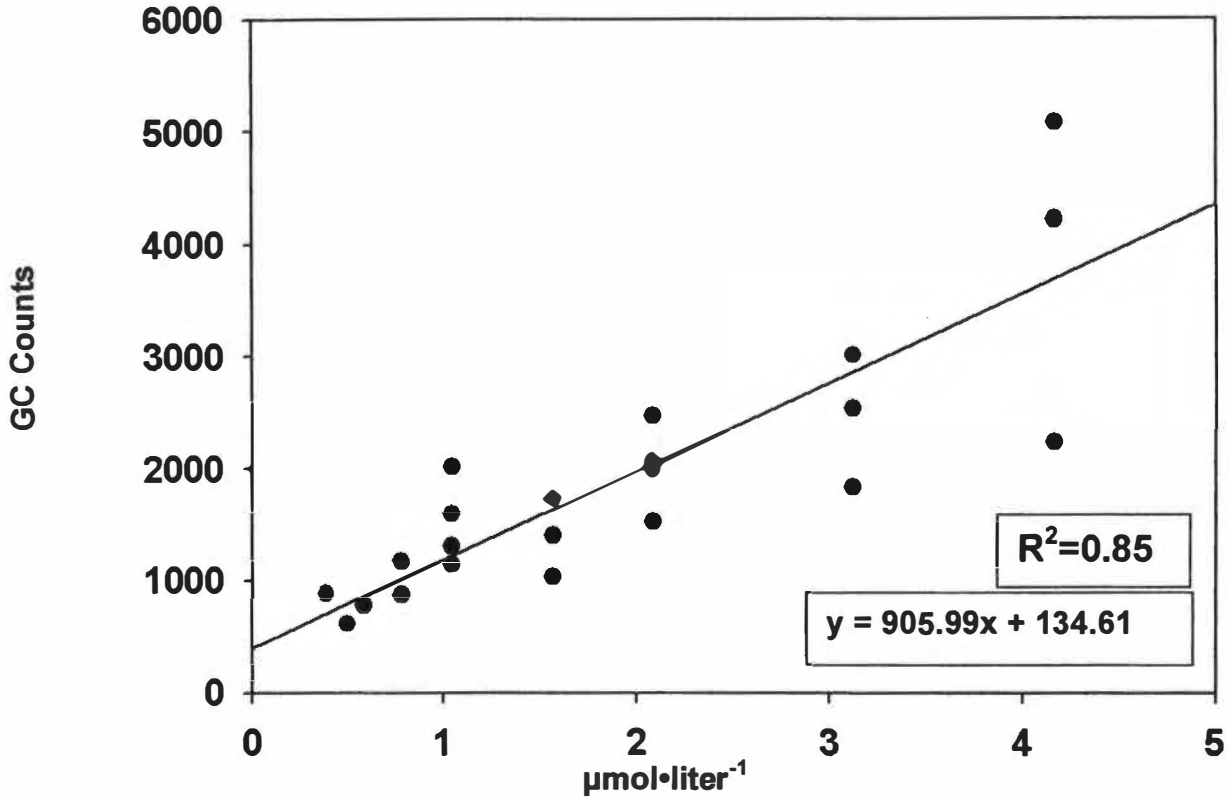

Fig. III-1. Standards were run with 490-cm^3 glass jars with a Teflon lid secured by a metal screw top. Headspace samples were taken from hole (0.635-cm-diam.) that was covered with adhesive tape. The equation above was obtained and used to calculate inhibiting concentrations (IC50 and IC90) of *Brassica juncea* seed meal

