CHAPTER 3

A PCR-BASED TEST FOR DIFFERENTIATING VARIETIES OF *Gaeumannomyces graminis*, THE TAKE-ALL PATHOGENS

The information presented in this chapter will be submitted to an appropriate journal with additional authors: C.L. Cramer, E.A. Grabau, G.H. Lacy, and E.L. Stromberg

3.1 ABSTRACT

A PCR-based diagnostic test for *Gaeumannomyces graminis* var. *tritici* (Ggt), var. *avenae* (Gga), and var. *graminis* (Ggg), the take-all pathogens of cereals and grasses, has been developed. The test is based on specific amplification of avenacinase-like genes present in all of the three varieties. Three 5' oligonucleotide primers specific to Ggt, Gga, and Ggg and a single 3' universal primer were used to generate PCR fragments of 870, 617, and 1,086 bp specific for Ggt, Gga, and Ggg respectively. Each 5'-specific primer showed high specificity towards its own DNA template in single and mixed populations of primers and templates. Sensitivity of PCR with these primers was quite high since detection of PCR products was as low as 50 pg of template DNA in 50 µl reactions with Gga-specific primer and 100 pg of template DNA for Ggt- and Ggg-specific primers. Nine out of sixteen isolates of Ggt produced Ggt-specific fragments and seven produced Ggg-specific fragments when all three variety-specific primers were added. Although the 16 isolates were originally isolated from wheat, some isolates may actually represent Ggg. All but one of Gga isolates produced Gga-specific fragments. Ggt- and Gga-specific fragments were observed from Gga isolate RB-W suggesting that our diagnostic test can differentiate each variety from a mixed populations of these fungi. In addition, no PCR products were amplified from any closely related fungi such as *Gaeumannomyces cylindrosporus* and *Phialophora* spp. This is a first diagnostic test capable of identification of all three varieties of *G. graminis* in a single PCR tube and with a basic PCR protocol. The test is rapid and specific. Interpretation of results is simple and conclusive based on differences in size of each variety specific fragment.

3.2 INTRODUCTION

Take-all is an economically significant and damaging root disease of cereals and grasses worldwide. It is very important in temperate areas where wheat and grass culture is intensive (Garrett, 1981). *Gaeumannomyces graminis* (Sacc.) Arx & Olivier, a soilborne ascomycete, is the causal agent of take-all of cereal and grasses. The fungus colonized the root systems of the susceptible plants producing characteristic symptoms which include stunted growth, white-heads, reduced and blackened roots, and prematurely ripening. Based on pathogenicity of host plants and cultural characteristics, three varieties of *G. graminis* have been recognized (Walker, 1972). *G. graminis* var. *tritici* Walker (Ggt) is the causative agent of take-all of wheat (*Triticum aestivum* L.), barley (*Hordeum vulgare* L.) and rye (*Secale cereale* L.) and the most economically important take-all pathogen. *G. graminis* var. *avenae* (E.M. Turner) Dennis (Gga) attacks oats (*Avena sativa* L.) and causes take-all patch of turfgrasses, particularly *Agrostis* spp. (Walker, 1981). *G. graminis* var. *graminis* (Ggg) is pathogenic on cereals and grasses (Elliott et al., 1991; Walker, 1972) and causes crown sheath rot of rice (*Oryza sativa* L.) (Walker, 1981) but mostly are non-pathogenic or weakly pathogenic on wheat. However, highly virulent isolates of Ggg isolated from pods and leaves of soybean [*Glycine max* (L.) Merr.] on wheat have been reported (Roy, 1982; Walker, 1981). In addition, other species of *Gaeumannomyces* and *Phialophora* are also capable of colonizing and cause root rot on cereals and grasses. For example, *G. cylindrosporus* and *G. incrustans* can cause root rot on wheat, barley, and turf grass respectively (Hornby et al., 1977; Walker, 1981) but are not considered take-all pathogens. In addition, some members of the genus *Phialophora*, the asexual state of *Gaeumannomyces*, are non-pathogenic on cereals and grasses. The *Gaeumannomyces-Phialophora* complex is confusing and this contributes to misidentification of the causal organism of take-all of wheat.

Conventional identification methods of the causal organism of take-all are usually laborious, time-consuming and indefinite. It is generally based on combination of the characteristic field symptoms, microscopic examination of morphological characteristics of the fungus such as the size of perithecia and ascospores, and type of hyphopodia, and

pathogenicity assay (Clarkson and Polley, 1981). While Ggt and Gga produce simple and non-lobed hyphopodia, Ggg is capable of producing two types of hyphopodia which are lobed and simple, non-lobed hyphopodia (Epstein et al., 1994; Nilsson, 1972). Variation in size of the ascospores can also be used to differentiate the three varieties, however, some overlap of this characteristic can make the specific identification difficult and inconclusive. Pathogenicity assay can be unreliable due to changes in virulence of long term storage culture of *G. graminis* (Asher, 1980). Moreover, other agents such as pathogenic *Fusarium* spp. and *Cochliobolus sativa* (Ito et Kuribayashi) Drechsler ex Dastur, insects infestations, and poor soil conditions can produce field symptoms similar to take-all that may also obscure specific pathogen identification (Clarkson and Polley, 1981).

Molecular techniques have played a significant role in recent development of specific and rapid diagnostic tests for *G. graminis,* especially Ggt. Most of the previous works were focused on the use of RFLPs identified by specific DNA probes (Bateman et al., 1992; Henson, 1989; Tan et. al., 1994; Ward and Gray, 1992), amplification of mitochondrial DNA fragments (Elliott, 1993; Henson, 1992; Schesser, et al., 1991), amplification of specific regions of the internal transcribed spacer (ITS) regions of the ribosomal DNA (Bryan et. al., 1995; Goodwin et al., 1995; O'Dell et al., 1992), and RAPDs (Bryan et al., 1999; Fouly et.al., 1996; Wetzel et al., 1996). Although these techniques are shown to have specificity toward varieties of *G. graminis* (Bryan at al., 1995; Goodwin et al., 1995; Ward and Gray, 1992) and advantages over the conventional methods, there are still some limitations in terms of interpretation of results and specificity to all varieties of *G. graminis*. For example, use of RFLP patterns can be time-consuming and expensive due to use of DNA probe hybridization and restriction endonucleases. In addition, all of these assays require multiple sets of reactions to distinguish all three varieties of *G.graminis* and are not very definitive on discrimination between Ggt and Ggg.

While others were interested in using the multicopy gene families present in the genome as targets for developing a specific identification of take-all pathogens, We proposed here a different approach for differentiation of these fungi. We have been interested in one of

the major distinct biochemical properties associated with pathogenicity of these fungi, the production of avenacinase, an avenacin-detoxifying enzyme. Avenacinase produced from Gga is very active towards avenacin A-1, which is the most abundant and fungitoxic substance present in oat roots (Crombie et al., 1986). The ability to detoxify avenacin A-1 to a less toxic substance enables Gga to be pathogenic on oats (Crombie et al., 1986; Osbourn et al., 1991). On the other hand, Ggt and Ggg also have the ability to produce avenacinase but the enzyme is not active towards the substrate (Crombie et al., 1986; Osbourn et al., 1991; Osbourn et al., 1994). Since Ggt, Gga, and Ggg have the ability to produce avenacinase or avenacinase-like enzymes, the genes encoding this enzyme in all three varieties must be closely related although the activity of the enzymes may be different. While avenacinase plays a role in pathogenicity of Gga but not in Ggt and Ggg, we believe that there should be sufficient divergence at the nucleic acid level affecting the enzyme activity that may be useful for identification purposes.

Isolation and partial characterization of the avenacinase-like genes of a few isolates of Ggt and Ggg were previously reported (see Chapter 2). Preliminary results on restriction endonuclease digestion profiles of the genes among three varieties confirmed our hypothesis that there was some divergence at the nucleic acid level. This corresponds to our sequencing data (Fig. 2.7 and 2.8, Chapter 2) that the genes were almost identical to that of Gga with limited regions of divergence on the nucleotide level.

We proposed exploitation of avenacinase gene as a marker for differential identification of Ggt, Gga, and Ggg. The rationale is that because the avenacinase gene is associated with pathogenicity on specific hosts, it should confer specificity particularly to the varieties of take-all fungi. We report a rapid, simple and specific identification test for take-all pathogens based on amplification of avenacinase or avenacinase-like genes. The principle was to design PCR primers specific to each variety of *G. graminis* from limited regions of nucleotide divergence among the avenacinase-like genes. It is the first identification tool designed to differentiate all three varieties of *G. graminis* in a single PCR reaction. Use of basic PCR protocols rather than nested PCR in combination with simple result interpretation with a presence of a single band contribute to a novel

diagnostic test for take-all pathogens. We demonstrate that the test is very sensitive and specific to each variety of *G. graminis* regardless of the amount and types of the fungal templates tested.

