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CHAPTER 1. LITERATURE REVIEW

1.1. TAKE-ALL DISEASE IN WHEAT

Take-all disease of wheat (*Triticum aestivum* L.) was first observed in Australia in 1852 and reported in 1868 (Garrett, 1981). It is a very wide spread disease in temperate climate region and is also found at high elevation regions in sub-tropical and tropical countries. The cause of the disease was established in 1890 as a fungus named *Ophiobolus graminis* Sacc. This is synonymous with the current name of *Gaeumannomyces graminis* (Sacc.) v. Arx & Olivier var. *tritici* Walker, abbreviated *Ggt*.

A characteristic symptom of take-all is blackening of roots, which may occur as early as the seedling stage (Clarkson and Polley, 1981). In severely affected plants, the root system is reduced. A black sock of mycelium may be seen on the stem base, especially during a wet season. Diseased plants appear as white patches in a field due to the bleached, empty heads, which are also called whiteheads. Diseased plants produce shriveled grain or even no grain. Other symptoms include reduced tillering, stunting, and uneven ripening. The yield loss caused by take-all is difficult to measure depending on several factors such as the types of soil and management practices. Take-all can cause yield losses up to 40% (E.L. Stromberg, personal communication).

Ggt is a root pathogenic ascomycete that survives by living saprophytically on grasses or plant residue between crop seasons (Skou, 1981). Mycelium, growing saprophytically on plant residue, is the main source of inoculum for disease initiation in a new season. The fungus grows from the residue towards plant roots. Once it reaches the plant, the mycelium can grow on all plant parts below the soil surface. If conditions are particularly wet, a sock of mycelium may be formed even up to a few centimeters above soil surface. Two different types of mycelia are recognized. Runner hyphae are brown, thick-walled macrohyphae that do not usually produce hyphopodia. Infection hyphae are hyaline, thin-walled microhyphae that produce hyphopodia. Runner hyphae grow on root surfaces or in root s outer cortex forming the mycelial sock. The runner hyphae branch off to give rise to infection hyphae, which penetrate epidermal cells.

Appressorium-like swellings are formed when infection hyphae contact plant cell wall, and enzymes are excreted to dissolve plant cell wall (Skou, 1981).

1.2. *GAEUMANNOMYCES-PHIALOPHORA* COMPLEX

There are two other varieties of *G. graminis* that are common on cereals and grasses, *G. graminis* (Sacc.) v. Arx & Olivier var. *avenae* (Turner) Dennis (*Gga*), a pathogen of oats (*Avena sativa* L.), and *G. graminis* (Sacc.) v. Arx & Olivier var. *graminis* (*Ggg*), a pathogen of seasonal grasses. To complicate identification, two non-pathogenic fungi, *Gaeumannomyces cylindrosporus* Hornby, Slope, Gutteridge & Sivanesan and *Phialophora* sp., which are very similar to *G. graminis,* are also found on roots of grasses and cereals. *G. cylindrosporus* is believed to be the teleomorph of *Phialophora graminicola*, while *Phialophora* sp. is believed to be the anamorph of *Ggg*. These pathogenic and non-pathogenic fungi form the *Gaeumannomyces-Phialophora* complex on the roots of cereals and grasses. *G. graminis* and *Phialophora* sp. produce hyphopodia on their host. A hyphopodium is a cell or swelling that functions as an organ of attachment and penetration and can be produced terminally, laterally, or intercalary. However, unlike an appressorium, a hyphopodium is produced from vegetative epiphytic hyphae (Walker, 1980). *Ggt* and *Gga* produce only the simple type hyphopodia while *Ggg*, *G. cylindrosporus*, and *Phialophora* sp. can produce both the simple and the lobed hyphopodia (Walker, 1981). Although all are common in grasses and cereals, *Ggt* is mostly pathogenic to wheat and barley (*Hordeum valgare* L.), *Gga* is pathogenic to oat and turf grasses, and *Ggg* is pathogenic to rice and grasses. *Phialophora* spp. is not pathogenic to grasses (Deacon, 1981). Some characteristics of these fungi are summarized in Table 1.

Table 1. Summary of characteristics of the fungi in *Gaeumannomyces-Phialophora* complex. (Walker, 1981).

Fungi	Perithecia	Asci	Ascospores	Hyphopodia	Phialospores	Cultures
Gaeumannomyces graminis var. *graminis* (*Ggg*)	Black, globose—oval, 200-400X150-300 µm	Unitunicate, elongated clavate, 100-135X10-15 µm, 8 spored	Hyaline to yellowish, slightly curved with rounded ends, 80-105X2.5-3 µm	Simple and lobed	Germinating (5-14X2-4 µm) and non-germinating (3-7X1-1.5 µm)	White to gray/black, dense short gray aerial mycelium, marginal hyphae curling back. Optimum temperature 20-25 °C
Gaeumannomyces graminis var. *avenae* (*Gga*)	Similar to *Ggg*, 300-500X250-400 µm	Similar to *Ggg*, 110-150X12-16µ m, 8 spored	Similar to *Ggg*, 100-130X2.5-3.5 µm	Simple	As in *Ggg*	Similar to *Ggg*. Optimum temperature 20-25 C
Gaeumannomyces graminis var. *tritici* (*Ggt*)	Similar to *Ggg*, 200-400X100-250 µm	Similar to *Ggg*, 80-130X10-15 µm, 8 spored	Similar to *Ggg*, 70-105X2.5-3 µm	Simple	As in *Ggg*	Similar to *Ggg*. Optimum temperature 20-25 C
Gaeumannomyces cylindrosporus		Similar to *Ggg*, 70-12-X9-16 m	Similar to *Ggg*, 40-70X3-5 m	Simple and lobed		Similar to *Ggg*. Optimum temperature 25 C
Phialophora sp.	Not found	Not found	Not found	Simple and lobed	Similar to *Ggg*	Similar to *Ggg*

1.3. PCR AS IDENTIFICATION TOOL FOR TAKE-ALL PATHOGEN

The similarities amongst *Gaeumannomyces* species and *G. graminis* varieties make it difficult to correctly and rapidly identify the take-all fungi. In addition to being difficult to isolate, colony morphology of *G. graminis* varieties is similar. Differences in hyphopodia type could be used but it is difficult and time consuming to produce the hyphopodia, although an improved method to produce them has been developed (Crozier, 1999). Ascospore lengths for the three varieties vary and are overlapping and therefore difficult to apply for identification. Pathogenicity testing is tedious and lengthy. On the other hand, accurate and rapid identification is very important in plant disease management.

Molecular approaches have been used to identify the fungus accurately and rapidly. Herdina *et al*., (1996) used DNA probes to identify and quantify *Ggt* in soil. Goodwin *et al*., (1995) used regions in the ribosomal RNA gene and internal transcribed spacer (ITS) regions to design primers for polymerase chain reaction (PCR) amplification to differentiate *G. graminis* from other fungi, including *Phialophora*. The PCR amplified DNA from *Gga* and *Ggg,* but not from *Ggt*.

PCR and its applications in plant disease diagnosis, such as nested PCR, multiplex PCR, random amplification of polymorphic DNA (RAPD), and amplified fragment length polymorphisms (AFLP), are rapid, sensitive and versatile. All of the PCR used to identify wheat take-all fungus are based on high redundancy of the families of DNA in the genome, such as the ITS of the rDNA. However, these methods are not associated with pathogenicity or host range of the pathogens. Manganese oxidation and avenacinase production are two metabolites activities that may be important to and correlate with pathogenicity of *G. graminis* varieties (Rachdawong, 1999).

Specific primers each for *Ggt*, *Gga* and *Ggg* have been developed based on single base variation among avenacinase genes present in all three varieties of *G. graminis* (Rachdawong, 1999). The avenacinase gene was chosen because it is associated with pathogenicity of the fungus. Avenacin is a fungitoxic substance present in the roots of oat. *Gga* is able to produce

avenacinase and detoxify avenacin allowing it to infect and colonize oats. *Ggg* and *Ggt* also produce avenacinase-like enzymes but they are not active against avenacin. The genes encoding avenacinase in all three varieties are highly similar in base composition but differ in enzyme activity. This allowed development of specific primers for the three varieties.

Based on avenacinase-like genes, three 5 oligonucleotide primers were developed for *Ggt*, *Gga*, and *Ggg*. The primers were specific to each of the *G. graminis* varieties, generating a 870 bp fragment for *Ggt*, a 617 bp fragment for *Gga*, and a 1,086 bp fragment for *Ggg* (Rachdawong, 1999). Differentiation of the three varieties is simple due to different sizes of the fragments generated. The primers are sensitive to their own DNA template even in mixtures of template and primer. This allows identification of all three varieties of *G. graminis* in a single PCR tube.

The high sensitivity of PCR makes it possible to identify fungi without isolating them. This is obviously an advantage since take-all fungus isolation is difficult. Henson *et al.*, (1993) detected *G. graminis* in plant and soil using nested PCR. Their reaction amplified a region in mitochodrial DNA specific for *G. graminis*, but they could not differentiate *G. graminis* varieties. Bryan *et al.*, (1995) were able to distinguish the three varieties of *G. graminis* and its *Phialophora*-like anamorphs. They amplified ITS region using nested PCR. Fouly and Wilkinson (2000) developed primers specific to *Ggt* and *Gga* that allow amplification of rDNA directly from infested plants. PCR with these primers generated fragments of different sizes from *Ggt* and *Gga*, but not from *Ggg* or other fungi.

1.4. DETECTION OF TAKE-ALL PATHOGEN FROM INFESTED PLANTS AND SOIL

PCR using avenacin specific primers permits detection and identification of the pathogen without the need for isolation. However, PCR conditions are optimized for amplification of purified fungal DNA (Rachdawong, 1999). DNA extraction from diseased plants or from infested soil co-purify substances that inhibit PCR, such as humic acids and phenolics. Wilson (1997) grouped the mechanism of inhibition into three categories, failure of lysis, nucleic acid degradation, and polymerase inhibition. Methods for DNA extraction determine if fungal DNA

present in diseased plant or infested soil is accessible for amplification. Phenolic compounds present in plants and soil inhibit lytic enzymes in the extraction method. In addition, phenolic compounds also bind or denature the polymerase. Humic acids inhibit DNA-DNA hybridization as well as inhibiting polymerase activity and lytic enzymes.

Henson *et al*., (1993) and Bryan *et al*., (1995) extracted wheat take-all fungal DNA from infested plants by boiling roots in buffer. Detection of the pathogen was achieved using nested PCR, which probably diluted the inhibitor concentration. Fouly and Wilkinson (2000) used Qiagen DNA extraction kit (Qiagen Inc., Valencia CA 91355). Volossiouk *et al*., (1995) successfully extracted *Verticillium dahliae* Kleb. DNA from soil and used the DNA in PCR. However, the extract needs to be diluted 50-fold to lower the concentration of inhibitor. McGregor *et al*., (1996) tested the ability of several proteins to overcome PCR inhibition by soil. They found carbonic anhydrase, ovalbumin, bovine serum albumin (BSA), and myosin as effective in overcoming the inhibition by including them in the PCR mixture. Niepold and Schober-Butin (1997) included BSA in their extraction procedure to adsorb impurities and then used the extract in PCR to detect *Phytophthora infestans* (Mont.) de Bary from potato (*Solanum tuberosum* L.). The type of extraction buffer used could play a big role in inhibitor build up. Heinz and Platt (2000) found proteinase K-ammonium acetate method better since the extract did not need to be diluted for PCR.

In addition to reducing the amount of PCR inhibitor, PCR conditions are important in ensuring a successful PCR. Some important factors are annealing time and temperature, magnesium ion concentration, and extension time and temperature (Ekman, 1999). All these factors need to be optimized so that the desired DNA is amplified despite the presence of contaminant or inhibitor.

1.5. OPTIMIZATION OF DNA EXTRACTION METHOD AND PCR CONDITION

Characteristic symptoms of take-all disease, such as root blackening and whiteheads, are not obvious or occur too late in the season, when disease control may be too late to apply. Therefore, accurate and rapid identification of wheat take-all fungus is important and needs to be

done early in the season. The avenacinase-based primers are very promising in early take-all fungus detection. However, they are still in the early stage of development and can only accurately identify the fungus in purified fungal DNA. Since isolation of the fungi is difficult, direct detection from infested plants and soil will be the ultimate objective in plant disease management. The objective of my research is to optimize DNA extraction methods from infested plants and soil in order to minimize the build up of inhibitor and to optimize PCR conditions in order to maximize the sensitivity of the reaction despite the presence of inhibitors.