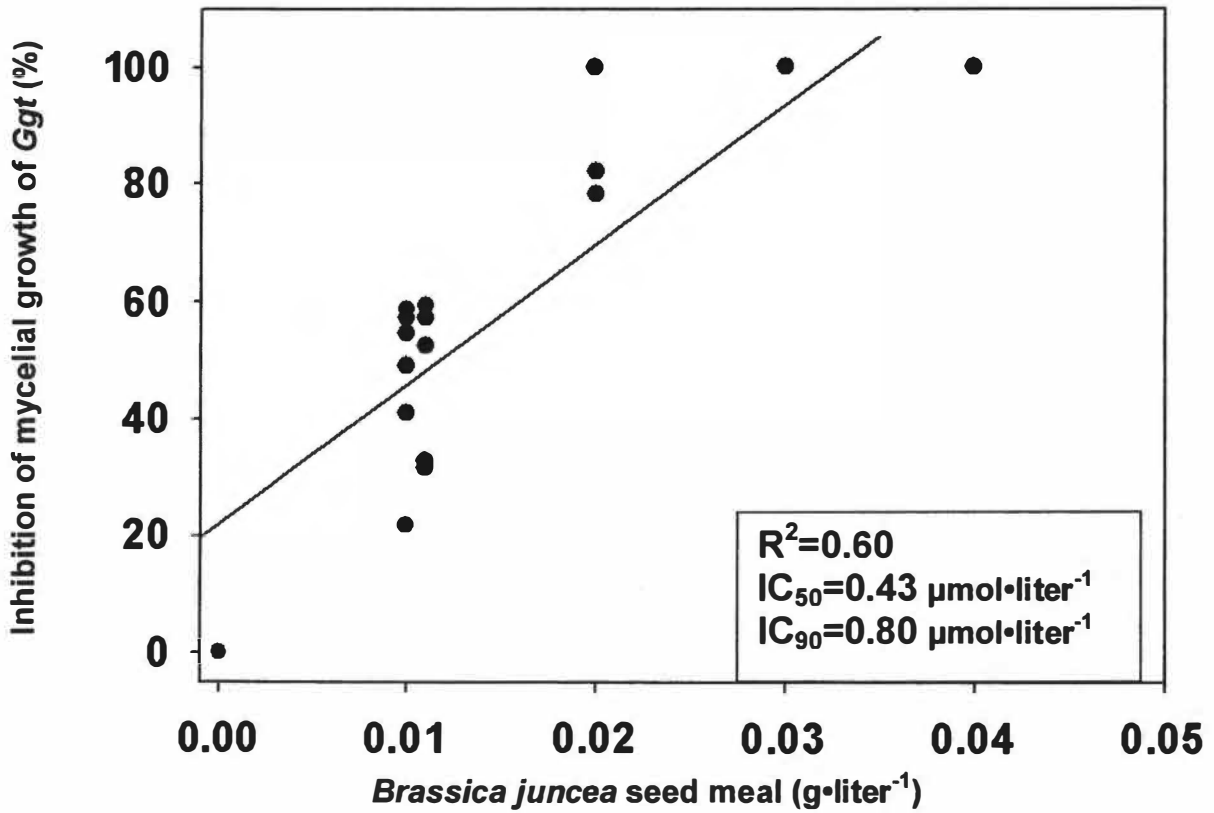

Fig. III-2. Inhibition of mycelial growth of *Ggt* with treatments of *B. juncea* seed meal. The IC50 and IC90 (inhibiting concentration at 50% and 90%) were calculated from the equation produced by the linear regression: $y = 905.99x + 134.61$ ($R^2=0.85$)

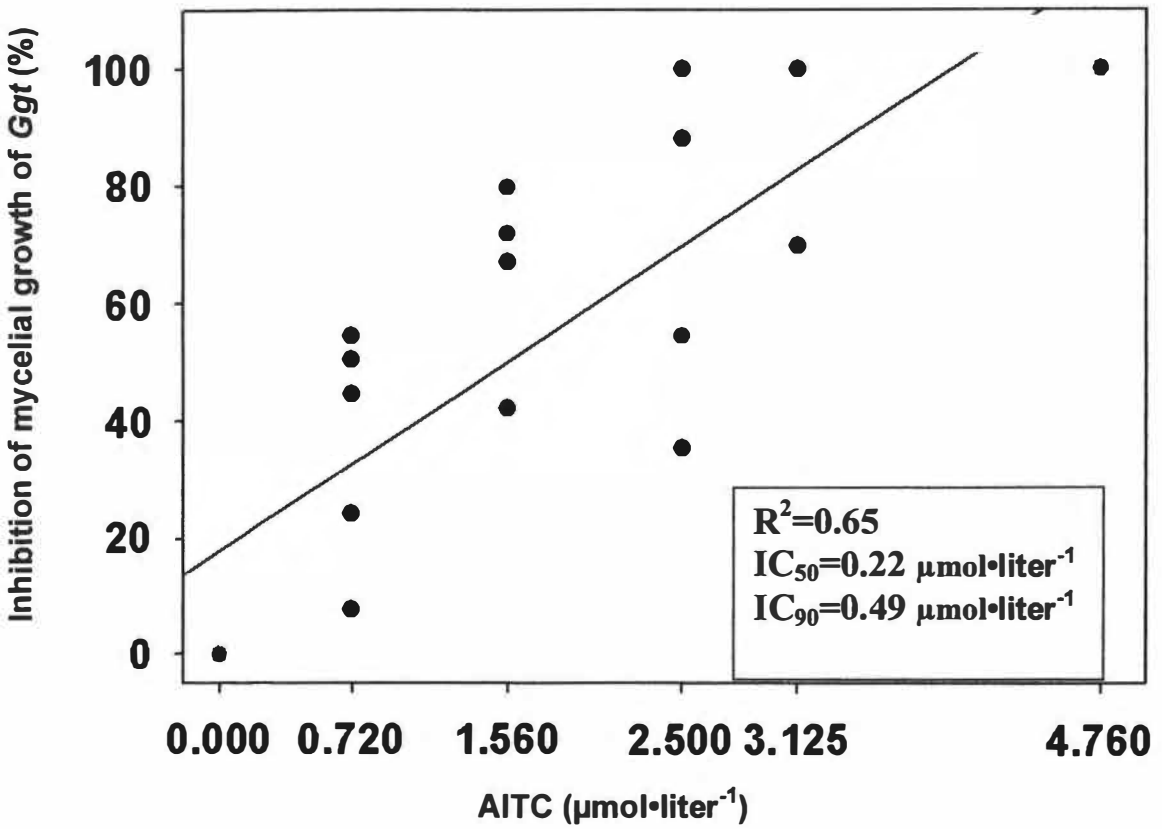

Fig. III-3. Inhibition of mycelial growth of *Ggt* with treatments of AITC diluted with 95% HPLC hexane. The IC50 and IC90 (inhibiting concentration 50 and 90%) were calculated from the equation produced by the linear regression: $y = 905.99x + 134.61$ ($R^2=0.85$)

Part IV

Effect of *B. juncea* 'Florida Broadleaf' Mulch Incorporated into Soil Infested with Wheat Roots and Crowns on Take-all Disease of a Subsequent Wheat Crop

Abstract

Mulches of *Brassica* spp. release glucosinolates during decomposition which are catalyzed to isothiocyanates, compounds that are toxic to soil microbes. Earlier we reported that *Brassica juncea* 'Florida Broadleaf' mulch was fungicidal *in vitro* to *Gaeumannomyces graminis* var. *tritici* (*Ggt*), which causes take-all of wheat. In this study, our aims were to determine if incorporating 'Florida Broadleaf' mulch into soil containing *Ggt*-infested wheat crowns and roots would reduce take-all in a subsequent wheat crop, and to evaluate phytotoxicity of *B. juncea* mulch to wheat seedlings. The experiment was a factorial in a split plot with two rates of *Ggt* (present or absent), two rates of *Brassica* mulch (present or absent) and ten replicates. *Brassica* mulch was the main plot and *Ggt* was the subplot. The study was repeated. Wheat 'AGS 2000' seeds were planted into soil in containers with and without *Ggt*. After 28 days, shoots were excised and wheat roots were left in soil. Soils with healthy and diseased roots were mulched with 'Florida Broadleaf' for five days; mulch was not applied to controls. Wheat 'AGS 2000' seeds were replanted into soil; take-all severity and shoot height were measured after 28 days. The main effects of mulch and *Ggt*, and the interaction were significant for disease in two trials. Treatment of soil containing *Ggt*-infected roots with *B. juncea* mulch significantly reduced take-all in the subsequent wheat planting in two tests. In one test, height of seedlings was reduced in soil with *B. juncea* mulch and seedling germination was reduced with *B. juncea* mulch regardless of the presence of *Ggt*.