3.3 MATERIALS AND METHODS

3.3.1 Fungal isolates, maintenance, and cultivation.

All fungi used in this study are listed in Table 3.1. All fungi except *Cercospora zea-maydis* were maintained on ¼ x potato dextrose agar (¼ x PDA; 6 g of potato dextrose broth and 1.5% w/v agar per liter) at 4°C and no more than three successive transfers were made to preserve their pathogenicity. Isolates other than those from American Type Culture Collection (ATCC, Manassas, VA 20110-2209) were purified by single hyphal tip isolation (Appendix A) prior to any DNA work. For long term storage, mycelium plugs were taken from the colony margin using a cork borer no. 3 (8-mm dia.) and stored at -80°C in 15% glycerol. Genomic DNA of *Cercospora zea-maydis* FOO1 was provided by V.K. Stromberg (Department of Plant Pathology, Physiology and Weed Science, Virginia Polytechnic Institute and State University, Blacksburg, VA 24061-0330).

For cultivation of fungi, three mycelial plugs taken from colony margin using cork borer no. 2 (5-mm diameter) were inoculated in a 50 ml ¼ x potato dextrose broth (¼ x PDB, Sigma, St. Louise, MO 63178) and grown in an orbital incubator (SanyoGallenkamp PLC., Loughborough, UK) for 7 days at 25°C, 120 rpm. Mycelia were ground in a Waring blender (Dynamics Corporation of America, New Hartford, CT 06057) and transferred to a 1 L flask containing 250 to 300 ml ¼ x PDB. The fungi grown for 7 to 10 days under the above condition were ready for harvest.

Table 3.1. Fungal isolates used in this study.

Isolate	Host	Source Location	Source/reference
Gaeumannomyces graminis var. *tritici*			
CB1	*Triticum aestivum* L.	Bedford Co., VA, USA	J.B. Crozier[1]
CD1	*T. aestivum.*	Dinwiddie Co., VA, USA	J.B. Crozier
CE1	*T. aestivum.*	Essex Co., VA, USA	J.B. Crozier
CH1	*T. aestivum*	Hannover, Co., VA, USA	J.B. Crozier
CHe1	*T. aestivum*	Henrico Co., VA, USA	J.B. Crozier
CK1a	*T. aestivum*	King and Queen Co., VA, USA	J.B. Crozier
CK1b	*T. aestivum*	King and Queen Co., VA, USA	J.B. Crozier
CS1	*T. aestivum*	Suffork Co., VA, USA	J.B. Crozier
M1	*T. aestivum*	Montana, USA	D. Mathre[2]
ATCC 28230	*T. aestivum*	United Kingdom	ATCC[3]
3053	*T. aestivum*	Washington, USA	M. Elliott[4]
3055	*T. aestivum*	Oregon, USA	M. Elliott
3056	*T. aestivum*	Indiana, USA	M. Elliott
3060	*T. aestivum*	Idaho, USA	M. Elliott
3066	*T. aestivum*	Montana, USA	M. Elliott
G. graminis var. *avenae*			
ATCC 15419	*Avena sativa* L.	United Kingdom	ATCC
PG-W	*Agrostis stolonifera* L.	Pinegrove, Canada	H.C. Wetzel III[5]
FR-W	*A.. stolonifera*	France	H.C. Wetzel III
RB-W	*A. stolonifera*	Delaware, USA	H.C. Wetzel III
RI-W	*A. stolonifera*	Rhode Island, USA	H.C. Wetzel III
WW-W	*A. stolonifera*	Ohio, USA	H.C. Wetzel III
G. graminis var. *graminis*			
ATCC 12761	*T. aestivum*	United Kingdom	ATCC
FL-19	*Cyanodon dactylon* (L.) Pers.	Florida, USA	M. Elliott
FL-39	*Stenotaphrum secundataum* (Walt.) Kuntze	Florida, USA	M. Elliott
FL-175	*Oryza sativa* L.	Florida, USA	M. Elliott
2033	*Glycine max* (L.) Merr.	Florida, USA	M. Elliott

Table 3.1 Fungal isolates used in this study (continued).

Isolate	Host	Source location	Source/reference
Gaeumannomyces cylindrosporus ATCC 64420	*Poa pratensis* L.	Rhode Island, USA	M. Elliott
Phialophora radicicola ATCC 64414	*P. pratensis* L.	Rhode Island, USA	M. Elliott
Phialophora sp.	unknown	unknown	M. Elliott
Cercospora zea-maydis FOO1	*Zea mays* L.	Wythe Co., VA, USA	V.K. Stromberg[6]

[1] Mr. James Brooks Crozier, Department of Plant Pathology, Physiology and Weed Science, Virginia Polytechnic Institute and State University, Blacksburg, VA 24061-0331

2 Dr. Don E. Mathre, Department of Plant Sciences, Montana State University-Bozeman, Bozeman, MT 59717-3150.

[3] American Type Culture Collection, 10801 University Boulevard, Manassas, VA 20110-2209

[4] Dr. Monica L. Elliott, Fort Lauderdale Research and Education Center, University of Florida, Ft. Lauderdale, FL 33314-7799.

[5] Mr. Henry C. Wetzel III, Department of Plant Pathology, Kansas State University, Manhattan, KS66506-5502.

[6] Mrs. Verlyn K. Stromberg, Department of Plany Pathology, Physiology and Weed Science, Virginia Polytechnic Institute and State University, Blacksburg, VA 24061-0330.

3.3.2 Preparation of fungal DNA.

After 7-10 days of cultivation, mycelial mass was collected on 4 layers of 46 x 91 cm cheesecloth wipes (Fisher Scientific, Pittsburgh, PA 15238) and transferred onto a sterile filter paper Whatman® no. 1 (Whatman International Ltd., Maidstone, UK). Wet mycelia were freeze-dried (FreezeMobile 6, The Virtis Company Inc., Gardiner, NY 12525) at 200 μτ overnight. Dry mycelia were collected on the next day and kept in a desiccator until used. Fungal DNA used as templates for PCR were prepared according to the protocol modified by Lee and Taylor (1990). Approximately 1 g of dry mycelia was powdered in liquid nitrogen and 10 ml of lysis buffer (50 mM Tris-HCl [pH, 7.2], 50mM EDTA, 3% v/v SDS, 1% v/v β-mercaptoethanol) was added immediately to the powder. The mixture was stirred with a 5 ml pipet tip and vortexed vigorously until it became homogeneous. The mixture was incubated for 1-2 hr at 65°C, extracted with phenol:chloroform until the aqueous phase was clear, incubated with RNase A (10 mg/ml) to obtain a final concentration of 50 μg/ml at 37°C for 3 hr, re-extracted once with phenol:chloroform, and the DNA was precipitated with 0.03 vol. of 3 M sodium acetate and 0.6 vol. of 99% isopropanol at 4°C for at least 30 min. The DNA pellet was subsequently obtained by centrifugation for 20 min (14,000 x g, 25°C), rinsed with 70% ethanol, dried, and re-dissolved in either 1 x TE or deionized water. DNA solutions were stored at 4°C.

3.3.3 PCR and PCR conditions.

DNA was amplified using three 5' oligonucleotide primers, each one specific for each variety of *G. graminis*, and a single 3' universal primer. Sequences of Ggt-, Gga-, and Ggg-specific primers were 5'-TCCTCGGCCCCGTAATTGGC-3', 5'-ACGGCGGTGGATGGCAAGAC-3' and 5'-CACCCCCGGTCCCTGCGTAA-3' respectively. While the universal 3' primer was 5'-TGCTCATGGTGGTTCCTGC-3'. Approximate locations of these primers on the avenacinase genes are shown in Fig. 3.1. All oligonucleotide primers used in this study were synthesized by GIBCO BRL Custom Primers (Life Technologies Inc., Gaithersburg, MD 20877).