1.6. CONTROL OF WHEAT TAKE-ALL

Cultural and chemical controls are available to control take-all in wheat (Stromberg, 1999). Plowing increases soil aeration that is conducive to the growth of soil microorganisms. These soil microbes can be inhibitory to the pathogen. Herbicides can be used to reduce nitrogen (N) competition by weeds hence increasing wheat ability to withstand take-all. This method can be troublesome since some herbicides can be toxic to the host plant, making them more susceptible to take-all. The use of N fertilizers, land reclamation, straw burning, and rotation are recommended for take-all control (Yarham, 1981). Some fungicides are also available to control take-all. However, these methods do not give complete disease control. Fungicides applied as seed treatment have limited ability since they protect seed environment only up to a certain area around the seed. Once a root is grown, it is going to grow beyond the protected area and hence become susceptible to disease. Chemicals applied after seed growth will be diluted as plant grows (Stromberg *et al*, 1999a). Repeated applications of chemicals are needed but this is not compliant with environmentally friendly agricultural practice.

Up to now, the best method to reduce take-all incidence is rotation with non-cereal crops (Stromberg, 1999; Yarham, 1981). This allows sufficient time to reduce the amount of inoculum growing saprophytically. Soybean (*Glycine max* (L.) Merril) has been used as a break crop in wheat farming but it is interesting that although soybean is not a host, *Ggt* can produce perithecia on soybean pods (Crozier, 1999). Another major disadvantage of crop rotation is that farmers may not be able to afford the break from wheat.

1.7. TAKE-ALL DECLINE (TAD)

Take-all decline (TAD) occurs following an outbreak of take-all in soil that has been planted continuously with wheat for years. It is a form of specific suppression where the decline is caused by a specific organism against a background of general suppression of take-all disease. General suppression is the combined action of soil microbiota that suppresses disease development (Campbell, 1989; Cook and Baker, 1983). The suppressive organism(s) probably operate in the rhizosphere or on the rhizoplane of wheat plants.

TAD led to the search for antagonistic organisms for disease control. Bacteria apparently are the main organisms responsible for TAD, although some fungi and viruses can also be antagonistic to the pathogen. Mechanisms of control include nutrient competition, antifungal antibiotic production (Keel *et al.*, 1992; Pierson and Thomashow, 1992; Weller, 1988), siderophore production (Hamdan *et al.*, 1991; Schippers *et al.*, 1987), and possible induction of host resistance.

1.8. BACTERIA AS BIOLOGICAL CONTROL AGENTS

Biological control is the use of an organism(s) other than humans to reduce the activity of a pathogen. Biological control of take-all is a potential alternative to fungicides, herbicides, or cultural control now available for take-all. The use of biological control can avoid pathogen resistance to chemicals such as herbicides and fungicides. Since it reduces the amount of chemicals in agriculture, biological control maintains a pollution free control (Cook and Baker, 1983). There are some potential biosafety effects of biological control but this will be discussed in the next section.

The organisms that have been shown to suppress *Ggt* include fungi, viruses, and bacteria. Of all the organisms, bacteria have been shown to be the most effective. Pseudomonads have been shown to be the cause of most take-all decline (Cook and Baker, 1983). There has been much research on the use of pseudomonads to control take-all (Duffy *et al.*, 1996; Duffy and Weller, 1995; Ownley *et al.*, 1992; Thomashow and Weller, 1990).

Bacillus spp. to a lesser extent are also potential as biological control agent of take-all. They have an advantage over pseudomonads since they produce endospores that are heat and desiccation tolerant. These properties make them desirable in formulation, storage and application. More *Bacillus* spp. are showing their potential as root colonizing and biological control agents (Kim *et al.*, 1997a; Kim *et al*., 1997b; Weller, 1988). However, since they are not as effective as pseudomonads in colonizing the rhizosphere, more research on biological control of take-all is directed towards pseudomonads.

Pseudomonads have the desirable attributes of biological control agent (Baker and Scher, 1987; Cook and Weller, 1987). They are rhizosphere-colonizing bacteria and able to use a wide range of substrates. They can be introduced via agricultural practice such as seed treatments. They produce a wide variety of antibiotics and siderophores that can suppress pathogen growth.

1.9. PROBLEMS IN BIOLOGICAL CONTROL

There are some essential attributes for biological control bacteria to be successful (Baker and Scher, 1987). They must be active at the same place and condition as the pathogen. If the pathogen lives in soil and can survive by saprophytic growth, bacteria need to be able to do the same since if they cannot live saprophytically, the pathogen is going to overcome them, especially between crop seasons. Bacteria must be compatible with soil biota or with other biological control agents that may be present. Introduction of several biological control agents must be careful so that the agents are not suppressive to the others. Since take-all is a root pathogen, the control agent must be root colonizing too. Additionally, bacteria have to survive formulation, storage and application.

Although bacteria as biological control agents hold many promises as alternative in plant disease control, they do not always work. Most research on biological control was initially performed in laboratory where conditions are favorable for bacteria. They have been cultured in favorable condition where their nutrients are provided and in nature they have to compete for this nutrient. Bacteria do not always maintain their ability to survive when re-introduced into nature.

The presence or absence of pathogen in soil can also affect bacterial survival (Mazzola and Cook, 1991). Bacteria may even need the presence of the pathogen. Under laboratory conditions, nutrients are not a problem since they are provided. In nature however, some bacteria may actually need the pathogen to provide their nutrition. For example, root pathogens provide nutrient leakage from host plant both to the pathogen and to the bacteria.

Experimental conditions in the laboratory or greenhouse usually only contain the biological control agents and the target pathogen. Therefore, although bacteria may be able to inhibit pathogen under laboratory condition and in greenhouse, when they are introduced into soil, they may not do so due to competition with other organisms in soil. Several authors reported the unreliability of antagonistic activity in laboratory experiments to predict activity in nature (de Boer *et al.*, 1999; Fukui *et al.*, 1994; Schottel *et al.*, 2001).

There are also some abiotic factors conferred by soil which can affect the performance of bacteria as biological control agents. Pseudomonads only produce siderophores in alkaline soil where iron is limiting. Other abiotic factors such as nutrient, mineral and soil type may play role. Andrade *et al*., (1994) used soil to include abiotic factor in screening potential strain to control take-all. They showed that different soils did affect antibiotic production by the same strain hence affected the biological control activity of the bacteria.

In addition to that, there are some potential biosafety concerns on the use of biological control agents. These are competitive displacement of a beneficial microorganisms, allergenicity, toxigenicity and pathogenicity towards non-target organisms (Mathre *et al*., 1999). However, these problems need to be compared to the problems created if biological controls are not used, especially the problem of too much or ineffective chemical use.

1.10. USE OF COMBINATIONS OF BACTERIA

Although many experiments have been done to utilize bacteria, fungi, or viruses to control the disease, researchers have shown that no single organism provides control as effective

as natural TAD. Mutational studies showed that no single mechanism of action provides complete control. Different strains of the pathogen from different sites had different sensitivity of an antibiotic produced by biological control agent (Mazzola *et al.*, 1995).

One way to overcome the inconsistent performance of biological control is by using combinations of agents. An example of this combined effect is the combination of *Pseudomonas fluorescens* (Trevisan) Migula strains 2-79 and 13-79. The combination of these two strains confers more inhibition than either of the strain alone (Weller, 1988). On the other hand, the combination of two strains of *Bacillus cereus* Frankland and Frankland is less inhibitory than the strain used alone (Ryder *et al.*, 1999).

Combinations of biological control agents, either bacterial mixtures or bacteria and fungi, works better than one type of bacterium. The fungus *Trichoderma* spp. is known for its biological control activity. The combination of *Trichoderma* spp. with pseudomonads increased wheat yield compared to when the agent was used alone (Duffy *et al.*, 1996). Even combinations of biological control agents with a different variety of the pathogen can increase biological control activity. An example of this is the utilization of pseudomonads and *Gaeumannomyces graminis* var. *graminis* to control take-all in wheat (Duffy and Weller, 1995).

The use of combinations of biological control agents would be more similar to the natural microbiota present in soil. This is probably the reason why combinations of agents can suppress disease more effectively, either by inhibiting pathogen growth or by competing for space and nutrients. Combinations of agents also provided more than one mechanism of action that work simultaneously to suppress pathogen growth. Since some strains of *Ggt* have different sensitivities to the antibiotics produced by pseudomonads, combining more than one mechanism of action is a good way to prevent resistance developing in the pathogen.

Several pathogen-antagonist combinations were studied to control different diseases. Some of them work better in combinations (de Boer *et al.*, 1999; Duijff *et al.*, 1999; Janisiewicz, 1996; Ram *et al.*, 1999) and some of them do not (de Boer *et al.*, 1999; Fukui *et al.*, 1994; Hervas *et al.*, 1998). This inconsistency implies that different bacteria and pathogen systems

have their own characteristic growth and generalizations about biological control agents cannot be made without extensive study. Each system needs to be studied carefully to determine whether biological control will work best in combination or singly.

One way to do this is by looking into nutrient and antagonism relationships amongst potential biological control agents. Bacteria that use same types of nutrients are likely to compete for them in the environment and probably will not work better in disease control. Additionally, bacteria that show antagonism towards each other are not expected to work better in disease control. These proposed hypothesis need to be examined for each biological control agent-pathogen system.

1.11. NUTRITIONAL AND ANTAGONISM STUDY OF BACTERIAL STRAINS AS BIOLOGICAL CONTROL AGENTS

Several bacteria have been isolated from roots of apparently healthy wheat growing in a field where severe symptoms of take-all occur (Stromberg *et al.*, 1999b). Research is being conducted to establish the potential of these strains in controlling take-all disease. Considering the problems in biological control, the potential of these strains that have been tested against take-all disease in culture, greenhouse, and field scale, both in combinations and singly, is being assessed. Some combinations showed better control of the disease and some did not (Stromberg *et al*., 1999b). Combination of strains should work if the strains do not compete for the same nutrition and space, and do not antagonize each other. Therefore, the other objective of my research is to study nutrient and antagonism profiles of potential biological control agents. This will help gain a better understanding of the relationship amongst the strains and predict which combinations will work.

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CHAPTER 2. PCR FOR TAKE-ALL FUNGUS IDENTIFICATION *IN PLANTA* AND SOIL

2.1. ABSTRACT

Avenacinase-based primers were used in polymerase chain reaction (PCR) to detect *Gaeumannomyces graminis* (Sacc.) v. Arx & Olivier var. *tritici* Walker (*Ggt*) in infested wheat and soil. To detect *Ggt* DNA *in planta* or in soil, annealing temperature of the reaction had to be reduced from 68°C, used with purified DNA, to 62°C. The lowest level of *Ggt* that could be detected in plant was when planted in soil containing 4 g/kg *Ggt*-infested millet. The lowest level of *Ggt* detected in soil was 16 μg of pure *Ggt* DNA per gram of soil.

2.2. INTRODUCTION

Take-all is a very damaging root disease of wheat (*Triticum aestivum* L.) that occurs wherever wheat is intensively grown (Garrett, 1981). The causal agent of the disease is an ascomycete, *Gaeumannomyces graminis* (Sacc.) v. Arx & Olivier var. *tritici* Walker (*Ggt*). The fungus grows on all plant parts below soil surface and produces characteristic symptoms of the disease, roots blackening, whiteheads, and stunted growth (Clarkson and Polley, 1981). Two other varieties of *G. graminis* are present on roots of cereals and grasses. *G. graminis* (Sacc.) v. Arx & Olivier var. *graminis* (*Ggg*) is a pathogen of seasonal grasses and *G. graminis* (Sacc.) v. Arx & Olivier var. *avenae* (*Gga*) is a pathogen of oat (*Avena sativa* L.). Other similar non-pathogenic fungi that are also common on cereal and grasses roots are *Gaeumannomyces cylindrosporus* Hornby, Slope, Gutteridge & Sivanesan and *Phialophora* sp. (Walker, 1981). All these fungi have similar teleomorphs and anamorphs, and they form the *Gaeumannomyces-Phialophora* complex. The presence of this complex on wheat roots makes pathogen identification difficult.

As conventional identification methods fail to provide rapid and accurate identification of wheat take-all fungus, molecular approaches are becoming the methods of choice. DNA probes are used to identify and quantify *Ggt* in soil (Herdina *et al.*, 1996). Polymerase chain reaction

(PCR) is used to amplify specific region in ribosomal RNA (Goodwin *et al.*, 1995), ribosomal DNA (Fouly and Wilkinson, 2000), internal transcribed spacer (Bryan *et al.*, 1995), and mitochondrial DNA (Henson *et al.*, 1993). These molecular methods allowed rapid and accurate differentiation of *Ggt* from the *Gaeumannomyces-Phialophora* complex. Except for Bryan *et al*. (1995), these methods only allowed differentiation of *Ggt* or *Gga*, but not *Ggg*.