Introduction

Gaeumannomyces graminis var. *tritici* is the most significant wheat disease worldwide, and U.S. yield losses exceed $1 billion/ year (USDA, 1994). The ascomycetous pathogen has a sexual stage but it is not important to disease transmission (Hornby and Cook, 1990). The fungus exists within soil, in old roots and tiller bases of previous wheat crops, and uses these plant remains as a food base for survival and to support growth onto new roots of the next crop (Cook and Veseth, 1991).

Take-all is usually more severe in sandy soils, with high pH and low fertility. Also, take-all is often found in regions with heavy rainfall, such as the Southeastern states, or in soils of dry wheat production under heavy irrigation, such as the Midwest and Pacific Northwest states. Infection by *Ggt* occurs through young seminal roots. Infections of these seedlings soon after planting result in the most severe yield losses.

Seed treatments with fungicides are effective in managing take-all (Duffy, 2000; Dawson, 2001). However, they are often economically impractical because take-all is sporadic in a field. Nutrient management is another form of take-all control. Maintaining adequate levels of nitrogen, phosphorus, and potassium for crop growth reduces take-all (Huber and McCay-Buis, 1993). Form of nitrogen is important in disease management. Nitrogen applied in the ammonium form is more efficient for suppressing take-all than urea nitrogen (Howard et al., 2002a; 2002b).

Crop rotation/alternative crops are often a practical, economical, and successful means of controlling soilborne pathogens. Crop rotation with other small grains, except oat, will not break the take-all disease cycle. In the southeast, wheat is often doubled-

cropped with soybean. However, an increase in disease following double cropping with soybean has been reported (Cook, 1981).

Brassica mulches have been reported to inhibit *Ggt in vitro* (Angus, 1994). Mulches of *Brassica* spp. such as *Brassica juncea* and *B. napus*, produce glucosinolates (GS). These are secondary metabolites found in intact plant tissues. When tissue is damaged the enzyme myrosinase (normally physically separated from GS) catalyzes the conversion of GS to isothiocyanates [ITC (Chew, 1988)], which have activity against plant pathogens (Charron and Sams, 1999). The ITC compounds are highly biocidal to a diverse range of organisms including nematodes, bacteria, fungi, insects, and germinating plant seeds (Brown and Morra, 1997; Borek, 1998).

The objectives of this experiment were to determine if incorporation of *B. juncea* 'Florida Broadleaf' mulch into soil containing *Ggt*-infested wheat crowns and roots would reduce take-all in a subsequent wheat crop, and to evaluate phytotoxicity of *B. juncea* 'Florida Broadleaf' to wheat seedlings.

Materials and Methods

Pathogen inoculum

Two isolates (WX and A2) of *Ggt* were used to produce inoculum in order to minimize possible effects from differential sensitivity of the pathogen to *Brassica* mulch. The isolates were cultured from diseased wheat roots in South Carolina (Mazzola et al., 1995) and Tennessee (our laboratory) respectively. Mycelia grown in 100-mm Petri dishes on potato dextrose agar (1/4 strength) were added to twice-autoclaved (120°C, 90 min) oat grain medium (150 cm^3 oats, 125 ml deionized water) in a 1-liter Erlenmeyer

flask that was plugged with a cotton swab wrapped in cheesecloth. Flasks were agitated every 24 h in order to distribute mycelia throughout the flask and allow mycelia to colonize oat seeds. As oat seeds became colonized with *Ggt* they turned black and began to clump together.

After 2 wk, oat inoculum was spread on heavy paper in a fume hood for 24 h to dry. Inoculum was stored at 38°C. As needed, whole colonized oat grains were ground in a blender, and sieved to obtain particles of 0.25 to 0.5-mm diam for use as inoculum.

Soil

Sequoia silty clay loam soil from the East Tennessee Research and Education Center Small Grain Unit, Blount County, TN was used for this test. Soil was autoclaved two times (120°C, 1.5 h) on consecutive days. Difficulty in obtaining infection from *Ggt* led to adjustment of pH and soil coarseness. Calcium carbonate was added to raise the pH of the soil from 5.5 to 7.0. A soil mineral and nutrient analysis of natural (Table IV-1; All tables and figures are located in Appendix IV) and autoclaved (Table IV-2) Sequoia silty clay loam was preformed by A&L Analytical Laboratories, Memphis, TN.

Coarseness of soil was adjusted by adding medium grained sand to soil. The final 3:1 ratio (wt/wt) of soil to sand was used for the disease assay.

Wheat Seed

Wheat seed used in this experiment was screened for viability and for susceptibility to take-all. Wheat cultivars (AGS 2000, USG 3209, OH 708, AST, and McCormick) were placed on 100-mm Petri dishes with moistened filter paper. Cultivars with less than 90% germination were not considered for the take-all susceptibility test.

Susceptibility to take-all was measured by planting seeds in a 1:1 mixture of *Ggt*

and soil. Plants grew for seven days and seedlings were rated for severity of take-all. Plant roots were washed free of soil and severity of take-all was evaluated on a scale of 0 to 8, where 0 = plant healthy (no disease evident); 1 = < 10% roots black; 2 = 10-25% roots black; 3 = 25-50% roots black; 4 = 50-100% roots black; 5 = all roots with lesions and lesions at base of stem; 6 = lesions moving up the stem; 7 = plant chlorotic and severely stunted; and 8 = plant dead or nearly so (Thomashow and Weller, 1988; Thomashow, 1990). Cultivar AGS 2000 was used in this experiment because it had the most severe take-all disease (Fig. IV-1).

Disease assay

Plastic containers (656 cm^3) with drainage holes were used in this experiment (Stuewe and Sons, Inc. Corvallis, OR). Eleven water absorbent cotton balls, 250-cm^3 vermiculite, 200-cm^3 soil with *Ggt* inoculum (6%, wt/wt), and 50-cm^3 vermiculite were layered in the containers for this assay. Seed were placed on the top of the soil/*Ggt* mixture and 50-ml of deionized water was added daily to simulate flood conditions. Cones were covered with clear plastic for 4 days to prevent excessive water evaporation during seed germination (Figure IV-2). Containers were placed in a growth chamber at 18°C for 28 days.