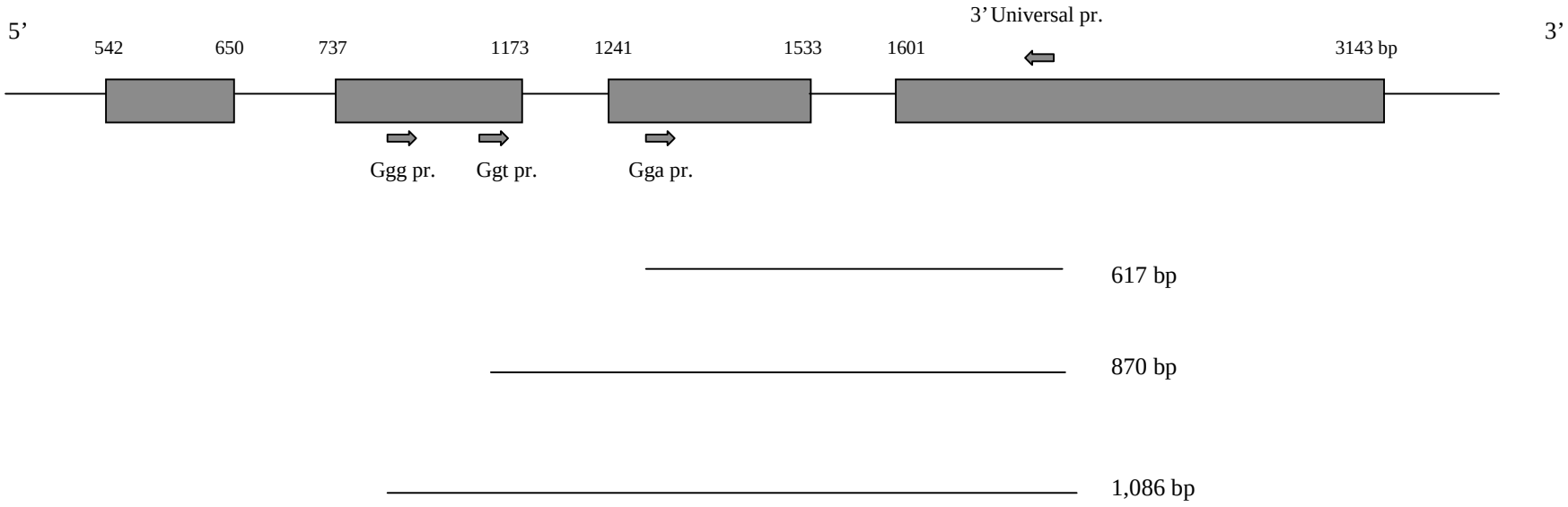


Fig. 3.1. Locations of varietiy-specific primers for *Gaeumannomyces graminis* var. *avenae* (Gga), var. *graminis* (Ggg), var. *tritici* (Ggt) flanking regions on the full-length avenacinase gene of Gga (Genbank accession number U35463). The filled boxes represent the coding regions and the lines connecting the boxes represent the non-coding regions of the gene. The arrows represent the approximate locations of the primers on the gene. PCR amplification with Gga-, Ggg-, and Ggt-specific primers and a 3’ universal primers give products of 617, 1,086, and 870 bp, respectively. See Fig. 3.2 for the exact position of the primers and the base composition of the primers.

To achieve high specificity and efficiency of amplification of each primer, optimization of PCR conditions such as $MgCl_2$ (1.5-9 mM) and annealing temperature (55-69°C) was always carried out with each variety-specific primer. For routine PCR assays, unless otherwise specified, each 50 µl reaction volume contained 50 pmol of each primer, 2.5 U of *Taq* DNA polymerase (Qiagen Inc. Valencia, CA 91355), 100 µm of each deoxynucleotide triphosphates, 1 x reaction buffer, 3 mM $MgCl_2$ and 50 ng of fungal DNA. Polymerase chain reactions 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, 68°C for 45 sec, and 72°C for 2 min. At the end of this incubation, an additional incubation for 10 min at 72°C was carried out to ensure complete extension of PCR products. PCR products were separated by electrophoresis (Small Horizontal Gel System, FB MSU-1, Fisher Biotech, Pittsburgh, PA, 15238) at 75 V for 1.5 hours in a 1.8% agarose gel (Ultrapure Agarose Electrophoresis Grade, Life Technologies, Inc., Gaithersburg, MD 20877) in 0.5 x TBE buffer (45 mM Tris-borate, 1 mMEDTA). Gels were later stained with ethidium bromide (0.5 µg/ml final concentration) for 15 min followed by destaining in deionized water, if necessary, and photographed with a Polaroid camera (with type 667 or 55 films) under 302 nm UV light using a Wratten 22A filter. Negative controls with no DNA template were included in all PCR experiments.

Verification of specificity of primers in PCR assay was carried out as described earlier with some modifications. If more than one primer was added to the reaction mixture, the concentration of each primer was reduced to 25 pmol of each primer in 50 µl reaction volume. If more than one template was added, the ratio of all templates was kept constant at 1:1. To demonstrate sensitivity of a variety-specific primer, PCR was carried out with a dilution series of fungal DNA template specific to each primer. Serial dilution of templates was done in sterile nanopure water and the concentration of total DNA used in PCR was ranged from 200 ng to 1 pg. PCR cycling parameters were as previously described.

In addition, differential titration of DNA templates was also carried out to demonstrate high sensitivity and specificity of all three variety-specific primers. In this experiment, a single reaction mixture contained all three variety-specific primers and two types of DNA templates (Ggt and Gga or Ggt and Ggg) were added. The ratio of two templates in each reaction was adjusted to represent a broad spectrum of possible proportions of the population of these fungi present in natural environments such as diseased tissues or in the soil. For PCR conditions, each 50 µl reaction volume contained 25 pmol of each variety-specific primer, 2.5 U of *Taq* DNA polymerase (Qiagen Inc., Valencia, CA 91355), 200 µM of deoxynucleotide triphosphates, 1x reaction buffer and 3 mM $MgCl_2$. Two types of DNA templates were added prior to cycling with the following ratios, 1:1, 1:10, 1:50, and 1:100 and vice versa where a ratio of 1 was always kept constant at 1 ng/µl. Polymerase chain reaction was carried out as described previously.

3.3.4 PCR-based test for *Gaeumannomyces graminis* varieties.

PCR-based test for differentiating *G. graminis* varieties was accomplished under the condition described earlier with slight modifications. In this experiment, each 50 µl reaction mixture contained 25 pmol of each variety-specific primer and 100 µM deoxynucleotide triphosphates. Polymerase chain reactions were performed as usual but only 30 cycles were done. PCR products were visualized by ehtidium bromide staining as described earlier. In case of ambiguity of the result as well as to prevent false-positive results, samples may be subjected to the second round of PCR with only a single variety-specific primer (50 pmol) added to the PCR cocktail.

3.3.5 DNA sequencing.

To confirm that each variety-specific fragment was the right fragment, fresh PCR products were directly cloned using TOPO™ TA Cloning® kit (Invitrogen Corporation, Carlsbad, CA 92008). Cloning and transformation of PCR products were carried out according to the manufacturer's protocol with slight modifications such that 35-75 µl of each transformation mixture was spread onto selective plates. White or light blue

colonies appeared on selective plates or positive clones were transferred to a second plate for further isolation of plasmid (Sambrooks et al., 1989). Restriction analysis of the plasmid with *Eco*RI was carried out for 1 h at 37°C and visualized on 0.8% agarose gel electrophoresis. In some cases, PCR analysis of positive clones was carried out. Each 25 µl reaction contained 10xPCR buffer, 4.5 mM $MgCl_2$, 200 µM dNTPs, 50 pmol of each primer and 2.5 U of *Taq* DNA polymerase (Qiagen Inc., Valencia, CA 91355). Positive colonies were picked directly from plates and individually resuspended in the PCR cocktail. PCR condition and detection of positive clones were performed as previously described. Plasmids of the size expected with the insert were purified (Qiaquick Gel Extraction Kit, QIAGEN, Inc., Valencia, CA 91355) and sequenced using a dye-terminator cycle sequencing reaction based on manufacturer's protocol (BigDye™ Terminator Cycle Sequencing, PE Applied Biosystem, Foster City, CA 94404). Sequencing was performed with an ABI Prism DNA sequencer model 373A Stretch (PE Applied Biosystem, Foster City, CA 94404). Sequence data were analyzed with the aid of the Lasergene Sequence Analysis Software (DNAStar Inc., Madison, WI 53715) and on-line basic BLAST search (BLAST 2.0) provided by National Center for Biotechnology Information (URL address: http://www.ncbi.nlm.nih.gov/BLAST).

3.4 RESULTS

3.4.1 Design and specificity of Ggt-, Gga-, and Ggg-specific primers.

Three variety-specific PCR primers were designed from DNA sequences of avenacinase-like genes of Ggt and Ggg and avenacinase gene of Gga. Although little sequence divergence among the genes of these fungi was found, it was prevalent enough to design specific primers for each variety. The rationale used in designing these primers was based on a single base difference at the 3' end of each 5' primer and a common universal primer flanking the 3' end of PCR product (Fig.3.2). Thus, each 5' primer can be designated as a variety-specific primer. With this strategy, PCR products amplified from Ggt-, Gga-, and Ggg-specific primers with 3' universal primer can be easily distinguished based on size differences which are 870, 617, and 1,086 bp, respectively (Fig. 3.1).