All PCR methods used to identify wheat take-all fungus utilized the presence of multicopy gene families in fungal DNA. Rachdawong (1999) developed specific primers for *Ggt*, *Gga*, and *Ggg* identification based on avenacinase genes, a gene associated with pathogenicity. Similar genes are present in *Ggt*, *Gga*, and *Ggg*. In *Gga*, this gene is responsible for producing avenacinase, an enzyme that detoxifies avenacin. Avenacin is a fungitoxic substance produced by oats and avenacinase of *Gga* allow it to be pathogenic on oats. *Ggt* and *Ggg* also produce avenacinase-like proteins but they are not active against avenacin (Crombie *et al*., 1986). The difference in enzyme activity suggested a difference in the nucleic acid composition of the genes, which the author used to develop the primers. The primers are sensitive to their own DNA template and the fragments generated are of different sizes, specific to the take-all pathogen *Ggt*, *Gga,* and *Ggg*. Take-all pathogen identification thus can be performed rapidly and accurately in a single tube PCR.

PCR with the avenacinase-based primers is still in its early stage of development and only works using purified fungal DNA. To be useful for rapid and accurate pathogen identification, PCR should be able to detect the pathogen directly from infested plants or soil. Several pathogens have been identified using PCR directly from infested plants or soil. Niepold and Schober-Butin (1997) extracted *Phytophthora infestans* (Mont.) de Bary DNA directly from infested potato (*Solanum tuberosum* L.) tubers. Fouly and Wilkinson (2000) extracted *G. graminis* DNA from infested wheat, oat, and turfgrass roots. Lee and Tewari (2001) extracted *Rhynchosporium secalis* (Oudem.) J.J. Davis DNA from infested barley (*Hordeum vulgare* L.) seed. Heinz and Platt (2000) and Volossiouk *et al.*, (1995) extracted *Verticillium* species and *Verticillium dahliae* (Kleb.) DNA from infested soil.

Although direct PCR from plant or soil extract is possible, DNA extraction from plant or soil co-purifies PCR inhibiting substances such as humic acids, polysaccharides, and phenolics. Wilson (1997) grouped the mechanisms of PCR inhibition into three categories, failure of lysis, nucleic acid degradation, and polymerase inhibition. Different methods or extraction buffers were used to minimize co-purification of PCR inhibiting substances. Volossiouk *et al.*, (1995) used skim milk powder in the extraction procedure to reduce DNA loss but the DNA extract needs to be diluted 50 times for PCR to work. Heinz and Platt (2000) used proteinase K-ammonium acetate extraction buffer to reduce inhibition. Cold extraction using extraction buffer that contains benzyl chloride, polyvinylpyrrolidone (PVP), and β-mercaptoethanol was carried out by Bahnweg *et al.*, (1998). They also eliminated humic acid by precipitating it with methanol and $CaCl_2$. Niepold and Schober-Butin (1997) included bovine serum albumin (BSA) in the extraction procedure to adsorb any impurities. BSA can also be included in the PCR reaction mixtures (Kreader, 1996; McGregor *et al.*, 1996).

In addition to extraction buffers or PCR mixtures, conditions of PCR are also important. Ekman (1999) listed some important parameters in PCR as annealing temperature, magnesium ion concentration, primer concentration, polymerase concentration and type, extension time, and even the possibility of nested PCR. Lowering annealing temperature reduces PCR sensitivity but may allow increase efficiency. Increasing magnesium (Mg) concentration has similar effect to lowering annealing temperature. Wilson (1997) listed primers, polymerase, Mg concentration, and reaction conditions as some reasons that can result in PCR failure. Formamide has been shown to improve PCR reaction by reducing DNA melting temperature (Comey *et al*., 1991). Dimethyl sulfoxide (DMSO) increases PCR sensitivity by eliminating non-specific amplification or improves annealing efficiency of the primers (Sidhu *et al*., 1996).

The objective of this research is to use the avenacinase-based primers to detect *Ggt* directly from infested plants and soil. Several extraction methods are going to be used in order to minimize the build up of PCR inhibiting substances. PCR conditions are going to be optimized by finding the optimum annealing temperature, magnesium ion and polymerase concentration, or by adding substances that relief PCR inhibition.

2.3. MATERIALS AND METHODS

2.3.1. DNA Extraction from Infested Plants

Fungal DNA was extracted from infested plants using the hot CTAB method (Rachdawong, 1999). Roots were washed, chopped into small pieces (approximately 2 mm in length), and ground in liquid nitrogen. Approximately 0.25 to 0.5 g of the ground sample was transferred into a 2 ml tube containing 0.5 ml of preheated extraction buffer (2% (w/v) CTAB, 1.4 M NaCl, 20 mM EDTA pH 8.0, 10 mM Tris-HCl pH 8.0, and 0.2% (v/v) β-mercaptoethanol added just before use). The mixture was incubated at 65°C for 1 hr, mixing every 10 to 15 min. An equal volume of chloroform:isoamyl alcohol (24:1) was added and the mixture was shaken horizontally on a minishaker MS 1 (IKA-WORKS, INC, Wilmington, NC 28405) at approximately 1,000 rpm for 30 min at room temperature (22-24°C). Chloroform mixture was separated by centrifugation for 10 min at 4°C (5,000g). RNAse A was added to the supernatant to a final concentration of 100 µg/ml and the mixture was incubated for 30 min at room temperature. DNA was precipitated by adding 0.6 volume of isopropanol (-20°C) and incubating the mixture at -20°C for 30 min. DNA was pelleted by centrifugation at 4°C for 10 min (2,000 g). The resulting pellet was washed in 300 µl of 70% ethanol/10 mM ammonium acetate and resuspended in 100 µl of TE buffer overnight at 4°C without agitation.

2.3.2. DNA Extraction from Infested Soil

Fungal DNA was extracted from soil using Ultra Clean Soil DNA Isolation Kit (Mo Bio Laboratories, Inc., Solana Beach, CA 92075) according to manufacturer s instructions.

2.3.3. Sensitivity Test for Plant Extraction

Sensitivity tests were performed to obtain the lowest level of DNA in plants that can be detected by PCR using the primers. After a week growth on PDA, ten 8-mm agar discs of *Ggt* were inoculated onto 152 g sterile German foxtail millet (*Setaria italica* L.) seeds in a 2 Liter Erlenmeyer flask. Several flasks were prepared and the flasks were incubated at room

temperature (22-24°C) for approximately two months. Inoculated millet was mixed with Kempsville loam soil to make 16 g millet/kg soil, 8 g/kg, 4 g/kg, 2g/kg, 1g/kg, 0.5g/kg, 0.25g/kg, and 0.125 g/kg. A cotton ball was inserted into the bottom of each Conetainer® (Ray Leach Conetainers®, Portland, Oregon, 4 X 21 cm) and approximately 150 g of the soil mixture was added to each cone. Four wheat seeds were planted into each cone, approximately 1 cm deep into the soil. Plants growing on soil without inoculated millet were used as controls. The plants were grown in the greenhouse. Disease symptoms were not recorded for this experiment but plants were pulled out after differences in plant heights between the control and the treatment was visible (approximately 4 weeks). Fungal DNA was extracted from the plant roots as described previously.

2.3.4. Sensitivity Test for Soil Extraction

To determine how sensitive the reaction is in detecting the fungi in soil, purified *Ggt* DNA were mixed with Kempsville loam soil (chemical and physical properties of the soil are summarized in Table 2), extracted, and amplified with the PCR. The concentrations of purified DNA were 4 µg/g, 8 µg/g, 16 µg/g, 32 µg/g, 64 µg/g, 128 µg/g, 256 µg/g, 512 µg/g, and 1024 µg/g soil. The purified DNA was mixed with 0.25 g soil and the whole mass of soil with the DNA was extracted as described previously.

2.3.5. PCR Conditions

DNA was amplified using the avenacinase-based primers (Rachdawong, 2000). The sequence of the 5 *Ggt*-specific primer was 5 -TCCTCGGCCCCGTAATTGGC-3 . The sequence of the 3 primer was 5 -TGCTCATGGTGGTTCCTGC-3 . Each 50 µl reaction volume contained 1 µl of plant extract or 5 µl of soil extract, 50 pmol of each primer, 1.25 U of *Taq* DNA polymerase (Qiagen Inc. Valencia, CA 91355), 100 µm of each deoxynucleotide triphosphates, 1 X reaction buffer, 3 mM $MgCl_2$. Volume was adjusted to 50 µl with nano-pure deionized water. PCR were performed in a thermal cycler (Mastercycler Gradient, Eppendorf Scientific Inc., Westbury, NY 11590) programmed for an initial denaturation of 3 min at 95°C, followed by 35 cycles, each consisting of 94°C for 45 sec, 62°C for 1 min, and 72°C for 2 min.

An additional incubation for 10 min at 72°C was carried out in the end. PCR products were separated by electrophoreses (Horizontal Gel Electrophoresis System, Bethesda Research Laboratories, Gaithersburg, MD) at 75 V for 1.5 hours in a 1.8% agarose gel in 0.5 X TBE buffer (45 mM Trisborate and 1 mM EDTA, pH 8.0).

Table 2. Selected chemical and physical properties of a Kempsville loam (fine-loamy, siliceous, thermic Typic Hapludult) soil utilized in greenhouse experiment.
(Crozier, 1999)

Parameter	Analyses	Method[1]
Textural classification	Sandy loam	
Sand %	59.7[2]	
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

[1]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 Socity of America.
[2]Average of three separately analyzed samples.

2.3.6. Optimization of PCR Conditions

Purified fungal DNA was used as the positive control in all experiments. Different annealing temperatures ranging from 61°C to 69°C were tested to determine the optimum annealing temperature. Two annealing times (45 sec and 1 min) were used.

2.3.7. Overcoming PCR Inhibition

To overcome PCR inhibition, 1% Igepal CA-630 (Sigma), 1 to 10% dimethyl sulfoxide (DMSO), 0.1 to 0.2% dry milk powder, 0.02 to 1% gelatin, 0.1% Tween 20, 5% formamide, and 5 to 50 μg bovine serum albumin (BSA) were included in the reaction individually at different concentrations. The amount of *Taq* Polymerase was also increased from 1.25 to 2.5 units.

2.3.8. DNA Sequencing

To confirm that the PCR-generated fragment was *Ggt*, PCR products were cleaned (Qiaquick PCR Kit, Qiagen, Inc., Valencia, CA 91355) and sequenced at the Core Laboratory Facility (CLF) at the Virginia Bioinformatics Institute. The product was cyclically labeled using dye terminator cycle sequencing kit (Big Dye Terminator Cycle Sequencing, PE Applied Biosystem, Foster City, CA) according to manufacture s instructions. Sequencing was performed using an ABI Prism 377 DNA Sequencer (Applied Biosystem, Foster City, CA). Sequence data were analyzed using the Lasergene Sequence Analysis Software (DNAStar Inc., Madison, WI 53715).

2.4. RESULTS

2.4.1. DNA Extraction from Plant

The hot CTAB method of Rachdawong (1999) for extracting fungal DNA from roots was the only method attempted that gave consistent PCR products. Several other DNA extraction methods were compared, including boiling root samples in NaOH (Bryan *et al.*, 1995) and extracting DNA with methanol-$CaCl_2$ and benzyl chloride (Bahnweg *et al.*, 1998). The method of Bryan *et al.,* (1995) was very simple and quick to perform but no PCR fragment was generated even when dried mycelia were included in the extraction process. Increasing the boiling time also did not work. The method of Bahnweg *et al.*, (1998) also did not generate any PCR fragment. This method was more complicated than that of Bryan *et al.*, (1995). The main disadvantage was that DNA pellet was washed in such high concentration of chloroform that

they float instead of sediment. The floating DNA was difficult to collect compared to the common method of centrifuging DNA into a pellet. A DNeasy Plant Mini Kit (Qiagen, Inc., Valencia, CA 91355) provided a simple and fast DNA extraction method, but the PCR results were inconsistent.

2.4.2. DNA Extraction from Soil

No DNA precipitate was formed when DNA was extracted from infested soil according to Volossiouk *et al.,* (1995) and Heinz and Platt (2000). No band was visible when 64 µg pure *Ggt* DNA per gram soil was included in the extraction procedure of both methods. The Ultra Clean Soil DNA Isolation Kit was able to amplify DNA in soil as low as 16 µg/g (see Sensitivity Tests). It proved to be the fastest and easiest extraction method and it was the only method that gave positive amplification of *Ggt* from infested soil. However, only two extractions worked and the repeated extraction did not yield amplified *Ggt* DNA.