Mulch incorporation

Brassica juncea leaves were harvested, dried (10% moisture), and stored (24°C) at the East Tennessee Research and Education Center, Knoxville, TN, before incorporation into the disease assay.

After 28 days of growth, wheat stems were excised (1-cm) above the soil line and discarded. For *Brassica* treatments, soil was separated into 3-cm sections, and kneaded

for 25 s in a sealable plastic bag. Dried *B. juncea* (8.8 g) was added, and soil was kneaded for an additional 25 s to ensure a homogeneous mixture. The same process was repeated for each treatment excluding the addition of dried *Brassica*. Soil sectioning and kneading were done to simulate tillage in a field system. For the treatments without *Ggt*, the same procedure was used except that soil with wheat roots and crowns were used instead of autoclaved soil. Biofumigation with *Brassica* mulch occurred for 5 days.

Data Collection

Ten wheat seeds were planted in each cone and seedlings were grown for 28 days. Soil was washed from plant roots and disease severity was determined (0-8 scale). Seed germination percentage and seedling height were measured also.

Design and Analysis

The study was designed as a 2 x 2 factorial in a split plot with two levels of *Ggt* inoculum (present or absent) and two soil treatments (untreated or *B. juncea* 'Florida Broadleaf' mulch). *Brassica* mulch was the main plot and *Ggt* was the subplot. There were 10 replicates per treatment and 10 plants per replicate. The study was repeated.

Data were analyzed for significance with the Mixed procedure of PC-SAS (Version 9.0, SAS Institute, Cary, NC). Significant effects were further analyzed with Fisher's protected least significant difference test at $P = 0.05$ or $P = 0.10$.

Results

Take-all disease severity

The interaction of soil treatment and pathogen was significant in both trials ($P < 0.0001$). In two trials, of soil containing *Ggt*-infected roots and crowns as inoculum, disease severity was significantly lower with incorporation of *Brassica* mulch in soil than in untreated soil (Fig. IV-3, IV-4). In the absence of *Ggt*, there was no evidence of take-all disease.

Shoot height

In the first trial (Fig. IV-5), the interaction of soil treatment and pathogen was significant. ($P < 0.0001$). Height of seedlings was less in *Ggt*-free soil mulched with 'Florida Broadleaf' than in soil without mulch, but with *Ggt* present. Plant height in untreated soil (no *Ggt*) with no mulch treatment was greatest. In the second trial (Fig. IV-6), the main effect of pathogen was significant ($P = 0.0476$). Height was lowest in soil without *Ggt* regardless of mulch addition.

Seedling germination

In the first trial (Fig. IV-7), the effect of pathogen was significant ($P = 0.0078$). Germination was greatest for seedlings in soil without *Ggt*. In contrast, in the second trial (Fig. IV-8), effect of soil treatment was significant ($P = 0.0547$). Germination was lower in soil mulched with *Brassica*, regardless of the presence of *Ggt*.

Discussion

In previous reports we have shown that fresh mulches of *Brassica* spp. have fungicidal activity *in vitro* against soilborne pathogens of wheat such as *Ggt* and *Fusarium graminearum*, *F. oxysporum* and *F. solani* (Breeden et al., 2002; Breeden et al., 2003). In this study, incorporation of dried mulch of *B. juncea* cv. Florida Broadleaf, at a rate of 4% wt/vol significantly reduced severity of take-all disease. However, *Brassica* mulch was associated with reduced shoot height and seed germination of wheat. It is possible that lower rates of 'Florida Broadleaf' mulch may suppress disease without causing phytotoxicity effects.

In addition to the release of ITC, incorporation of dried *Brassica* mulch may alter the microbial population dynamics of soil. Addition of *Brassica napus* seed meal, with little ITC activity, suppressed *Rhizoctonia* root rot of apple caused by *R. solani* and increased specific groups of microorganisms, such as total bacteria, actinomycetes and fluorescent *pseudomonads*. The authors concluded that disease suppression could have resulted from enhanced populations of microorganisms that were antagonistic to *R. solani* (Mazzola et al., 2001). Although effects of *B. juncea* mulch on microbial populations were not evaluated in this study, it is likely that incorporation of mulch altered microbial populations which in turn could have contributed to disease suppression of *Ggt*.

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Appendix IV

Table IV-1. Analysis of Sequoia silty clay loam soil.

Nutrient	Concentration
Phosphorus (P)	35.0 ppm
Potassium (K)	200.0 ppm
Calcium (Ca)	697.0 ppm
Magnesium (Mg)	145.0 ppm
Sulfur (S)	7.0 ppm
Boron (B)	0.7 ppm
Copper (Cu)	1.8 ppm
Iron (Fe)	65.0 ppm
Manganese (Mn)	314.0 ppm
Zinc (Zn)	2.9 ppm
Sodium (Na)	17.0 ppm
Organic Matter	1.4%
CEC	8.1 meq/100g

Table IV-2. Analysis of autoclaved Sequoia silty clay loam soil.

Nutrient	Concentration
Phosphorus (P)	31.0 ppm
Potassium (K)	194.0 ppm
Calcium (Ca)	660.0 ppm
Magnesium (Mg)	146.0 ppm
Sulfur (S)	8.0 ppm
Boron (B)	0.7 ppm
Copper (Cu)	1.8 ppm
Iron (Fe)	51.0 ppm
Manganese (Mn)	385.0 ppm
Zinc (Zn)	60.1 ppm
Sodium (Na)	18.0 ppm
Organic Matter	1.7%
CEC	8.7 meq/100g