Several PCR conditions were designed to demonstrate the specificity of each primer. Results in Figs. 3.3 and, 3.4 (panels a and b) and 3.5 strongly confirmed that my Ggt-, Gga-, and Ggg-specific primers showed high specificity to their own DNA templates. Only a variety-specific PCR product was always observed regardless of numbers of primers used. Although more than one specific primer was added in PCR mixture, efficiency of amplification in each reaction was as high as that of a single template (Fig. 3.4 panels a and b). We did not observe any competition for templates from any combination of primers added, especially, with all three variety-specific primers (Fig. 3.4a). In addition, we did not see any non-specific product present in any of these PCR experiments and each experiment was repeated twice. Final confirmation was carried out as shown in Fig. 3.5. This experiment was set up to demonstrate the efficacy of the primers in a mixed DNA population of take-all pathogens. Undoubtedly, each variety-specific primer annealed to its own template as shown in lanes 2-4 in Fig. 3.5. When more than one 5'-primer was added to the reaction, two or three specific PCR fragments were observed depending on combination of primers used. However, competition of substrates such as dNTPs was observed with the mixed population resulting in less efficient amplification of longer PCR products, *i.e.*, Ggg- and Ggt-specific fragments (Fig. 3.5, lanes 5 and 8).

Fig. 3.2. Nucleotide sequence alignments of regions of avenacinase and avenacinase-like genes of *Gaeumannomyces graminis* var. a*venae* (Gga), var. *graminis* (Ggg), and var. *tritici* (Ggt), respectively, showing the design strategy for variety-specific primers and locations of primers on the gene. Nucleotide sequence of Gga was obtained from Genbank (accession number U35463). Variations in nucleotide sequence are indicated in red. Numbers at the beginning of each block represent base-pair position on the gene.

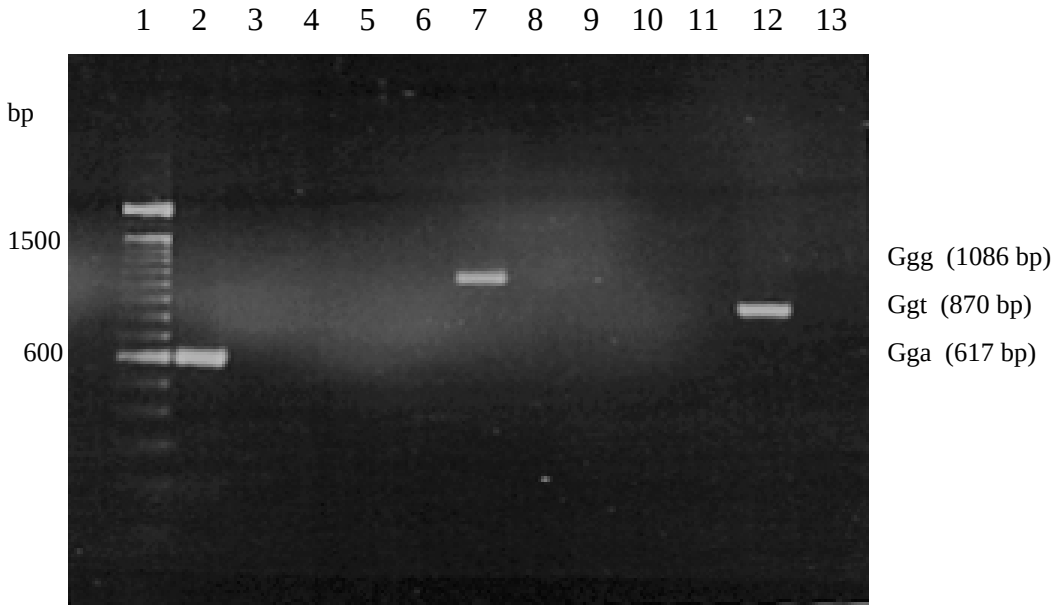
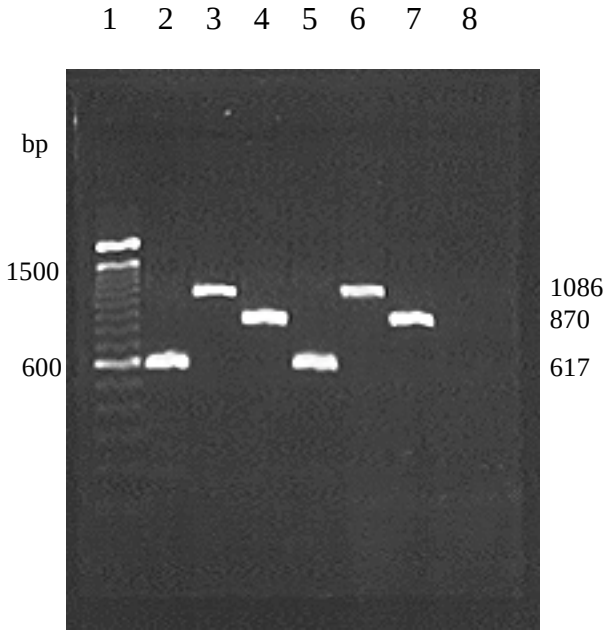
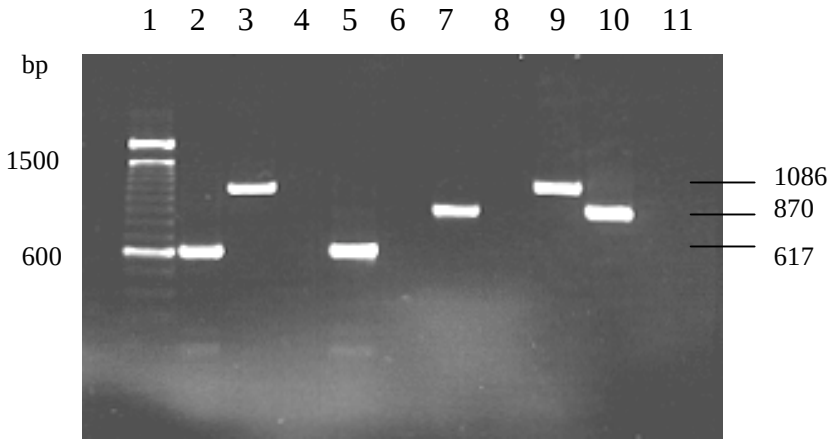


Fig. 3.3. Specificity of *Gaeumannomyces graminis* var. *tritici* (Ggt), var. *avenae* (Gga), and var. *graminis* (Ggg) variety-specific primers from PCR amplification of Ggt, Gga, and Ggg genomic DNA. Each lane contains a single template with a variety-specific 5' primer and the universal 3' primer. All templates were amplified with all types of primers. PCR products were electrophoresed on 1.8% agarose gel, stained with ethidium bromide, visualized and photographed with 302 nm UV light using a Wratten 22A filter. Lane 1 contains 100 bp DNA ladder as molecular weight standards. PCR products in lanes 2-5, 6-9, and 10-13 were loaded, respectively, in the following order; Gga, Ggg, Ggt and no DNA control. Lanes 2-5 are products with Gga-specific primer. Lanes 6-9 are products with Ggg-specific primer. Lanes 10-13 are products with Ggt-specific primer.



(a)

Fig. 3.4. panel (a). Amplification of genomic DNA of *Gaeumannomyces graminis* var. *tritici* (Ggt), var. *avenae* (Gga), and var. *graminis* (Ggg) with single and all three variety-specific primers in a single PCR reaction. PCR products were electrophoresed on 1.8% agarose gel, stained with ethidium bromide, visualized and photographed with 302 nm UV light using a Wratten 22A filter. Lane 1 contains 100 bp DNA ladder as molecular weight standards. Lane 2 contains genomic DNA of Gga amplified with Gga-specific primer. Lane 3 contains genomic DNA of Ggg amplified with Ggg-specific primer. Lane 4 contains genomic DNA of Ggt amplified with Ggt-specific primer. Lanes 5-8 contain genomic DNAs of Gga, Ggg, Ggt, and no DNA control, respectively, amplified with combined Gga-, Ggg-, and Ggt-variety specific primers.