The first extraction was from take-all infested field soil that has been frozen at —20C for a year. Wheat plants pulled from the soil was frozen together with some soil around the roots. The soil used for the extraction was scraped from the roots very near the base of the plants. Repeated extraction from the same soil did not result in a positive amplification for *Ggt*. The second extraction was from infested soil used in the greenhouse experiment (see Chapter 3). This soil was inoculated with 10 g *Ggt*-infested millet per kg soil and had wheat grown on it for approximately a month. Extraction from the soil resulted in *Ggt* amplification only when performed right after wheat was harvested. Extraction from the same soil that had been frozen for a few days without prior drying did not result in *Ggt* amplification.

2.4.3. Sensitivity Tests

The lowest level of DNA detected by PCR was from plants grown in 4g *Ggt*-infested millet/kg soil (Figure 2.1). However, the DNA extract had to be diluted two-fold to get a clear gel band and reduce background. This is probably due to the high plant DNA content in the extract.

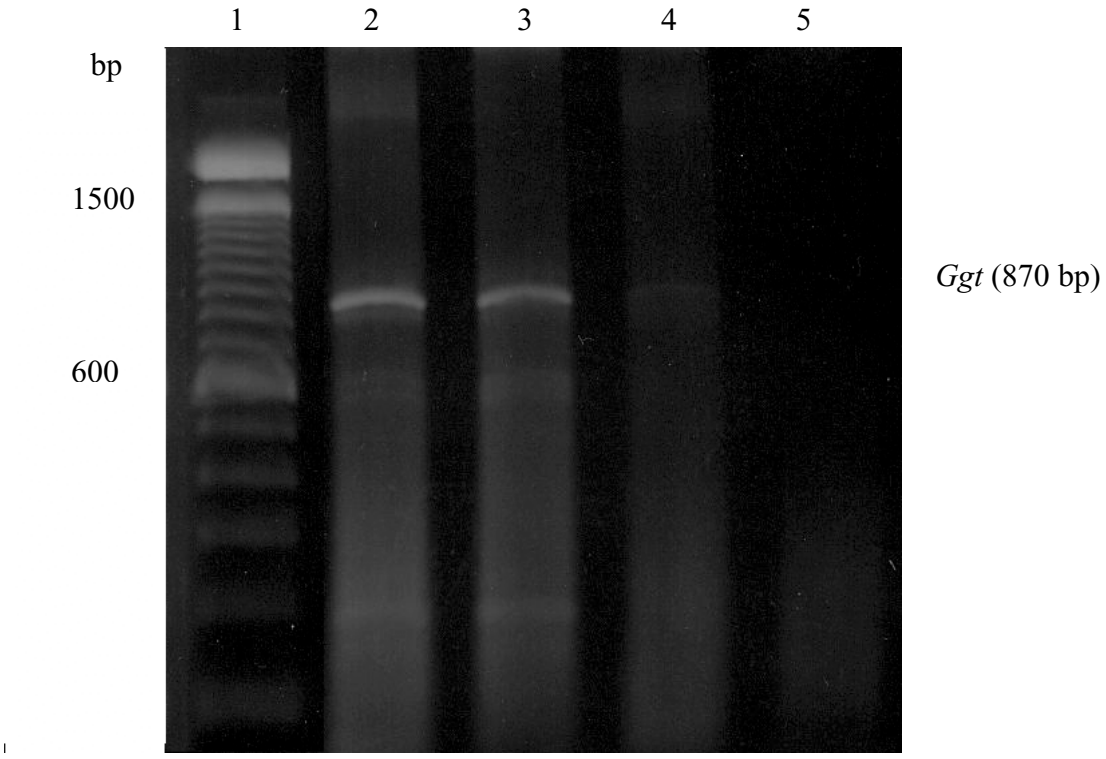

Figure 2.1. Sensitivity test for plant extraction.
Each lane contains PCR products of DNA extracted using the hot CTAB method from roots of plants grown in soil containing *Gaeumannomyces graminis* var. *tritici (Ggt)*-infested millet seeds. Extracted DNA was used as template with *Ggt*-specific primers for 35 cycles of PCR amplification as described in text. PCR products were separated in 1.8% agarose gel by electrophoresis, stained with ethidium bromide and visualized under UV light. Lane 1 is a 100 bp DNA ladder, pertinent molecular sizes are indicated as basepairs (bp). Lanes 2 to 5 represent 16 g *Ggt*-infested millet/kg soil, 8 g/kg, 4 g/kg, and 2 g/kg, respectively.

The lowest level of DNA in soil that could be detected was 16 μg/g (Figure 2.2). However, this was only achieved when 4 μg of pure DNA was mixed with 0.25 g soil and all of the soil was used in the extraction. Detection failed with the same concentration of DNA when less than the total amount of soil (0.25 g) was used in the extraction.

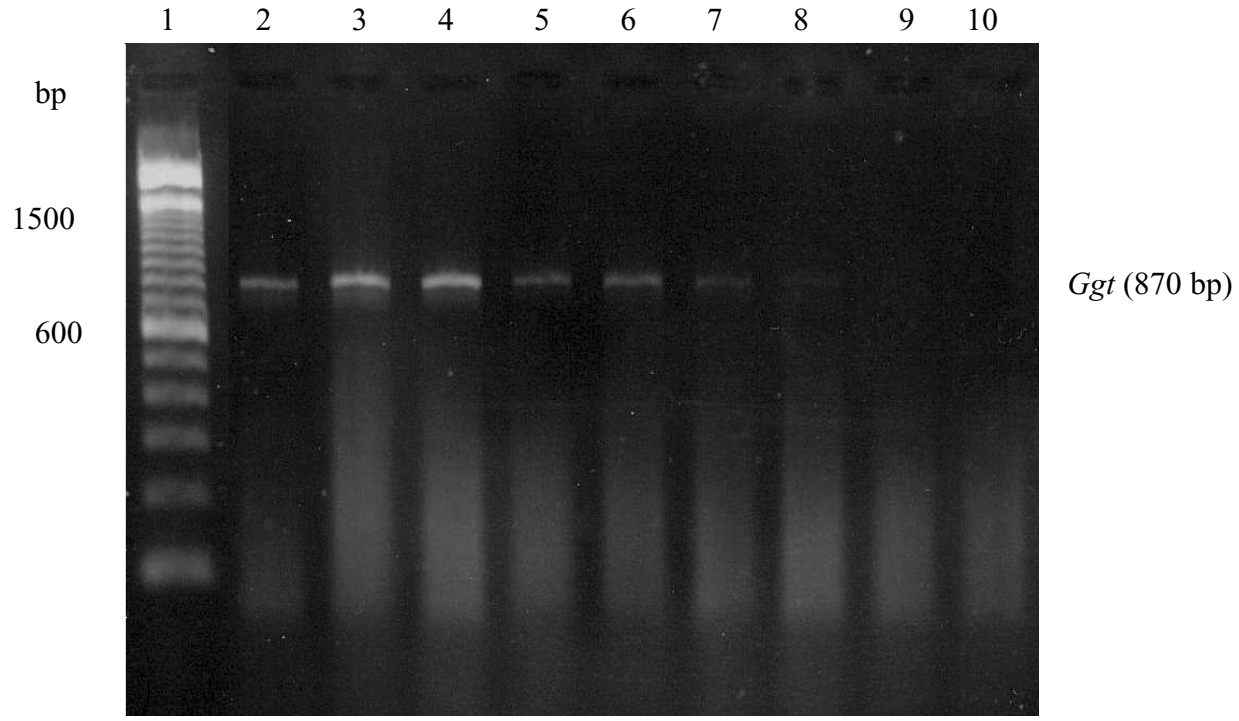

Figure 2.2. Sensitivity test for soil extraction.
Soil artificially contaminated with *Gaeumannomyces graminis* var. *tritici (Ggt)* DNA was extracted using a soil DNA isolation kit. Extracted DNA was used as template with *Ggt*-specific primers for 35 cycles of PCR amplification as described in text. PCR products were separated in 1.8% agarose gel by electrophoresis, stained with ethidium bromide and visualized under UV light. Lane 1 is a 100 bp DNA ladder, pertinent molecular sizes are indicated as basepairs (bp). Lanes 2 to 10 represent DNA-soil mixtures of 1024 µg /g, 512 µg/g, 256 µg/g, 128 µg/g, 64 µg/g, 32 µg/g, 16 µg/g, 8 µg/g and 4 µg/g, respectively.

2.4.4. PCR Optimization

Fungal DNA extraction from plants with the hot CTAB method (Rachdawong, 1999) generated a *Ggt*-sized fragment when the annealing temperature was reduced to 62°C (Figure 2.3). No fragment was generated at annealing temperature of 68°C (results not shown). In addition, annealing time was increased from 45 s to 1 min (results not shown).

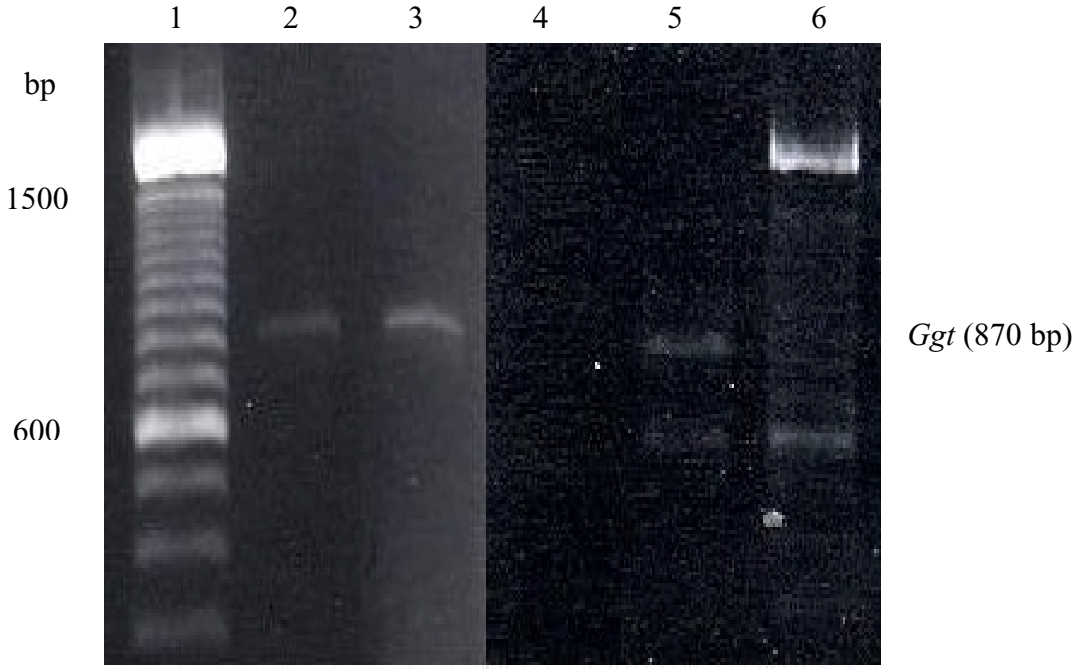

Figure 2.3. Comparison of *Gaeumannomyces graminis* var. *tritici (Ggt)* DNA amplification with avenacinase-based primer in PCR at different annealing temperatures.
Each lane contains PCR products of DNA extracted using the hot CTAB method from roots of plants grown in soil containing *Gaeumannomyces graminis* var. *tritici (Ggt)*. Extracted DNA was used as template with *Ggt*-specific primers for 35 cycles of PCR amplification as described in text. PCR products were separated in 1.8% agarose gel by electrophoresis, stained with ethidium bromide and visualized under UV light. Lanes 1 to 3 are PCR products at 62°C annealing temperature while lanes 4 to 6 are at 68°C. Lanes 1 and 6 contain 100 bp DNA ladder, pertinent molecular sizes are indicated as basepairs (bp). Lanes 2 and 5 represent PCR products of purified *Ggt* DNA as positive controls. Lanes 3 and 4 represent PCR products from DNA extracted from the roots of a plant grown in *Ggt*-infested soil.

Lowering the annealing temperature from 68°C to 62°C and increasing annealing time from 45s to 1 min were found to be sufficient to overcome PCR inhibition. Additional enhancer such as Igepal CA-630 (Sigma), dry milk powder, BSA, DMSO, or Tween 20 did not increase sensitivity. However, the amount of *Taq* Polymerase had to be doubled from 1.25 units to 2.5 units to amplify fungal DNA extracted from soil.