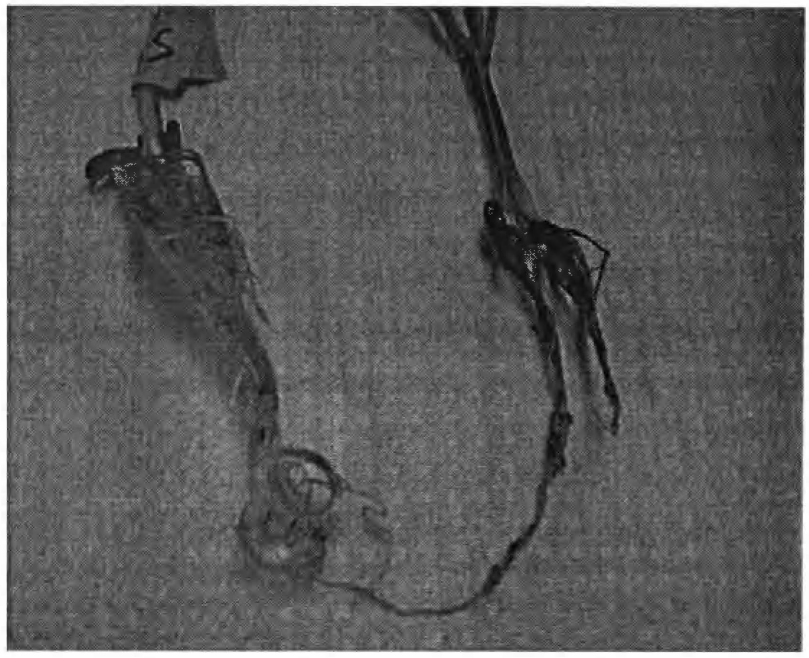

Figure IV-1. Wheat cv. AGS 2000 was very susceptible to take-all disease. Healthy wheat roots not exposed to *Ggt* (left) and roots with severe take-all (right).

Figure IV-2. Containers were covered with plastic to prevent excessive moisture evaporation during seed germination.

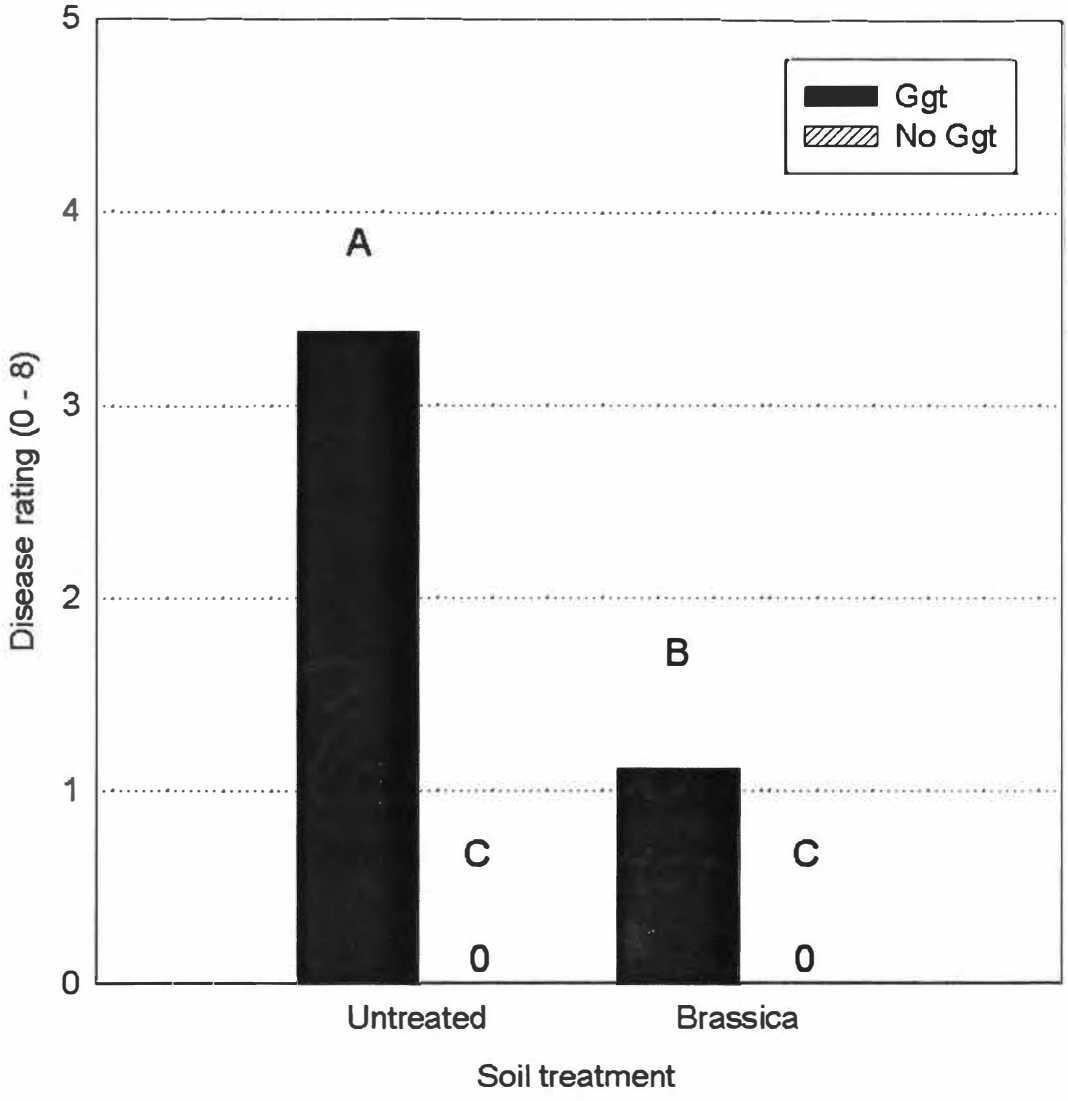

Fig. IV-3. Effect of the interaction of soil treatment and soil infestation with *Gaeumannomyces graminis* var. *tritici* on disease rating (0-8) of wheat seedlings. Standard error of the mean = ± 0.2335. Bars with different letters are significantly different according to a F-protected least significant difference test at $P = 0.05$. Trial 1.