(b)

Fig. 3.4 panel (b). Amplification of genomic DNA of *Gaeumannomyces graminis* var. *tritici* (Ggt), var. *avenae* (Gga), and var. *graminis* (Ggg) with pairwise combination of Ggt-, Gga-, and Ggg-variety specific primers in a single PCR reaction. PCR products were, electrophoresed on 1.8% agarose gel, stained with ethidium bromide, visualized and photographed with 302 nm UV light using a Wratten 22A filter. Lane 1 contains 100 bp DNA ladder as molecular weight standards. Lanes 2-4 contain genomic DNA of Gga, Ggg, and Ggt, respectively, amplified with combined Gga- and Ggg-specific primers. Lanes 5-7 contain genomic DNA of Gga, Ggg, and Ggt, respectively, amplified with combined Gga- and Ggt- specific primers. Lanes 8-10 contain genomic DNA of Gga, Ggg, and Ggt, respectively, amplified with combined Ggg- and Ggt-specific primers. The reaction mixture in lane 11 contained no DNA.

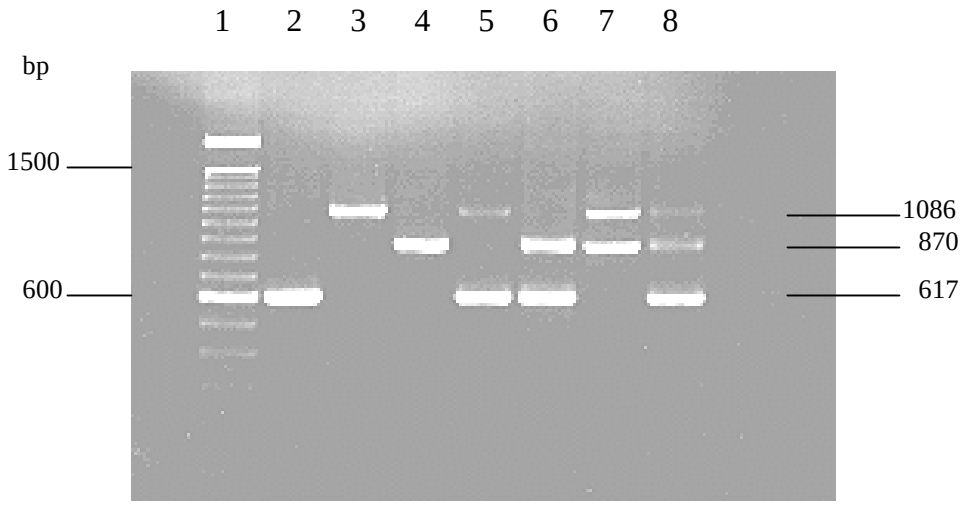


Fig. 3.5. Amplification of mixed DNA populations of *G. graminis* with *Gaeumannomyces graminis* var. *tritici* (Ggt), var. *avenae* (Gga), and var. *graminis* (Ggg) variety-specific primers to confirm sensitivity of primers. Genomic DNA of Ggt, Gga and Ggg were used as templates in all lanes with a ratio of 1:1:1. Single, double and triple combinations of primers were tested as follows. PCR products were electrophoresed on 1.8% agarose gel, stained with ethidium bromide, visualized and photographed with 302 nm UV light using a Wratten 22A filter. Lane 1 contains 100 bp DNA ladder as molecular weight standards. Lanes 2 contains PCR product from Gga-specific primer. Lane 3 contains a product from Ggg-specific primer. Lane 4 contains a product from Ggt-specific primer. Lane 5 contain the products from Gga + Ggg- specific primers. Lane 6 contain the products from Gga + Ggt- specific primers. Lane 7 contain the products from Ggg + Ggt- specific primers. Lane 8 contains the products from all three variety-specific primers.

3.4.2 Sensitivity of PCR-based differentiation test.

Fig. 3.6 panels a, b, and c shows titration of genomic DNA of Gga, Ggg, and Ggt with its variety-specific primer. According to the results obtained, Gga-specific primer exhibited the highest sensitivity among all of the primers in which PCR products were obtained from template DNA as low as 50 pg. Since PCR product generated from Gga-specific primer is the smallest specific fragment generated in this study (617 bp), it is reasonable that the sensitivity of PCR would be greater than that of Ggg and Ggt. While the lowest concentration of DNA templates that generated products with Ggg- and Ggt-specific primers was 100 pg although I anticipated higher sensitivity from Ggt-specific primer.

We also conducted sensitivity assays with mixed populations of Ggt, Gga, and Ggg DNA (Figs. 3.7 and 3.8). In Fig. 3.7, genomic DNA of Ggt was titrated against Gga with all three variety-specific primers added to each reaction and results showed that efficiency of amplification favored template of higher concentration. Little or no PCR product was generated from Gga template when it was present in an abundance of Ggt template, (lanes 2-5) and vice versa (lanes 9-11). However, when both species of template DNA were present at the same concentration, shorter products were more prevalent. Similar results were observed in Fig. 3.8 when genomic DNA of Ggt was titrated against Ggg. In this case, when DNA of Ggg was presented at low concentration, no products were generated (lanes 2-5) while, in opposite reactions, little product was observed (lanes 8-11). This was due to the fact that the shorter fragment was more efficiently amplified than longer fragment.

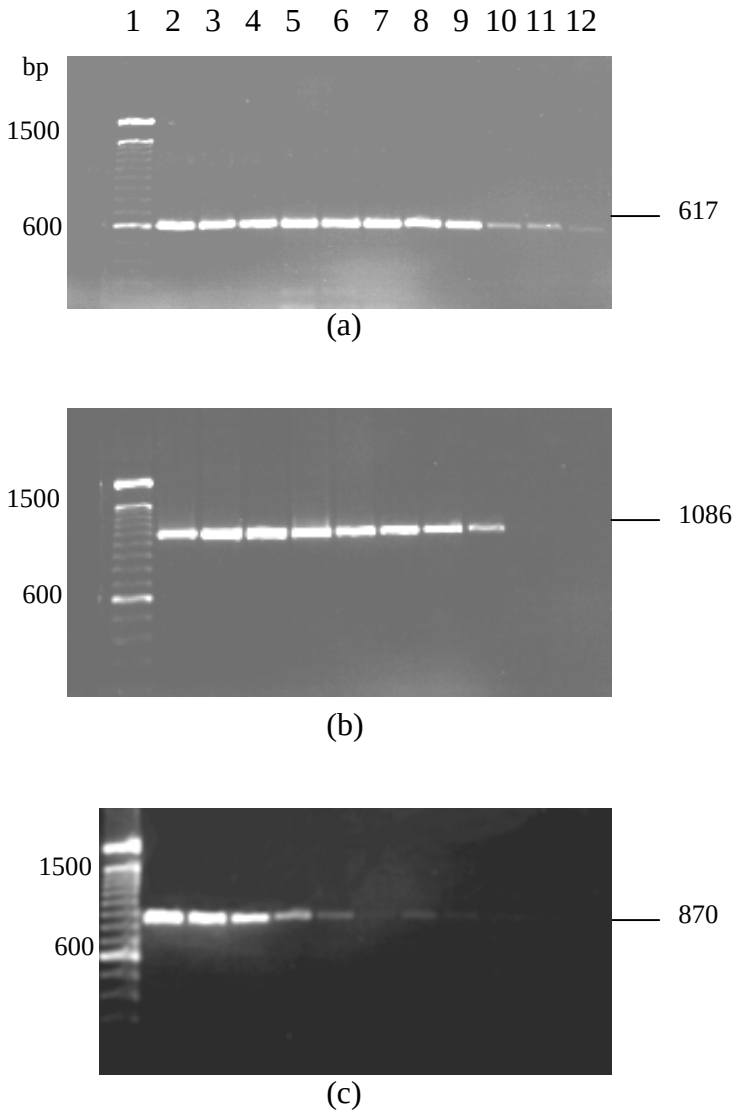


Fig. 3.6. PCR titration of *Gaeumannomyces graminis* var. *avenae* (Gga) (panel a), var. *graminis* (Ggg) (panel b), and var. *tritici* (Ggt) (panel c) genomic DNA with a variety-specific primer in each panel to show sensitivity of amplification. PCR products were electrophoresed on 1.8% agarose gel, stained with ethidium bromide, visualized and photographed with 302 nm UV light using a Wratten 22A filter. In all panels, lane 1 contains 100 bp DNA ladder as molecular weight standards. Lanes 2-12 are PCR products generated from DNA templates ranging from 200ng, 100ng, 50ng, 10ng, 5ng, 1ng, 0.5ng, 0.1ng, 50 pg, 10 pg, and 5 pg, respectively.