2.4.5. Sequencing

Sequence alignment with *Ggt* isolate from Montana (M1) and *Ggt* isolate from the ATCC (ATCC28230) DNA confirmed the identity of the PCR fragment (98% match). The complete sequence alignment is shown in Appendix A.

2.5. DISCUSSION

The avenacinase-based primers specific for each *Ggt*, *Ggg*, and *Gga* were used to detect take-all pathogen in infested wheat and soil. Before this, the primers worked only on purified fungal DNA. This research showed that the *Ggt*-specific primer can be used to detect the pathogen from infested wheat and soil. Further optimization will be necessary before the procedure can be applied in practical situations. Previously, the annealing temperature of the PCR was 68°C and this did not generate any fragment. This temperature was the optimum temperature when using purified fungal DNA template. However, the high annealing temperature may be inefficient when the target DNA is present in smaller proportion against a background of other DNA, such as in plant or soil extracts. In this research, 62°C allowed detection of amplified products.

Faster DNA extraction methods from plants are available but they did not work for these primers. Boiling root samples in NaOH (Bryan *et al*., 1995; Henson *et al*., 1993) was a very fast and easy method but apparently was not sufficient. Wilson (1997) explained that boiling samples may release DNA from cells but the DNA may fail to separate from any structural or DNA-binding proteins.

The primers used by these researchers amplified ribosomal DNA (rDNA) and mitochondrial DNA (mtDNA) instead of nuclear DNA. mtDNA may be more accessible for amplification than nuclear DNA because of the different compartmentalization and protein complexes involved (Henson *et al*., 1993). Moreover, these DNA are present in more copies than nuclear DNA resulting in more targets and more sensitivity (Fouly and Wilkinson, 2000).

Johnston and Aust (1994) were able to amplify the ITS region of *Phanerochaete chrysosporium* (Burdsall) easily but the sensitivity was much reduced when amplifying a ligninase H8 DNA of the same organism, even when extraction was made from pure cultures. They also suggested that the high copy number of ribosomal DNA make them a much more abundant target for amplification.

The *Gga* genome contains a single copy of avenacinase gene (Bowyer *et al*., 1995). Similarly, *Ggt* genome most likely also contains only a single copy of avenacinase-like gene. Compared to the number of rDNA genes that is more than 50 copies per genome (Fouly and Wilkinson, 2000), the avenacinase gene is a very low target for PCR. This might account for the false negative results.

The DNeasy Plant Mini Kit (Qiagen, Inc., Valencia, CA 91355) provided a clean and fast way of obtaining DNA from plant. However, the PCR results from this extract were inconsistent (results not shown). One possible explanation for this is the small sample load (100 mg) that results in more plant DNA being extracted than fungal DNA.

Failure to repeat the soil extraction may result from variations in inoculum loads in the sample, as suggested by Lee and Tewari (2001). The small sample size (0.25 g) may also contribute to this. Another possibility is the loss of DNA due to degradation by DNase (Wilson, 1997). The repeated extraction was performed several days after the first extraction. DNA may have been degraded then since the sample was directly frozen without drying.

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CHAPTER 3. COMBINATIONS OF BACTERIAL BIOLOGICAL CONTROL AGENTS BASED ON ANTAGONISM AND NUTRIENT PROFILES

3.1. ABSTRACT

Several bacterial biological control strains were tested for antagonism towards each other. Five strains showed antagonism towards some other strains. Nutrient profiles of the strains were assessed using BIOLOG. Based on these results, a greenhouse experiment was conducted to test several combinations of bacteria against take-all. Six treatments had statistically significant difference in root weight. However, the greenhouse experiment did not correspond to the antagonism test and nutrient profiles.

3.2. INTRODUCTION

Take-all is probably the most important disease of wheat (*Triticum aestivum* L.), especially since it attacks wheat roots and hence is not recognized until the disease is too late to control. It is also widespread, occurring everywhere wheat is intensively grown. Characteristic symptoms of the disease are roots blackening, stunting, and patches of white-heads. The cause of the disease is an ascomycete fungus, *Gaeumannomyces graminis* (Sacc.) v. Arx & Olivier var. *tritici* Walker, abbreviated as *Ggt*.

Several control measures are available in reducing take-all incidence. Herbicide, fungicide, land reclamation, straw burning, soil management are some examples (Yarham, 1981). Herbicides reduce weed competition, increase plant fitness and reduce its susceptibility to disease. Fungicides applied as seed coating provide early disease control by protecting seed from the pathogen and allowing it to grow without competition from the pathogen. However, as the roots grow out of the zone of protection, the plant is exposed to the pathogen (Roberts *et al*., 1999). Land reclamation and straw burning reduce the amount of inoculum present in the field but these practices are not environmentally sound. The most effective mean of controlling the disease is by rotation with non-cereal crops (Stromberg, 1999; Yarham, 1981). Most farmers,

however, cannot economically afford the break from wheat. Therefore, an alternative control for take-all is needed.

Take-all decline (TAD) is a natural phenomenon that reduces the incidence of take-all disease in a field that has been planted with wheat continuously for years. The cause of the decline is most likely biological (Cook and Baker, 1983). *Gaeumannomyces graminis* (Sacc.) v. Arx & Olivier var. *graminis* (*Ggg*) and *Phialophora* spp. were found to reduce the incidence of take-all (Duffy and Weller, 1995; Zriba *et al*., 1999). These two fungi make *Gaeumannomyces-Phialophora* complex together with *Ggt*, *G. graminis* (Sacc.) v. Arx & Olivier var. *avenae* (Turner) Dennis (*Gga*), and *Gaeumannomyces cylindrosporus* Hornby, Slope, Gutteridge & Sivanesan. Other fungi such as *Trichoderma* (Duffy, 1996) and some viruses were also found to attack *Ggt* and can be exploited to control take-all. However, most detailed studies of TAD implicated bacteria as the biological agent responsible for the decline (Cook and Baker, 1983). All these findings led to the search of biological agent for disease control as other cultural and chemical measures do not provide adequate control.

There have been many research attempts to control take-all using bacteria. Almost all these bacteria were isolated from wheat fields that have undergone TAD. Some examples of bacterial strains that have been studied to control take-all are *Pseudomonas fluorescens* 2-79 (Trevisan) Migula (Weller, 1988), *Pseudomonas aureofaciens* (Kluvyer) 30-84 (Pierson and Thomashow, 1992), *Pseudomonas fluorescens* (Trevisan) Migula Q8r1-96 (Raaijmakers and Weller, 1998), and *Pseudomonas fluorescens* (Trevisan) Migula CHAO (Keel *et al.*, 1992). One mechanism of action of these strains involves antibiotic production. These strains produce the antibiotics Phenazine 1-carboxylic acid (PCA) or 2,4-Diacetylphloroglucinol (Phl).

Another mechanism of action is siderophore production. Siderophores are bacterial secondary metabolites produced under iron-limiting conditions to scavenge iron (Schippers *et al*., 1987). Plant pathogens are suppressed by the presence of siderophores due to the iron competition. Siderophores have been suggested to play an important part in TAD. However, siderophores are only produced in alkaline soil when iron is limiting (Cook and Weller, 1987). Therefore, siderophores production is not the only mechanism working in a natural TAD.

Experiments with mutants had shown that the bacteria can still provide biological control even when the ability to produce these secondary metabolites is deleted (Hamdan *et al.*, 1991). This indicated that bacteria have more than just one gene that affects disease control.

In addition to that, the fungal strains that cause disease at different places have different sensitivity to a certain bacterial strain. Different isolates of the pathogen show different sensitivity to antibiotics produced by bacteria. In an experiment using different *Ggt* isolates from United Kingdom, France, Australia, Poland, Germany, Denmark, Washington, Georgia, South Carolina, Arkansas, Indiana, Missouri and Kansas, Mazzola *et al*., (1995) showed that almost all *Ggt* isolates from Washington are sensitive to PCA produced by pseudomonads although they have different level of sensitivity. However, *Ggt* isolates from Europe show less sensitivity compared to the Washington isolates. This would mean that a single bacterial strain would only provide control to certain strains of the fungus.

Despite considerable research on bacteria as biological control agents, no single bacterium can provide control as occurs in natural TAD. Failure to colonize roots and persist in the environment, and interference by non-target pathogens, are some problems. An alternative approach is to use a combination of bacteria to more resemble the natural condition of TAD. A mixture of bacteria, which is closer to the natural environment, is expected to work better than a single bacterial strain. Bacterial combinations will likely provide more than just one mechanism of action that may provide more protection against wider range of pathogens. They will also have better root colonization and persistence in the environment, better survival in a wider range of environment conditions (Pierson and Weller, 1994).

Several factors need to be addressed when using mixture of bacteria. First of all, the bacteria must not be antagonistic to each other. For example, one of the mechanisms of action that bacteria use against the pathogen is antibiotic production. For a mixture to work, bacteria must also be tolerant to any antibiotic that is produced by other bacteria.

Secondly, bacteria should have different nutrient requirements so that they will not compete for the same nutrients. Siderophore, an iron-chelating agent produce by bacteria, is

another mechanism by which bacteria suppress disease. Different bacteria produce siderophores with different affinity towards iron. Bacteria in mixture should not compete with each other for this iron, especially under alkaline condition when iron in soil is solubilized and siderophore production may be induced.

Another factor to be considered is avoiding niche competition amongst bacterial strain. Bacteria growing on different sites along wheat roots may also prevent any antagonistic or nutrient competition that they would have if they grow on the same site.

The objective of this research is to use antagonism test and nutrient profiles to predict which combination of bacteria will work against the pathogen, based on antagonistic properties and nutrient requirement of the bacteria. Growing two bacterial strains on the same plate will provide information on antagonistic properties of the bacteria. Nutrient profile of the bacteria was assessed by using BIOLOG. A greenhouse experiment was used to test whether antagonism test and nutrient profile provide accurate prediction of *in situ* condition.

3.3. MATERIALS AND METHODS

3.3.1. Antagonism Test

Several bacteria were isolated from apparently healthy plants in a field that had take-all symptoms (Stromberg *et al*., 2000). The antagonism test was performed by first growing the strains on tryptic soy agar (TSA) at 28°C. One bacterial strain was suspended in sterile water until the water was just visibly turbid. This suspension was streaked using a sterile cotton swab onto the middle of a Petri plate containing dilute tryptic soy medium in 1.5% agar (1/10 TSA), making a line across the plate. After incubation at 28°C for 48 hours, other bacterial strains were streaked in lines perpendicular very close but not in contact with the previous strain (Figure 3.1). The plates were incubated further at 28°C for 48 to 96 hours. The bacterial strain in the middle line was said to be antagonistic if it inhibited the growth of other bacterial strains. Shorter lines of bacterial growth were interpreted as antagonism between the bacterial strains.

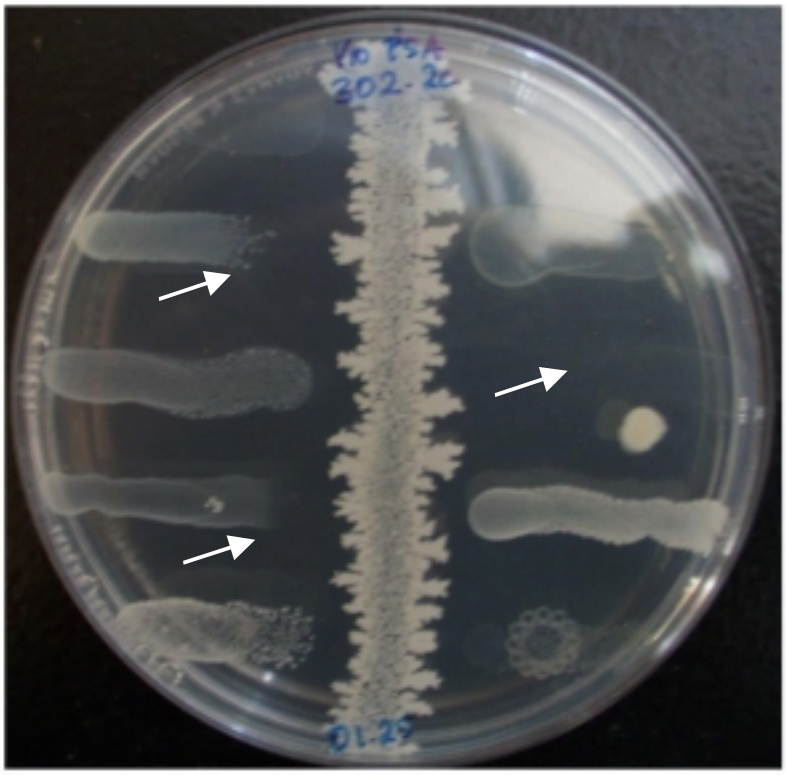

Figure 3.1. Antagonism test between bacterial strains.
The bacterial strain (antagonist strain) streaked on 1/10 TSA from top to bottom in the middle of the agar plate was grown at 28°C for approximately 48 hours before the other bacterial strains (target strains) were streaked on the plate. Antagonism by the antagonist strain against other target strain is indicated by shorter lines of growth or no growth of the target bacteria (arrows).