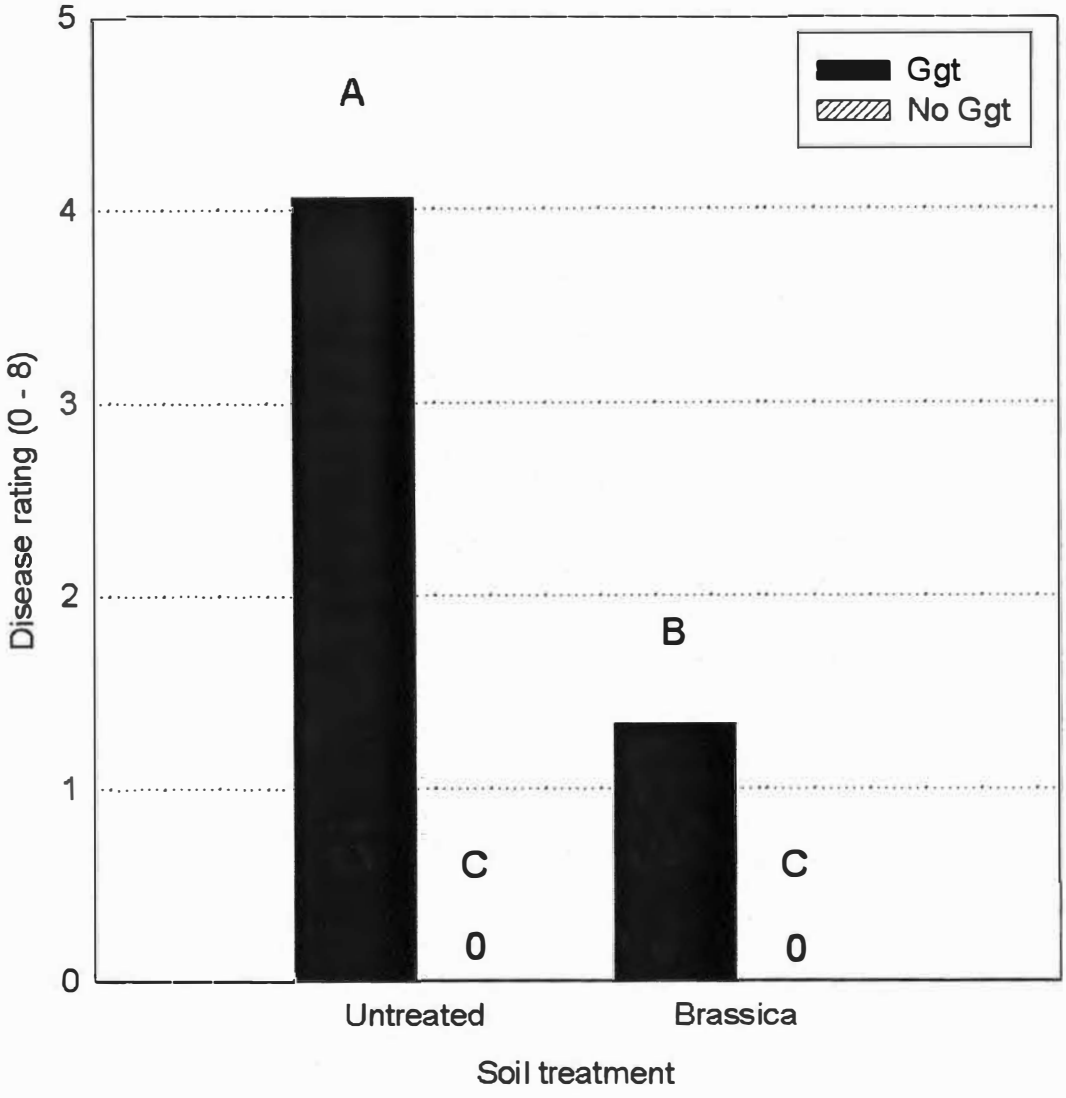

Fig. IV-4. Effect of the interaction of soil treatment and soil infestation with *Gaeumannomyces graminis* var. *tritici* on disease rating (0-8) of wheat seedlings. Standard error of the mean = ± 0.1992. Bars with different letters are significantly different according to a F-protected least significant difference test at $P = 0.05$. Trial 2.