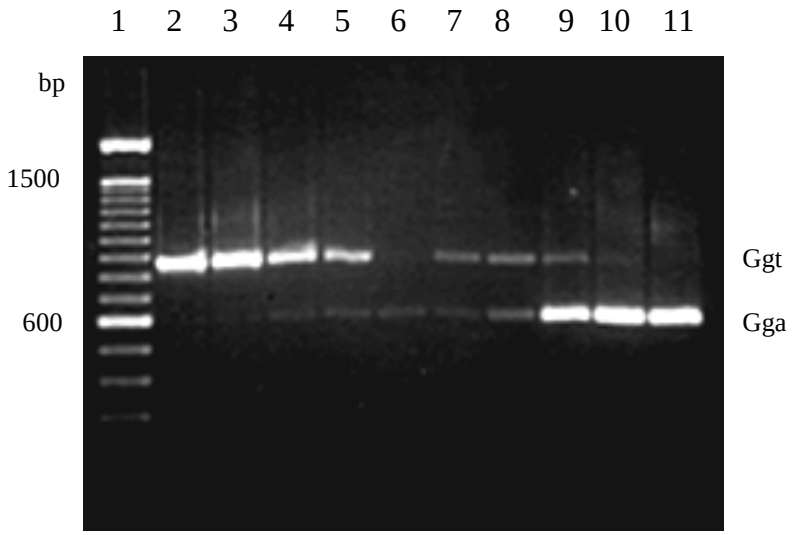


Fig. 3.7. Differential titration of genomic DNA templates between *Gaeumannomyces graminis* var. *tritici* (Ggt) and var. *avenae* (Gga) mimicking possible ratios of these fungi in natural environment. Each lane contains genomic DNA of Ggt and Gga in differential ratios ranging from 1:1, 1:5, 1:10, 1:50 and 1:100 where 1 equals to 1 ng of DNA. PCR products were electrophoresed on 1.8% agarose gel, stained with ethidium bromide, visualized and photographed with 302 nm UV light using a Wratten 22A filter. Lane 1 contains 100 bp DNA ladder as molecular weight standards Lanes 2-6 are PCR products from differential concentration of Ggt templates with constant Gga templates with ratios (Ggt:Gga) ranging from 100:1, 50:1, 10:1, 5:1, and 1:1, respectively. Lanes 7-11 are the reverse of lanes 2-6 with ratios (Ggt:Gga) ranging from 1:1, 1:5, 1:10, 1:50 and 1:100, respectively.

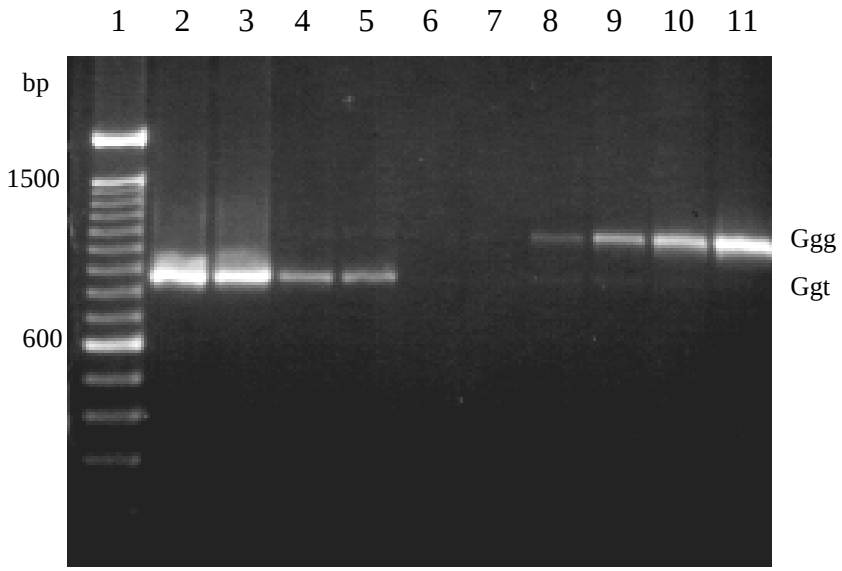


Fig. 3.8. Differential titration of genomic DNA templates between *Gaeumannomyces graminis* var. *tritici* (Ggt) and var. *graminis* (Ggg) mimicking possible ratios of these fungi in natural environment. Each lane contains genomic DNA of Ggt and Ggg in differential ratios ranging from 1:1, 1:5, 1:10, 1:50 and 1:100 where 1 equals to 1 ng of DNA. PCR products were electrophoresed on 1.8% agarose gel, stained with ethidium bromide, visualized and photographed with 302 nm UV light using a Wratten22A filter. Lane 1 contains 100 bp DNA ladder as molecular weight standards. Lanes 2-6 are PCR products from differential concentration of Ggt templates with constant Ggg templates with ratios (Ggt:Ggg) ranging from 100:1, 50:1, 10:1, 5:1, and 1:1, respectively. Lanes 7-11 are the reverse of lanes 2-6 with ratios (Ggt:Ggg) ranging from 1:1, 1:5, 1:10, 1:50 and 1:100, respectively.

3.4.3 PCR-based test for differentiating *Gaeumannomyces graminis* varieties.

All isolates of *G. graminis* and related fungi test in this study are listed in Table 3.1. When DNA of all Ggt isolates were amplified with only Ggt-specific primer, PCR products were obtained from M1, 6 Virginia isolates and 2 isolates from other regions. No Ggt-specific fragments were generated from ATCC isolate 28230, Virginia isolate CHe1 and CK1a, 3056, 3060 and 3066 (panel a of Figs. 3.9 and 3.10). Those isolates were then subjected to the diagnostic PCR containing all of the three variety-specific primers in a single reaction. Interestingly, all of the isolates that gave negative result with Ggt specific primer strongly produced Ggg-specific PCR fragments (panel b of Fig. 3.9 and 3.10). All of these isolates efficiently produced Ggg-specific fragments, when amplified with only Ggg-specific primer (data not shown). These results corresponded well with our previous study on DNA polymorphisms of the avenacinase-like genes of these isolates (Chapter 2). Previous study on pathogenicity of isolates ATCC 28230, CHe1 and CK1a on wheat seedlings also strengthened our results because these isolates were described as weakly virulent or non-pathogenic on wheat (Crozier, personal communication). Although these isolates were isolated from colonized wheat tissues, it is very convincing that they are more likely to be Ggg rather than Ggt as previously suspected. Results in Fig. 3.10 a and b also demonstrated that our diagnostic PCR was very specific to only varieties of *G. graminis*. No PCR product was detected from samples containing DNA of *G. cylindrosporus, P. radicicola*, which has been considered an anamorph of Ggg, *Phialophora* sp. and *C. zea-maydis*. This suggested that the diagnostic PCR was specific.

Similar PCR-based differentiation tests were achieved with all of Gga and Ggg isolates listed in Table 1. All of Gga isolates produced Gga-specific fragments when amplified with Gga-specific primer alone (Fig. 3.11, panel a). Unexpectedly, when all three primers were added to each sample, one isolate, RB-W, produced 2 fragments of 870 and 617 bp specific for Ggt and Gga, respectively, while others showed strong identity of Gga (Fig. 3.11, panel b). Ggg isolates other than those isolated from wheat did not amplify efficiently with Ggg-specific primers (Fig. 3.12, panel a). Ggg isolates FL-39, FL-175

and 2033 produced little Ggg-specific product when amplified with both single Ggg-specific primer and all of the three variety-specific primers (Fig. 3.12, panel b). Other non-specific products were visualized from all of them. Ggg isolate FL-19, which was isolated from bermudagrass generated a PCR product of 300 bp that was not related to any of variety-specific fragments. In this case, more Ggg isolates from wheat and other host plants are needed before conclusions can be made.

Confirmation of identities of Ggt-, Gga-, and Ggg-specific PCR fragments was performed by sequencing of these PCR fragments and comparing their avenacinase DNA sequences separately. DNA sequence alignment demonstrated that both of Ggt- and Ggg-specific fragments were 100% homology to their avenacinase-like genes. However, the Gga-specific fragment showed slight divergence to the published sequence of avenacinase gene. This is not unexpected since the Gga isolate used in this study was not the same isolate originally used by Osbourn et al. (1994).