3.3.2. Bacterial Nutrient Profile

Nutrient profiles of the strains were identified using BIOLOG GN2 MicroPlate“ (BIOLOG, Hayward, CA 94545). Bacteria strains were grown in TSA at 28°C for 24 hours before transferred onto BIOLOG s BUG agar containing 5% sheep blood. BIOLOG GN2 MicroPlates“ were inoculated according to manufacturer s instructions. After incubation at 28°C overnight, the microplates were read using the MicroStation“ reader (BIOLOG, Hayward,

CA 94545). Identification of bacterial strains was performed using the BIOLOG MicroLog 3.0 Software (BIOLOG, Hayward, CA 94545).

Based on the microplates reading, niche overlapping index (NOI) for each bacterium was calculated as the number of nutrients used by both bacteria divided by the number of nutrients used by the targeted bacteria (Wilson and Lindow, 1994).

3.3.3. Seed coating

Based on BIOLOG identification and the result of the antagonism test, several bacterial combinations were tested against the pathogen. For the greenhouse experiment, each bacterial strain was grown in two 250 ml flasks containing 50 ml tryptic soy broth (TSB) at 28°C for 48 hours. Bacteria were collected by centrifugation at 6,000g for 1 min. The cells were washed with sterile water and then suspended in 8 ml of sterile water. Wheat seeds were coated with the bacterial suspension by mixing 2.4 g of seeds with 2 ml of bacteria suspension and 8 ml of sterile 10% aqueous gelatin. Seeds were dried in a laminar flow hood overnight and were kept in the refrigerator until used.

Two seeds from each single treatment were inoculated into 5 ml TSB in a test tube. The tubes were incubated at 28°C for 48 hours. To determine the number of colony forming units per seed, 100 µl of the 10^6 dilution of the broth was plated onto TSA.

Seed coating for the field experiment was a scale up from the greenhouse experiment. Each strain was grown in three 250 ml flask containing 50 ml nutrient broth (NB) at room temperature for 48 hours. Cells were washed and suspended in 8 ml sterile water. Seeds were coated by mixing 24 g seeds with 8 ml bacteria suspension and 2 ml of 10% gelatin, and dried overnight. A total of 640 g seeds per treatment was prepared. Some treatments were also made in half strength by mixing half amount of bacterial suspension with sterile water.

3.3.4. Greenhouse Experiment

Ggt isolates were grown on PDA at room temperature (22-24°C) for approximately a week. Ten 8-mm discs of *Ggt* were inoculated onto 152 g sterile German foxtail millet (*Setaria italica* L.) seeds in a 2 liter Erlenmeyer flask. The flasks were incubated at room temperature for approximately 1 month.

The soil used in the experiment was Kempsville loam soil (chemical and physical properties of the soil are summarized in Table 3.1). Approximately 5 minutes before use, calcium phosphate monobasic and potassium chloride were mixed into the soil (25 µg P/g soil and 25 µg K/g soil), as well as 15 g of calcium carbonate per 30 kg soil. At the time of planting, inoculated millet was mixed with soil at a rate of 10 g millet / kg soil. A cotton ball was inserted into the bottom of each Conetainer® (Ray Leach Conetainers®, Portland, Oregon, 4 X 21 cm) and approximately 150 g of the soil mixture was added to each cone. An ammonium nitrate solution was prepared so that 30 ml solution was added to each cone to yield 30 µg N/g soil. Two seeds were planted into each cone, approximately 1 cm deep into the soil. Seeds coated with gelatin only were used as controls. Five replications for each treatment were performed. Plants were thinned to one plant per cone after a week. The plants were grown in the greenhouse until differences in plant heights between the controls and the treatments were visible (approximately 4 weeks).

At harvest, plants were removed intact from cones. Soil was washed from roots under running water. Roots were excised, washed in 70% ethanol, air dried for 10-12 mins, and weighed. Roots were rated from 0 to 5 (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). Shoots were weighed after drying in a paper bag overnight at 70°C.

Table 3.1. Selected chemical and physical properties of a Kempsville loam (fine-loamy, siliceous, thermic Typic Hapludult) soil utilized in greenhouse experiment.
(Crozier, 1999)

Parameter	Analyses	Method[1]
Textural classification	Sandy loam	
Sand %	59.7[2]	
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

[1]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 Socity of America.
[2]Average of three separately analyzed samples.

3.3.5. Field Experiment

Soft red winter wheat cultivar Roane was planted on October 12, 2000 at the Eastern Virginia Agricultural Research and Education Center at Warsaw, VA. Seeds were hand planted approximately 3 cm deep in a 1 meter row at a rate of 100 seed/m. Before planting, fertilizer containing 30 lb N, 80 lb P_2O_5, 120 lb K_2O per A was broadcast and disk-incorporated. Seeds were treated with Gaucho 480F (1.0 oz ai/cwt) using a Hage II Seed Treater before coated with bacterial strains. At planting, an equal amount of *Ggt*-infested, non-viable wheat seed was added to the 1 meter row. Seeds treated with Gaucho only were used as controls. On June 29, 2001 the total above ground biomass from a one meter row was collected with a sickle bar mower and immediately weighed.

3.4. RESULTS

3.4.1. Antagonism Test

Only 5 out of 19 strains tested showed antagonism towards other bacterial strains (Table 3.2). Strain 302-2c was antagonistic to most bacterial strains, inhibiting the growth of 12 out of 19 strains. This strain was identified as *Acinetobacter johnsonii/*genospecies 7 (0.569 similarity index) with BIOLOG and as *Paenibacillus macerans* with FAME (0.813 similarity index).

3.4.2. Nutrient Profile

The GN2 BIOLOG MicroPlate“ identification of the bacterial strains are summarized in Table 3.3. The niche overlapping index (NOI) of the bacterial strains are presented in Table 3.4. Most of the strains had low NOI suggesting that they use different nutrients and would not be competing for nutrients in the environment and would, most likely, colonize different regions of wheat rhizosphere. Only a few of the strains had NOI equal to or greater than 0.9.

Table 3.2. Antagonism among strains of biological control bacteria.

Antagonism (+) was determined as described in Fig. 3.1.

Antagonist bacteria (strain numbers)	Target bacteria (strain numbers)												
	98-30	98-58	98-62	98-79b	98-79c	99-58	302-2c	302-5a	302-6a	302-7b	302-11a	302-19b	303-6c
98-61		+	+	+	+						+		
98-62				+	+						+		
302-2c	+	+	+	+	+	+	+	+	+	+		+	+
302-7c							+						
302-11a	+		+			+			+			+	

Table 3.3. Identification of bacterial strains with GN2 BIOLOG Microplate“ and Fatty Acid Methyl Esters (FAME).

Strain Number	BIOLOG Identification	Similarity index for BIOLOG[1]	FAME Identification[2]	Similarity index for FAME
98-20	*Aeromonas sobria* DNA group 7	0.521	*Pseudomonas putida*	0.755
98-30	*Flavobacterium johnsoniae*	0.577[3]	Unknown or contaminated	
98-58	*Acinetobacter baumanii*/genospecies 2	0.927	*Acenitobacterbaumanni*	0.647
98-61	*Pseudomonas marginalis*	0.621	*Pseudomonas chloroaphis*	0.857
98-62	Unknown or contaminated		*Bacillus thuringiensis canadensis*	0.630
98-79	*Flavobacterium tirrenicum (Chryseobacterium)*	0.790	*Chryseobacterium indologenes*	0.815
99-58	Burkholderia cocovenenans	0.571	*Arthrobacter globiformis*	0.676
302-2c	*Acinetobacter johnsonii*/genospecies 7	0.569	*Paenibacillus macerans*	0.813
302-3b	Unknown or contaminated		*Micrococcus luteus* subgroup C	0.479
302-5	*Burkholderia vietnamiensis*	0.589[4]	*Arthrobacter aurescens*	0.649
302-6	*Pseudomonas putida* biotype B	0.588	*Flavobacterium mizutaii*	0.452
302-7b	*Pseudomonas putida* biotype B	0.637	*Pseudomonas mendocina*	0.911
302-7c	*Pseudomonas putida* biotype B	0.665	*Pseudomonas mendocina*	0.818
302-11a	*Aeromonas jandaei* DNA group 9	0.705	*Bacillus lentimorbus*	0.833
302-19b	*Pseudomonas putida* biotype B	0.550[3]	*Pseudomonas mendocina*	0.842
303-2a	Unknown or contaminated		*Bacillus lentimorbus(Paenibacillus macerans* subgroup A*)*	0.657 (0.624)
303-2b	Acidovorax delafieldii	0.694	*Variovorax paradoxus*	0.755
303-6c	Unknown or contaminated		*Paenibacillus polymixa (Alcaligenes paradoxus)*	0.594
SB40	*Pseudomonas putida* biotype B	0.658[3]	*Pseudomonas putida*	0.830

[1]The similarity index must be at least 0.5 to be considered acceptable.
[2]FAME identification was performed in the Biocontrol of Plant Diseases Laboratory, Beltsville, MD 20705.
[3]Strains were grown on tryptic soy agar (TSA) instead on BUG + blood agar.
[4]The microplates were incubated for 48 hours instead of 24 hours.

Table 3.4. Niche Overlapping Index (NOI) for bacterial strains.

Strain number	98-20	98-30	98-58	98-61	98-79b	98-79c	99-58	302-2c	302-5	302-6	302-7b	302-7c	302-11a	302-19b	303-2a	303-2b	SB 40
98-20		0.3	0.2	0.2	0.2	0.3	0.6	0.3	0.4	0.3	0.3	0.3	0.3	0.3	0.2	0.2	0.3
98-30	0.5		0.4	0.4	0.7	0.5	0.6	0.8	0.7	0.4	0.4	0.4	1	0.4	0.4	0.4	0.4
98-58	0.6	0.5		0.6	0.5	0.8	0.7	0.8	0.5	0.7	0.7	0.7	0.8	0.7	0.6	0.7	0.7
98-61	0.8	0.7	0.7		0.5	0.9	0.7	0.3	0.6	0.9	0.9	0.9	0.3	0.9	0.7	0.7	1.0
98-79b	0.5	0.8	0.4	0.4		0.5	0.5	0.8	0.4	0.4	0.4	0.4	1	0.4	0.5	0.5	0.5
98-79c	0.1	0.1	0.2	0.1	0.1		0.2	0.8	0.2	0.2	0.2	0.2	0.8	0.2	0.1	0.2	0.2
99-58	0.6	0.3	0.3	0.2	0.2	0.4		0.8	0.7	0.3	0.3	0.3	0.8	0.3	0.3	0.3	0.2
302-2c	0.1	0.1	0.1	0.02	0.1	0.4	0.2		0.1	0.1	0.1	0.8	0.1	0.1	0.1	0.1	0.1
302-5	0.4	0.4	0.2	0.2	0.2	0.4	0.8	0.5		0.3	0.3	0.3	0.5	0.3	0.2	0.3	0.2
302-6	0.7	0.5	0.7	0.7	0.4	0.9	0.8	0.5	0.7		0.9	1	0.5	1.0	0.6	0.7	0.8
302-7b	0.7	0.5	0.7	0.7	0.4	1	0.9	0.8	0.7	1.0		1.0	0.8	1.0	0.6	0.7	0.8
302-7c	0.7	0.5	0.7	0.7	0.4	0.9	0.8	0.5	0.7	1.0	0.9		0.5	1.0	0.5	0.6	0.8
302-11a	0.1	0.5	0.1	0	0.1	0.4	0.2	0.8	0.1	0.1	0.1	0.1		0.1	0.1	0.1	0.1
302-19b	0.7	0.5	0.7	0.6	0.5	0.9	0.8	0.5	0.6	1.0	1.0	1.0	0.8		0.5	0.6	0.8
303-2a	0.8	0.8	0.8	0.8	0.8	0.8	0.9	1.0	0.7	0.8	0.8	0.8	0.8	0.8		1.0	0.8
303-2b	0.6	0.7	0.7	0.6	0.6	0.9	0.8	0.8	0.7	0.7	0.7	0.7	0.8	0.8	0.8		0.8
SB40	0.8	0.6	0.8	0.7	0.5	1	0.8	0.7	0.6	0.8	0.8	0.8	0.5	0.8	0.6	0.7	

Niche Overlapping Index (NOI) was calculated as the number of nutrients used by both bacteria divided by the number of nutrients used by the targeted bacteria (first row), as observed on BIOLOG microplates. The NOI indicates nutrient profiles of the target bacteria. Each pair has two NOI depending on which strain act as the target. For example the pair 98-61 and 98-79c has an NOI of 0.9, meaning that 98-79c uses similar nutrient that 98-61 uses. However, the pair 98-79c and 98-61 only has an NOI of 0.1, meaning that 98-61 have broader nutrient use than 98-79c, using other nutrients as well as those also used by 98-79c.
Numbers with shading are NOIs that are at or above 0.9 indicating similar nutrient profiles between the pair of bacteria.