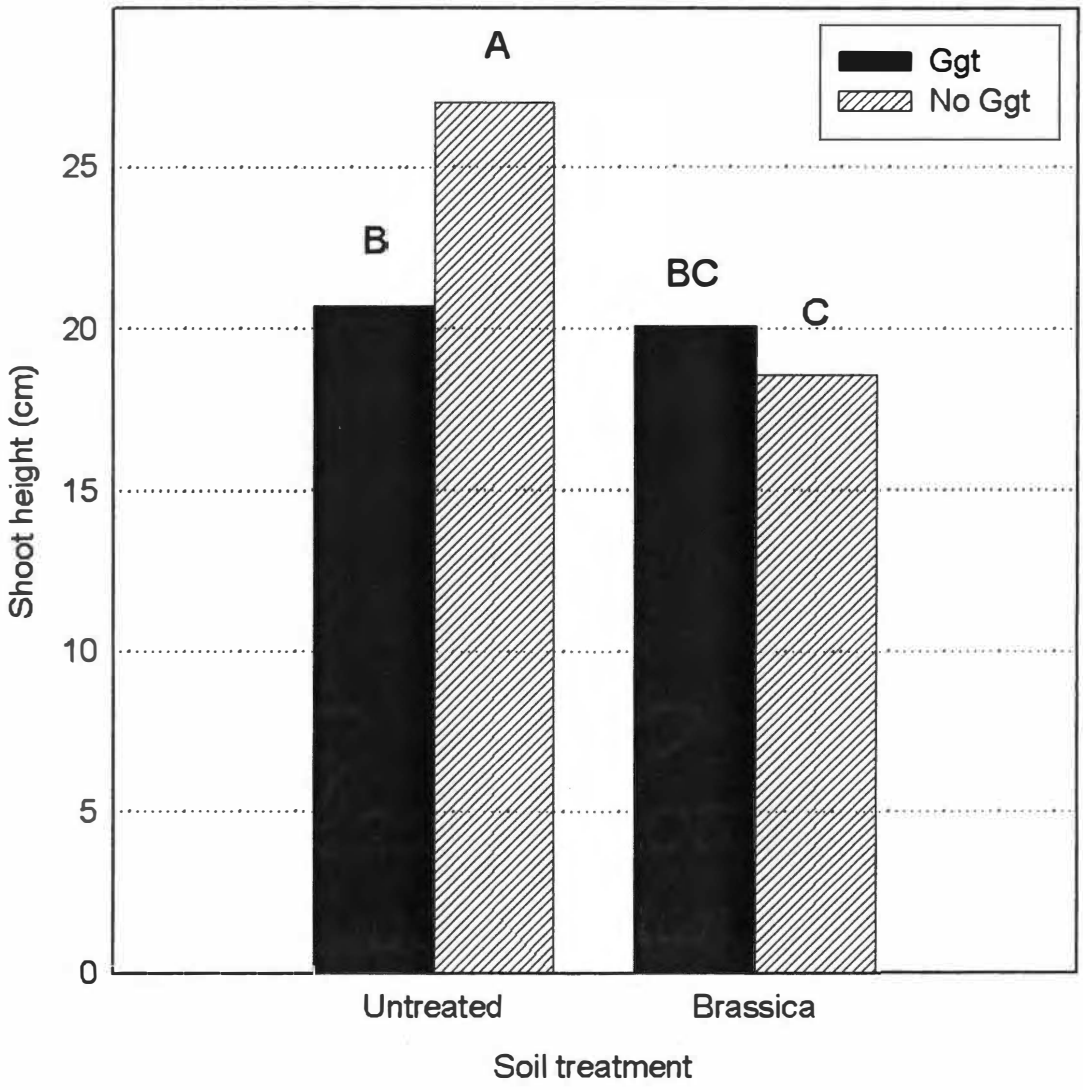

Fig. IV-5. Effect of soil treatment (untreated or *Brassica* mulch) on shoot height of wheat seedlings planted in untreated soil or soil infested with *Gaeumannomyces graminis* var. *tritici*. Standard error of the mean = ± 0.6668. Bars with different letters are significantly different according to a F-protected least significant difference test at $P = 0.05$. Trial 1.

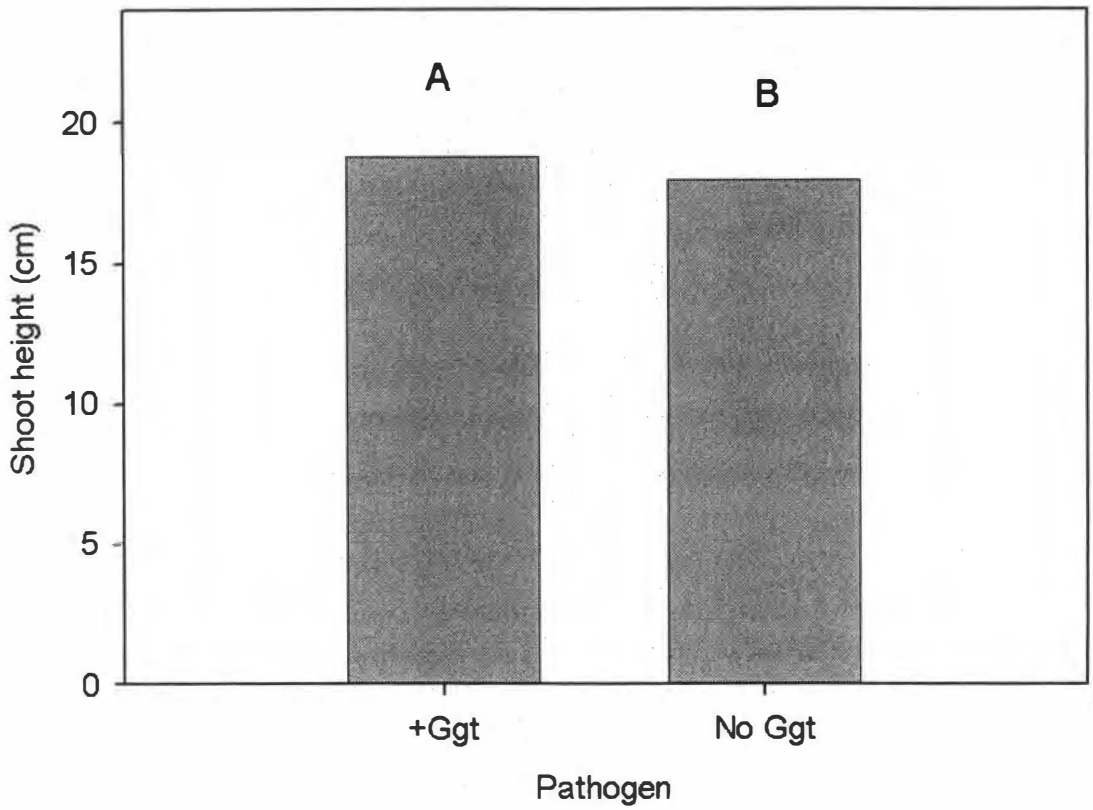

Fig. IV-6. Effect of pathogen treatment (+*Ggt*, no *Ggt*) on shoot height of wheat seedlings planted in untreated soil or soil mulched with *Brassica juncea* 'Florida Broadleaf'. Standard error of the mean = ± 0.3123. Bars with different letters are significantly different according to a F-protected least significant difference test at $P = 0.05$. Trial 2.