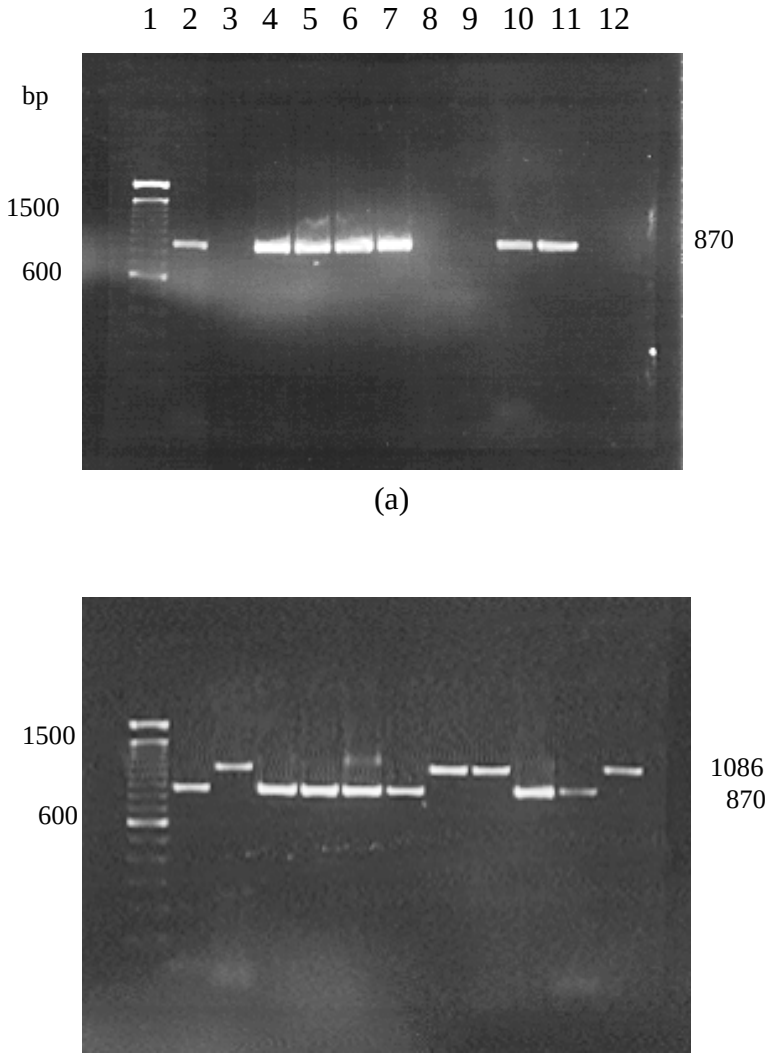


Fig. 3.9. PCR-based differentiation test for Virginia isolates of *Gaeumannomyces graminis* var. *tritici* (Ggt) from wheat with Ggt-variety specific primer (panel a) and all three variety-specific primers (panel b). PCR products were electrophoresed on 1.8% agarose gel, stained with ethidium bromide, visualized and photographed with 302 nm UV light using a Wratten 22A filter. Lane 1 contains 100 bp DNA ladder as molecular weight standards. For both panels, lanes 2-11 contain isolates M1, ATCC 28230, CB1, CD1, CE1, CH1, CHe1, CK1a, CK1b and CS1. Lane 12 present only in panel b contains *G. graminis* var. *graminis* ATCC 12761 as a control.

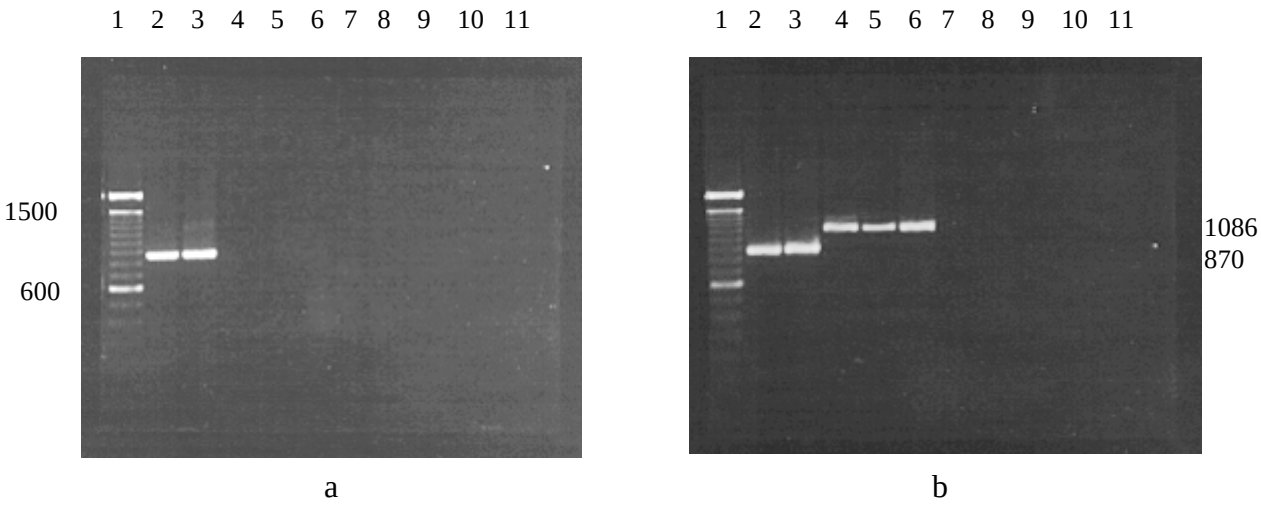


Fig. 3.10. PCR-based differentiation test for isolates of *Gaeumannomyces graminis* var. *tritici* (Ggt) and other closely related fungi with Ggt-specific primer (panel a) and all three variety-specific primers (panel b). PCR products were electrophoresed on 1.8% agarose gel, stained with ethidium bromide, visualized and photographed with 302 nm UV light using a Wratten 22A filter. Lane 1 contains 100 bp DNA ladder as molecular weight standards. For both panels, lanes 2-6 are Ggt isolates 3053, 3055, 3056, 3060, and 3066, respectively. Lanes 7-11 are *Gaeumannomyces cylindrosporus* ATCC 64420, *Phialophora radicicola* ATCC 64414, *Phialophora sp.*, *Cercospora zea-maydis* and no DNA template.

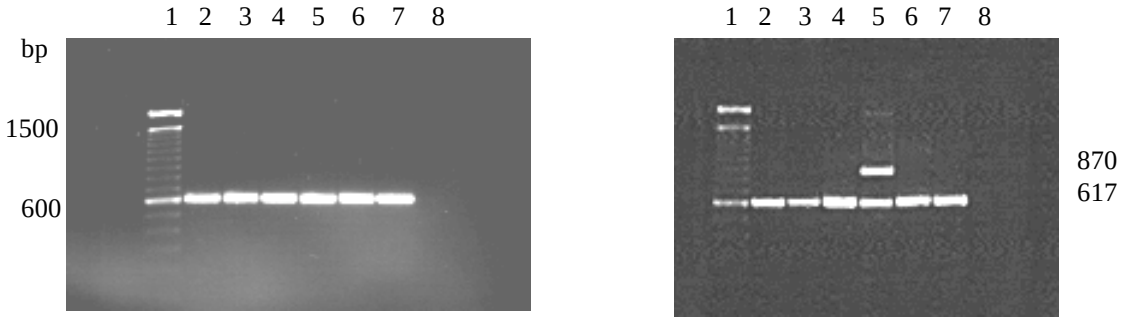


Fig. 3.11. PCR-based differentiation test for *Gaeumannomyces graminis* var. *avenae* (Gga) isolates with Gga-specific primer (panel a) and all three variety-specific primers (panel b). PCR products were electrophoresed on 1.8% agarose gel, stained with ethidium bromide, visualized and photographed with 302 nm UV light using a Wratten 22A filter. Lane 1 contains 100 bp DNA ladder as molecular weight standards. For both panels, lanes 2-8 are Gga isolates ATCC 15419, FR-W, PG-W, RB-W, RI-W, WW-W and no DNA respectively.

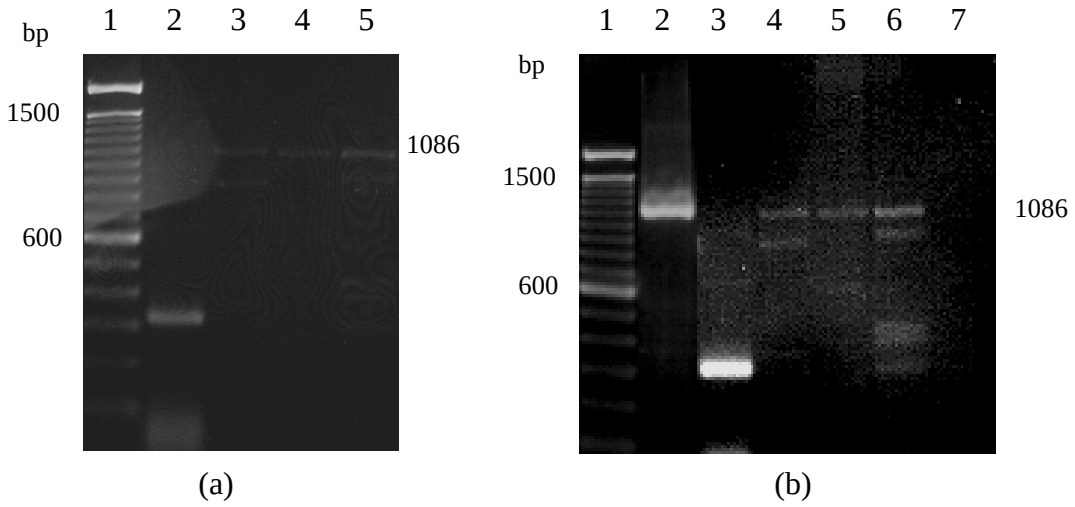


Fig. 3.12. PCR-based differentiation test for *Gaeumannomyces graminis* var. *graminis* (Ggg) isolates with Ggg-specific primer (panel a) and all three varieties-specific primers (panel b). PCR products were electrophoresed on 1.8% agarose gel, stained with ethidium bromide, visualized and photographed with 302nm UV light using a Wratten 22A filter. In both panels, lane 1 contains 100 bp DNA ladder as molecular weight standards. In panel (a), lanes 2-5 contain Ggg isolates FL-19, FL-39, FL-175, and 2033, respectively. In panel (b), lanes 2-7 contain Ggg isolates ATCC 12761, FL-19, FL-39, FL-175, 2033 and no DNA, respectively.