3.4.3. Greenhouse Experiment

The number of colony forming units/seed of the coated seeds range from 15 X 10^7 to 53.5 X 10^7 and are presented in Table 3.5. The results of the greenhouse experiment are summarized in Table 3.6. All roots were necrotic although some are less necrotic than others (see root rating in Table 3.6). Several treatments showed better root ratings (< one LSD below control), root and shoot weight than the control (> one LSD above control). Better root weight did not always correlate with better shoot weight although all of the better shoot weights (> one LSD above control) have better root weight. The plants were only grown for a month, which may explain the lack of correlation. If the plants were grown in the field, or for longer period of time, longer growth would allow greater disparity in biomass between healthy and diseased plants.

Table 3.5. Number of colony forming units/seed (cfu/seed) used in greenhouse experiment

Seed coating	Cfu/seed (X 10^8)
302-2c	2.9
SB40	1.9
98-58	5.4
302-19b	1.5
302-6	2.3
302-11a	1.6
302-7c	2.5
303-2b	3.1
302-5	2.5
98-61	5.2

Two seeds per treatment were inoculated into 5 ml tryptic soy broth (TSB) in a test tube and incubated at 28°C for 48 hours. The broth was diluted 10^6 times and 100 µl of the diluted broth was plated onto two tryptic soy agar (TSA) plates. The numbers of colonies on the two plates were averaged to obtain the number of cfu/seed.

Table 3.6. Influence of bacteria combination against take-all

Treatment[1]	Greenhouse experiment			Field experiment
	Mean Root Rating[2]	Mean Root Weight (g)[3]	Mean Shoot Weight (g)[4]	Yield (g/m)[5]
Control[6]	3.2 c-h	0.14 f-i[7]	0.08 abc	175.43 f-k
302-11a	3.6 b-f	0.24 cde[8]	0.10 abc	202.08 a-k
302-11a+303-2b	3.8 a-e	0.08 hij	0.04 c	---[10]
302-19b	3.0 e-h	0.3 bc	0.10 abc	168.20 h-k
302-19b (0.5 X)[9]	---	---	---	203.45 a-k
302-19b+98-61	2.6 gh	0.16 e-h	0.10 abc	218.35 a-j
302-19b+SB40	2.5 h	0.26 cd	0.10 abc	207.88 a-k
302-2c	3.2 c-h	0.22 c-f	0.08 abc	192.90 b-k
302-2c (0.5 X)	---	---	---	238.40 a-e
302-2c+302-11a	3.0 e-h	0.3 bc	0.14 a	---
302-2c+302-5	3.0 e-h	0.14 f-i	0.08 abc	---
302-2c+302-7c	2.8 fgh	0.18 d-g	0.10 abc	---
302-2c+98-58	2.7 gh	0.38 ab	0.14 a	---
302-2c+98-61	2.8 fgh	0.26 cd	0.12 ab	203.10 a-k
302-5	3.6 b-f	0.18 d-g	0.10 abc	201.90 a-k
302-5+302-11a	3.0 e-h	0.10 g-j	0.06 bc	---
302-5+302-7c	4.4 a	0.04 j	0.04 c	---
302-5+303-2b	3.0 e-h	0.14 f-i	0.08 abc	---
302-6	3.0 e-h	0.24 cde	0.08 abc	199.32 a-k
302-6 (0.5 X)	---	---	---	246.43 ab
302-6+302-19b	3.9 a-d	0.10 g-j	0.04 c	---
302-6+302-7c	3.1 d-h	0.10 g-j	0.08 abc	---
302-6+98-61	4.0 abc	0.10 g-j	0.08 abc	240.40 a-e
302-6+SB40	3.2 c-h	0.16 e-h	0.08 abc	174.23 g-k
302-7c	3.0 e-h	0.26 cd	0.12 ab	241.95 a-d
302-7c (0.5 X)	---	---	---	191.23 b-k
302-7c+302-11a	3.3 c-h	0.10 g-j	0.08 abc	---
302-7c+302-19b	3.0 e-h	0.14 f-i	0.10 abc	---
302-7c+303-2b	2.6 gh	0.10 g-j	0.06 bc	---
302-7c+98-61	3.0 e-h	0.10 g-j	0.06 bc	198.17 a-k
303-2b	3.4 c-g	0.18 d-g	0.08 abc	---
98-58	3.4 c-g	0.24 cde	0.14 a	204.45 a-k
98-58 (0.5 X)	---	---	---	222.03 a-i
98-58+302-5	3.7 a-e	0.08 hij	0.06 bc	---
98-58+302-7c	3.9 a-d	0.08 hij	0.04 c	165.40 ijk
98-58+302-11a	4.4 a	0.06 ij	0.06 bc	---
98-58+303-2b	4.2 ab	0.14 f-i	0.06 bc	---
98-61	3.0 e-h	0.28 c	0.10 abc	233.20 a-f
98-61+SB40	2.5 h	0.3 bc	0.12 ab	217.95 a-j
SB40	2.8 fgh	0.28 c	0.08 abc	184.35 d-k
SB40 (0.5 X)[7]	---	---	---	197.65 a-k
SB40+302-2c	2.6 gh	0.16 e-h	0.06 bc	197.93 a-k
SB40+302-7c	2.6 gh	0.42 a	0.14 a	---
LSD	0.669	0.08	0.058	43.468
Standard deviation	0.534	0.064	0.046	37.370

[1]Treatments for the greenhouse experiment consist of either 2 ml of bacteria suspension mixed with 8 ml of gelatin solution and applied to 2.4 g of wheat seeds or gelatin only. Treatments for the field experiment consist of either 8 ml of bacteria suspension mixed with 2 ml of gelatin solution and applied to 24 g of wheat seeds or Gaucho only.
[2]Mean root ratings are from 0 to 5 (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).
[3]Roots were excised from plants, washed in 70% ethanol, air dried for 10-12 mins, and weighed.
[4]Shoots were put in a paper bag, dried overnight at 70°C, and weighed.
[5]All above ground tissue removed from 1 meter row and expressed as fresh weight in grams.
[6]The control was seeds treated with gelatin only for the greenhouse experiment and with Gaucho only for the field experiment.
[7]Means with letter in common do not differ significantly by Duncan s MRT (P=0.05 for greenhouse experiment and P=0.10 for field experiment).
[8]Means in shading are greater than the least significant difference (LSD) above the control, except for root ratings it is less that the LSD (root weight > 0.22 g; shoot weight > 0.138 g; yield > 218.90 g; root ratings < 2.53).
[9]Bacteria seed coating is at half the concentration.
[10]Means no data was available.

3.4.4. Field Experiment

Results of the field experiment are summarized in Table 3.6. Only two treatments gave better yield than the control and these include the strain 98-61. There are other treatments that gave better yield and these are the half strength treatment (see Table 3.6). It may be that the half strength is the optimum concentration for the bacteria to provide disease control but this needs to be proved.

3.5. DISCUSSION

The antagonistic properties of bacterial strains shown by the antagonism test did not reflect what occurs *in situ*. In the greenhouse experiment, for example, the strain 302-11a gave better root weight but did not when combined with strain 303-2b. This combination gave a mean root weight even less than strain 303-2b alone. Neither of the strain was antagonistic to each other, nor had similar nutrient profile. Some other combinations that gave less mean root weight when combined are 302-6 and SB40, 302-7c and 302-11a. The antagonism test and nutrient

profile provided no indication to what might have cause the loss of control activity. The combination of strains 98-58 and 302-2c gave better mean root weight than either of the strain alone, although the antagonism test showed strain 302-2c to be antagonistic to strain 98-58. However, since 302-2c and 98-58 share an NOI of less than 0.9, they may be spatially separated on plant roots. On the other hand, some combinations are in correlation with the nutrient profile. For example, the combination 302-7c and 302-19b gave less mean root weight than either of the strain alone. In this case, the nutrient profile of the two strains showed that they are similar (NOI=0.98), indicating possible nutrient competition between the two resulting sequentially in reduction in the area of roots colonized and reduced disease control.

Results from the field experiment showed that the combination 302-6 and 98-61 gave better yield. However, they have similar nutrient profile. These results suggest that laboratory experiments, such as the antagonism test and nutrient profiles, and *in situ* results do not always show any relationship.

Several factors may explain the lack of correlation. Firstly, the antagonism test was performed by allowing the first strain to grow approximately two days before subjected to other strains. This strain had a chance to produce its secondary metabolites that probably are responsible for any antagonism activities. In the greenhouse and field experiments, however, the strains were mixed at the same time so no secondary metabolites are present. Secondly, unlike in Petri plates where bacterial growth is confined, growth in roots is less restricted. Any secondary metabolites may not affect the growth of other strains simply because they are not in sufficient proximity (Fukui *et al*., 1994). Likewise, any bacteria pair that has similar nutrient profile may not compete for nutrient *in situ* due to the unrestricted space of growth.

Furthermore, the nutrient profile generated using the BIOLOG GN2 MicroPlate“ was only for 95 carbon sources. NOI was established for phyllosphere bacteria (Wilson and Lindow, 1994) and BIOLOG was sufficient for that purpose. Our bacterial strains, however, are rhizosphere bacteria and the nutrient sources in the rhizosphere are very different. Therefore, NOI obtained from BIOLOG may not represent the true nutrient profile of these bacteria. This

has to be proved by using additional nutrient sources that represent rhizosphere nutrient conditions and comparing the results with BIOLOG.

Following the number of bacteria after inoculation may provide more information on the persistence of the strain *in situ*. The population can be viewed as increasing in number, decreasing, or constant. However, this does not necessarily support the antagonism and nutrient test results. If a certain strain decreases in number, several possible reasons might occur, antagonism, nutrient competition, or site competition. A replacement series experiment (Wilson and Lindow, 1994) would be useful in deciding which combination would persist best in the environment.

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CHAPTER 4. FUTURE DIRECTION

4.1. INTRODUCTION

Since there are two main objectives in my research, namely the early pathogen detection from plants and soil using polymerase chain reaction (PCR) and the study on interactions among bacterial biological control agents, there will be two main directions for future research. This will be discussed in two sections.

4.2. DETECTION OF *GAEUMANNOMYCES GRAMINIS* VAR. *TRITICI* (*GGT*) *IN PLANTA* AND SOIL USING PCR

Unlike other primers that identify the take-all fungus *Gaeumannomyces graminis* var. *tritici* (*Ggt*) by amplifying its mitochondrial DNA or the internal transcribed spacer (ITS) regions, the avenacinase primers amplify the genes associated with pathogenicity. Fast and accurate identification using polymerase chain reaction (PCR) with these primers would be very useful in disease control management. On the other hand, since nuclear DNA as the target is present in lower concentration in cell than ribosomal or mitochondrial DNA, better DNA extraction method is needed to increase PCR sensitivity. Alternatively, PCR conditions have to be optimized to overcome any inhibition that may be caused by any carry over from the extraction. Early identification of the fungus using PCR would benefit control management practices by controlling the disease as early as possible, as would be achieved by identification of the fungus in infested soil before planting season begins.

4.2.1. Sample Concentration Prior To DNA Extraction

Since the target DNA for amplification with the avenacinase-based primers are probably present in cell in low concentration, a concentration or an enrichment method may provide a way to increase it in the sample before extraction. Sample concentration was done by Jacobsen (1995) using a Magnetic Capture Hybridization (MCH)-PCR method. This method concentrates the target DNA by hybridizing it with a single stranded DNA probe conjugated to magnetic

beads. A single stranded DNA specific to *Ggt* can be used as probe. Alternatively, a common probe to *Ggt*, *G. graminis* (Sacc.) v. Arx & Olivier var. *graminis* (*Ggg*), and *G. graminis* (Sacc.) v. Arx & Olivier var. *avenae* (*Gga*) would identify all three fungi. This single stranded DNA should be within the region that is amplified by the avenacinase-based primers. Samples with sheared target DNA are hybridized with magnetic beads conjugated with the probe. This specifically selects the target DNA and concentrating it on the beads. By hybridizing the target DNA onto the beads, this method also minimizes the amount of PCR inhibitors, such as humic acids. The beads are resuspended in water or buffer and used in PCR.