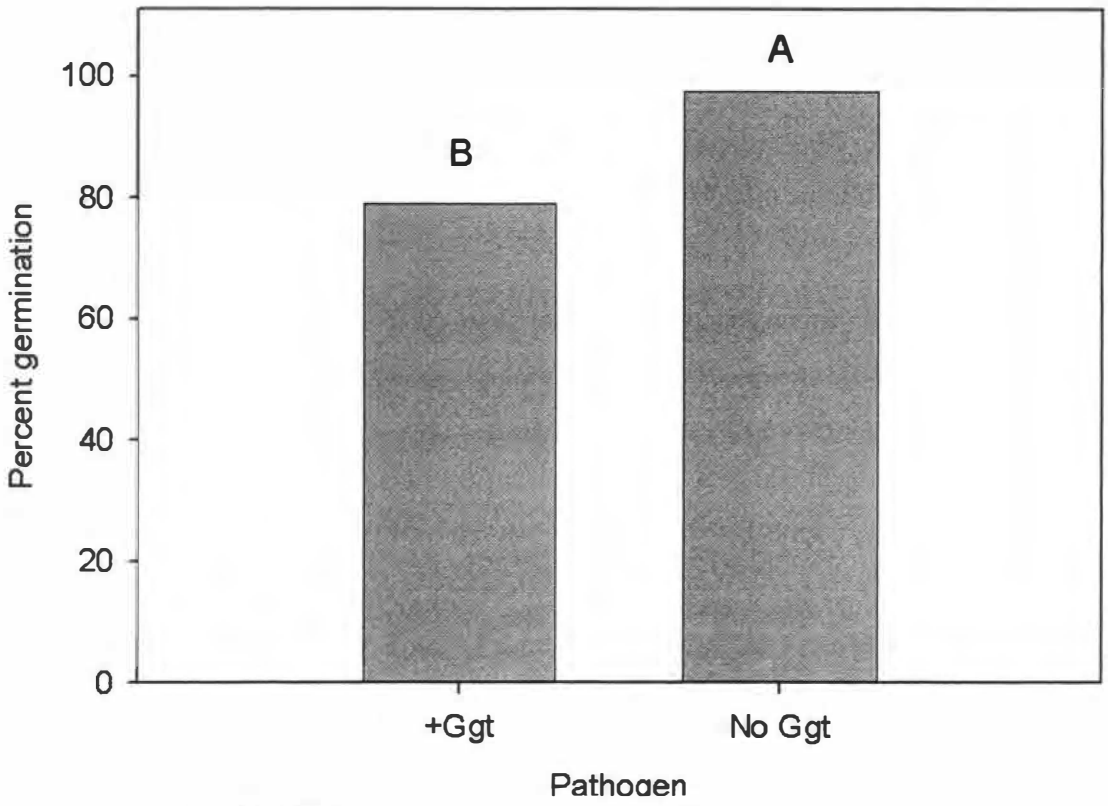

Fig. IV-7. Effect of pathogen (+*Ggt*, no *Ggt*) on germination of wheat seedlings planted in untreated soil or soil mulched with *B. juncea* 'Florida Broadleaf'. Standard error of the mean = ± 5.0254. Bars with the same letters are not significantly different to an F-protected least significant difference test at $P = 0.05$. Trial 1.

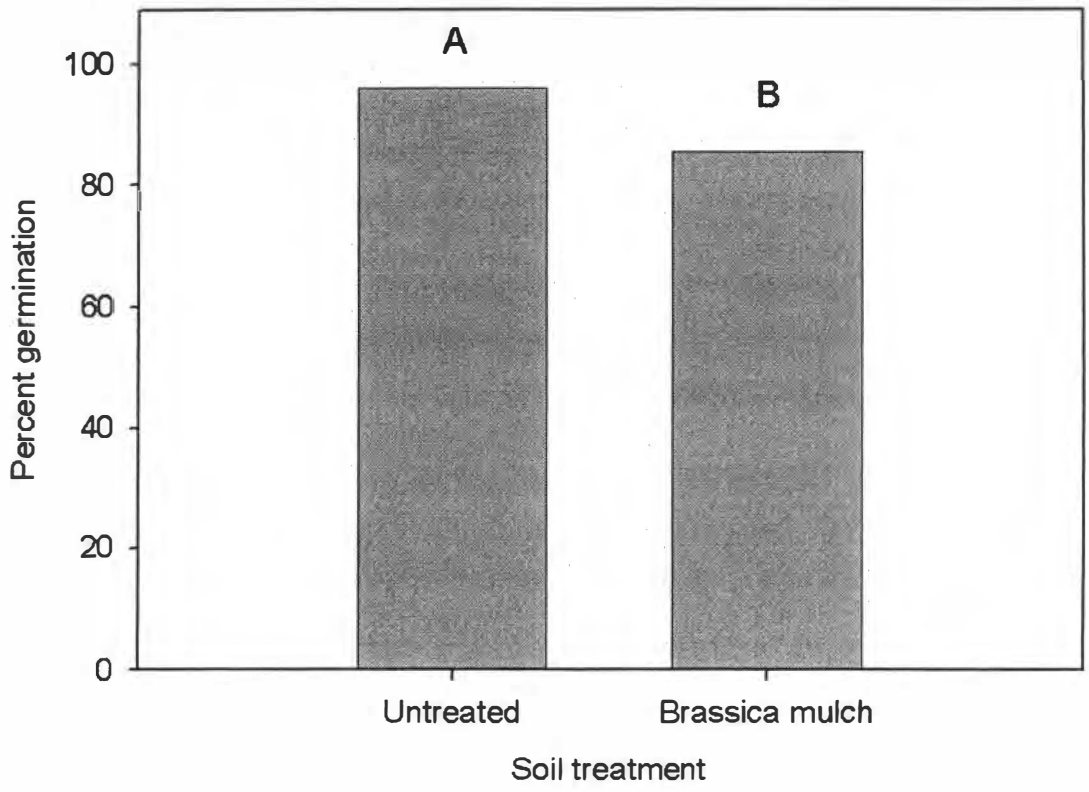

Fig. IV-8. Effect of soil treatment (untreated or *Brassica* mulch) on germination of wheat seedlings. Standard error of the mean = ± 3.4312. Bars with the same letters are not significantly different according to an F-protected least significant difference test at $P = 0.10$. Trial 2.

Vita

Thomas Samuel Breeden grew up in Decatur, TN. He was very active in agriculture and sports growing up. Thomas graduated from Meigs County high school in Decatur. He attended the University of Tennessee (UTK), Knoxville, TN where he played football for the VOLS and obtained his B.S. in Agriculture Education. After his undergraduate study, Thomas entered graduate school in Entomology and Plant Pathology at UTK. He married his good friend Angela (Cosgrove) Breeden and took a job teaching high school science in Raleigh, NC. Thomas' career is wide open. Him and his wife look forward to starting a family and perhaps working overseas.