4.2.2. Sample Enrichment Prior to DNA Extraction

Another method is an enrichment method used by Pradhanang *et al*., (2000) to detect the presence of *Ralstonia solanacearum* in soil. A selective medium is needed in the enrichment method and Juhnke *et al*., (1984) has developed one for *Ggt*. Soil samples are inoculated to the selective medium and incubated to increase the number of cells present in the samples. The incubation time needs to be considered before doing this enrichment. Overnight incubation was sufficient for Pradhanang *et al*., (2000) because they were enriching bacteria. *Ggt* enrichment may take longer since fungi generally grow slower than bacteria. Therefore, this method should be taken only if better DNA extraction or more optimized PCR conditions cannot be established.

4.3. SELECTION OF BIOLOGICAL CONTROL STRAINS FOR TAKE-ALL

One method to control take-all that is being considered is biological control. The biological control of take-all, although very promising, has been confronted with many challenges, inconsistency being the main factor. Incomplete understanding of the complex ecology of the rhizosphere complicates the problem. To our knowledge, no bacterial strain has been commercially successful to control take-all in wheat.

As an ongoing research in biological control towards take-all, the best approach seems to be using combinations of bacteria. Combinations of bacteria are expected to have more complete root colonization, more persistence in the environment, and more than one mechanism

of action providing disease suppression in a wider range of conditions (Pierson and Weller, 1994). This would hopefully lead to better consistency. In addition, combinations of bacteria may provide control to more than one disease, which could make disease control more economical.

On the other hand, use of bacterial combinations is not without problems. Bacteria suppress disease by producing secondary metabolites such as antibiotics or siderophores, and by competing for nutrients and space. The same mechanism that bacteria use to suppress disease can result in suppression of other bacteria in the mixture. Therefore, proper assessment of which bacteria to be used in the combination is needed. The best combination would be the one in which all the bacteria strains can live and survive *in situ*, as well as providing control to the disease.

The *in situ* status needs to be stressed here since many research have shown that laboratory and *in situ* survival of bacteria in mixture differ considerably (de Boer *et al*., 1999; Fukui *et al*., 1994). In our antagonism tests, differences in inoculation time and limited growing space allowed for antagonism to occur. As Fukui *et al*., (1994) explained, these two factors do not happen *in situ* and may account for the different results. For instance, antagonists with low NOI may not colonize the same part of the roots and, therefore, may not antagonize each other *in situ* in the rhizosphere.

Another factor to be considered is the fact that so far only two bacterial strains are used in a mixture. The rhizosphere contains so many types of organisms interacting with each other. Therefore, using more than two bacterial strains in a combination may provide further buffering capacity, better performance, and consistency to persist in the environment.

4.3.1. De Wit Replacement Series

In order to find the best combination of bacteria, an *in situ* method is needed. De Wit replacement series is a method that has been adapted to microbiological application by Wilson and Lindow (1994) to understand ecological similarity and coexistence of bacteria. Although the

original adaptation was made for phyllosphere bacteria, it should also work for rhizobacteria. This method, together with the antagonism tests and nutrient profile, should give us better understanding on the coexistence of bacteria in the rhizosphere.

The result of a replacement series indicates various types of interaction, equal competitiveness, unequal competitiveness, growth limitation by the same resource, and niche differentiation. Only the last interaction signifies a high level of coexistence between two strains. The advantage of a replacement series is that it is done *in situ*. Furthermore, the bacterial population number is monitored so the results of any interaction between the bacteria can be known with certainty. Antagonism tests and nutrient profile can then be related to the replacement series to predict which factor contributes to the low or high level of coexistence between the bacteria.

As disease control is the ultimate goal, the information obtained from the replacement series, antagonism test, and nutrient profiles should be used to predict the best combination of bacterial strains. This prediction should then be tested against the pathogen, in a greenhouse or field experiment. More importantly, the population number of the bacteria in the mixture should be followed (Fukui *et al*., 1994; Janisiewicz, 1996) and related to disease severity.

4.3.2. Use of More than Two Bacterial Strains

Since the rhizosphere contains a diverse array of microorganisms, using two bacterial strains to control plant disease may not be sufficient. More than two strains will mimic the natural environment more closely. In this case, several bacterial strains that have shown potential disease control should be used together, for example as a seed coating. Then one of the strains is taken out of the mixture to see which strain is not needed in disease control. This way instead of figuring out which pair or mixture works best by adding one strain after another, the strains are all used and then one after another is taken out. The same method was used by Fukui *et al*., (1999) in their search for the best bacterial mixture in controlling anthurium blight.

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APPENDIX A

Sequence alignment of PCR fragments with *Ggt* DNA from isolate M1 and ATCC 23230.

Ggt M1	TGGAAGGTCCCCGTACGCCGGGCGCAACTGGGAGGGATTC	40
Ggt ATCC	TGGAAGGTCCCCGTACGCCGGGCGCAACTGGGAGGGATTC	40
Ggt IND	ACGACC–TGAGCAGACGCNGCGCCCAGGAGGGAGGAANAC	39
Ggt M1	TCCCCCGACTCGTACCTCGCGGGCGTCCTGGCAGAGCAGA	80
Ggt ATCC	TCCCCCGACTTGTACCTCGCGGGCGTCCTGGCAGAGCAGA	80
Ggt IND	TCCCCCGGCTCGTACCTCGCGGGCGTCCTGGCAGAGCAGA	79
Ggt M1	CGGTCAAGGGGATGCAGGTAAGGAGCCCTCTCCAGCAACA	120
Ggt ATCC	CGGTCAAGGGGATGCAGGTAAGGGGCCCTCTCCAGCAACA	120
Ggt IND	CGGTCAAGGGGATGCAGGTAAGGAGCCCTCTCCAGCAACA	119
Ggt M1	TGTCGGCGCCGAGCCTATTCCCTGTAATACTGACACTTTG	160
Ggt ATCC	TGTTGGCGCCGAGCCTATTCCCTGTAATACTGACACTTTG	160
Ggt IND	TGTCGGCGCCGAGCCTATTCCCTGTAATACTGACACTTTG	159
Ggt M1	ACAGTCGGTCGGCGTGCAAACCTGCACCAAGCACTACATC	200
Ggt ATCC	ACAGTCGGTCGGCGTGCAAACCTGCACCAAGCACTACATC	200
Ggt IND	ACAGTCGGTCGGCGTGCAAACCTGCACCAAGCACTACATC	199
Ggt M1	GGCAATGAGCAGGAGGAGCAGCGCAACCCCACGACGGTGG	240
Ggt ATCC	GGCAATGAGCAGGAGGAGCAGCGCAACCCCACGACGGTGG	240
Ggt IND	GGCAATGAGCAGGAGGAGCAGCGCAACCCCACGACGGTGG	239
Ggt M1	ATGGCAAGGGGGTTGAGGCCATCTCGTCCAACATTGACGA	280
Ggt ATCC	ATGGCAAGGGGGTTGAGGCCATCTCGTCCAACATTGACGA	280
Ggt IND	ATGGCAAGGGGGTTGAGGCCATCTCGTCCAACATTGACGA	279

Ggt M1	CCGCACAATGCACGAGACCTACCTGTGGCCCTTTTACAAC	320
Ggt ATCC	CCGCACAATGCACGAGACCTACCTGTGGCCCTTTTACAAC	320
Ggt IND	CCGCACAATGCACGAGACCTACCTGTGGCCCTTTTACAAC	319
Ggt M1	GCCGTCAGGGCCGGCACCACGTCCATAATGTGCTCTTACC	360
Ggt ATCC	GCCGTCAGGGCCGGCACCACCTCCATAATGTGCTCTTACC	360
Ggt IND	GCCGTCAGGGCCGGCACCACGTCCATAATGTGCTCTTACC	359
Ggt M1	AGAGGATCAACGGCAGCTACGGCTGCCAGAACAGCAAGAC	400
Ggt ATCC	AGAGGATCAACGGCAGCTACGGCTGCCAGAACAGCAAGAC	400
Ggt IND	AGAGGATCAACGGCAGCTACGGCTGCCAGAACAGCAAGAC	399
Ggt M1	CCTCAACGGGCTTC—TCAAGACCGAGCTCGGCTTCCAGGG	439
Ggt ATCC	CTTCAACGGGCTTCTTCAAGACCGAGCTCGGCTTCCAGGG	440
Ggt IND	CCTCAACGGGCTTC—TCAAGACCGAGCTCGGCTTCCAGGG	438
Ggt M1	CTTCGTCGTGTCGGACTGGTGCGTGGCTACCTCCCTCTAC	479
Ggt ATCC	CTTCGTCGTGTCGGACTGGTGCGTGGCTACCTCCTTCTAC	480
Ggt IND	CTTCGTCGTGTCGGACTGGTGCGTGGCTACCTCCCTCTAC	478
Ggt M1	CAGATGAAACATGCAGTGCCTTG—TTTTTGCTAATGGCCA	518
Ggt ATCC	CAGATGAAACATGCAGTGCCTTGTTTTTTGCTAATGGCCA	520
Ggt IND	CAGATGAAACATGCAGTGCCTTG—TTTTTGCTAATGGCCA	517
Ggt M1	TAACAGGGCCGCTACCCATTCCGGAGTCGCCTCCATTGAG	558
Ggt ATCC	TAACAGGGCCGCTACCCATTCCGGAGTCGCCTCCATTGAG	560
Ggt IND	TAACAGGGCCGCTACCCATTCCGGAGTCGCCTCCATTGAG	557

Ggt M1	GCTGGTCTGGACATGAACATGCCCGGACCGCTCAATTTTT	598
Ggt ATCC	GCTGGTCTGGACATGAACATGCCCGGACCGCTCAATTTTT	600
Ggt IND	GCTGGTCTGGACATGAACATGCCCGGACCGCTCAATTTTT	597
Ggt M1	TTGCCCCAACCCTCGAGTCTTACTTTGGCAAGAACATCAC	638
Ggt ATCC	TTGCCCCAACCCTCGGGTCTTACTTTGGCAAGAACATCAC	640
Ggt IND	TTGCCCCAACCCTCGAGTCTTACTTTGGCAAGAACATCAC	637
Ggt M1	CACTGCGGTCAATAACGGCACACTCTCCTCCCGGAGGGTC	678
Ggt ATCC	CACTGCGGTCAACAACGGCACACTCTCCTCCCGGAGGATC	680
Ggt IND	CACTGCGGTCAATAACGGCACACTCTCCTCCCGGAGGGTC	677
Ggt M1	GACGACATGATTGAGCGCATCATGACTCCCTACTTCGCCC	718
Ggt ATCC	GACGACATGATTGAGCGCATCATGACTCCCTACTTCGCCC	720
Ggt IND	GACGACATGATTGAGCGCATCATGACTCCCTACTTCGCCC	717
Ggt M1	TGGGTCAGGACAAGGACTACCCCCCTG—TCGACGGCTCCA	757
Ggt ATCC	TGGGTCAGGACAAGGACTACCCCCCTG—TCGACGGCTCCA	759
Ggt IND	TGGGTCAGGACAAGGACTACCCCCCTGGTCGACGGCTCCA	757
Ggt M1	CGGTGCCCAT—CGGCTACTTGCAGCCCGACGCCTGGAACC	796
Ggt ATCC	CGGTGCCCAT—CGGCTTCTTGCAGCCCGACGTCTGGAGCC	798
Ggt IND	CGGTGCCCATacGGTTACTTGNAGCCGCACG	788

VITA

Indira Genowati was born on January 28, 1973, in Surabaya, Indonesia. She finished high school in Jakarta and got a scholarship to pursue her degree in the United Kingdom. She obtained a Bachelor of Science in Microbiology/Biotechnology from the University of Sheffffield in 1997. Her final projects developed her interests in environmentally friendly practice. She had to isolate bacteria that can utilize coal as a nutrient source for her laboratory project. This introduced her to the concept of bioremediation. She had to write on the utilization of agricultural waste for her library project. As part of her Bachelor degree, she did a one year co-op with the British Sugar Technical Center where she was entrusted with maintaining tissue cultures of genetically modified potatoes. Along with that, she had to isolate and identify bacteria that were causing problems in sugar thick juice. After graduation, she became a Research Scientist in a government-based research agency in Indonesia working in the development of *Trichoderma* sp. as biological control agent for plant pathogens. She initiated the work on utilizing fungi as biological control of insect and weeds. After two years she got married and went to Blacksburg, VA, to pursue her Master s degree.