3.5 DISCUSSION

PCR technology has played a major role as a sensitive method for identification of plant pathogens. It offers a number of advantages over conventional methods of identification. Several groups have developed a PCR-based tests for differentiation of varieties of *G. graminis*, however, almost all of them were based on amplification of the conserved regions of the intergenic spacer regions of ribosomal DNA with either specific or random primers (Bryan et al., 1995; Fouly et al., 1996; Goodwin et al., 1995, and Wetzel et al., 1996). In contrast, we have shown that by using a single gene as a target for PCR amplification, our PCR-based identification test is simple and specific for each variety of the take-all pathogen. It is the first successful PCR-based test for fungal pathogens without exploitation of high abundance of ribosomal DNA, mitochondrial DNA and DNA probe hybridization.

Specificity of PCR identification of Ggt, Gga, and Ggg is based on critical nucleotide sequence differences among the genes encoding avenacinase or avenacinase-like proteins. Three upstream primers, annealing closer to the 5' end of the genes, provide variety-specificity. A single universal downstream primer, closer to the 3' end of the gene, was paired with each of the variety-specific primers. Variety-specific primers were designed to incorporate three to four mismatched bases compared to the sequences of the non-target varieties. Most important to specificity was a mismatch at the 3' end of each primer, which prevents DNA polymerase extension during PCR. Primer annealing efficiency was manipulated and specificity was increased by including two to three other variety-specific base mismatches in each primer (experiments not shown). The sequence of the universal primer was based on sequence completely conserved among all three varieties. Bryan et al. (1995) successfully used this rationale to design primers for Gga and Ggt from the internal transcribed spacer regions (ITS) of the ribosomal DNA of the fungi. However, Ggt and Gga isolates obtained from different geographical regions were placed into several subgroups instead of being identified as a single group due to minor sequence divergence within a variety at the primer annealing site. This strategy has also been useful for detection of point mutations within the gene. PCR-based characterization of resistance of benomyl in *Venturia inaequalis* (Cooke) Wint., causal agent of apple

scab, is based on a single base pair mutation in the β-tubulin gene in combination with allele-specific oligonucleotide probe replaces conventional DNA sequencing analysis (Koenraadt and Jones, 1994).

An important feature of our differential identification test is that all three variety-specific primers may be used in a single PCR reaction. If there are multiple bands present on the gel, that sample will be further tested with each individual primer to prevent false-positive results. It is worth noting that some fungi isolated from wheat tissues showing characteristic symptoms of take-all turned out to be Ggg, a common saprophyte of wheat. It is difficult to differentiate between Ggt and Ggg from cultural appearance, especially since both of them grow on selective medium for Ggt. Use of ascospore size and shape of hyphopodia has been unreliable as variations of these characteristics are common and many *G. graminis* isolates were placed under the intermediate group (Ward and Akrofi, 1994; Yeates, 1986). For instance, Ggt isolate ATCC 28230 also produced Ggg specific PCR product and the result was confirmed several times. This fungus was isolated from the *Gaeumannomyces-Phialophora radicicola* complex since 1974 and identification was based on morphological, physiological and pathological assays (Deacon, 1974). Hyphopodia production of all Ggt isolates producing Ggg-specific PCR products was subsequently carried out in our laboratory and none produced lobed hyphopodia. Since Ggg can produce both lobed and non-lobed hyphopodia (Deacon, 1981; Nilsson, 1972 and Henson personal communication), it is very possible that the ATCC isolate was mis-identified as Ggg.

Other closely related fungi such as *G. cylindrosporus*, *Phialophora* spp., and *P. radicicola* failed to amplify with any of the three variety-specific primers demonstrating that all of the primers are specific. This is one of the most important criteria for a test since these fungi are present together in the soil as a *Gaeumannomyces-Phialophora* complex (Deacon, 1974; Deacon, 1981). The *Gaeumannomyces-Phialophora* complex also includes other morphologically similar fungi that are non-pathogenic on cereal roots (Walker, 1981; Ward and Akrofi, 1994). *P. radicicola* produces lobed hyphopodia while *Phialophora* spp. produces both lobed and simple hyphopodia which cannot be

distinguished from those of Ggg. Further confirmation with more isolates of Ggt, Gga, Ggg, *Phialophora* spp. and other closely related fungi such as *G. incrustan* and *Magnaporthe* spp. need to be conducted to confirm specificity of these primers.

The results obtained using the variety-specific primers indicate possible genetic differences among populations. The Gga- and Ggt-specific PCR products recovered from diverse isolates indicated a great deal of genetic homology among Gga and Ggt populations except for Gga isolate RB-W (see below). However, results with Ggg-specific primers indicated a significant amount of variation among the PCR products obtained from diverse Ggg isolates. Three possibilities exist for this result. First, the variation may mirror genetic variation in the avenacinase-like genes of Ggg. Perhaps, this gene is either inactive or not conserved by functions in host pathogenicity in Ggg. Second, because Ggg may cause pathogenesis on several grassy weeds, the variation may reflect co-evolution with different compatible hosts. Perhaps, the Ggg-specific primer may be used to separate these pathogenic groups. Third, the variation may indicate sub-optimal priming from other parts of the genome. DNA sequencing of the atypical PCR products will confirm if they have similarity to avenacinase-like genes.

PCR products recovered from Gga isolate RB-W are more complex. Using all three variety-specific primers, Ggt- and Gga-specific products were observed. When individual variety-specific primers were used, expected PCR products were recovered from Gga- and Ggg-specific primers: Gga-specific product and no product, respectively. When Ggt-specific primer was used, surprisingly, only a Ggt-like product was recovered. Three possible explanations exist. First, isolate RB-W may not be a pure culture although it was purified by hyphal tipping. Second, isolate RB-W may be a heterokaryon produced by anastomosis among *G. graminis* varieties. Differences in band intensity may indicate the relative “dose” of nuclei. In other experiments in our laboratory, anastomosis among fungicide-resistant mutants of Ggt apparently occurs. Third, the variation in isolate RB-W may indicate sequence differences with sub-optimal priming accounting for the various products. Further purification of the culture and sequencing of the products may determine the correct hypothesis.

Sensitivity of detection of PCR with multi-copy target sequences is believed to be higher than with a single copy target sequence such as avenacinase gene (Henson and French, 1993). Detection level for our diagnostic test was 100 pg for Ggt and Ggg, and 50 pg for Gga in a single population DNA sample. In mixed DNA populations, sensitivity of PCR was decreased (Figs. 3.7 and 3.8) to 1 ng of total DNA of each variety. These levels are adequate for direct detection of the presence of the fungi in diseased tissues and soil samples according to Herdina et al. (1996). These authors were able to detect Ggt directly from diseased wheat roots and infested soil samples using slot-blot hybridization with a mitochondrial DNA probe. However, the detection level was as high as 1 and 0.3 ng in roots and soil samples, respectively. In contrast, Hu et al. (1993) showed that, with autoradiography, detection level of DNA of *Verticillium albo-atrum* (Rienke & Berthold) from inoculated alfalfa (*Medicago sativa* L.) by amplification of the intergenic spacer regions of rDNA was down to 10 pg.

Although our PCR-based differentiation test provides several advantages over previous tests, it is still in the first stage of development. Additional experiments are needed to develop this test for plant tissue and soil applications. We hope that, in the near future, this test will provide a breakthrough in diagnosis of the take-all pathogens due to the simplicity and reliability of the assay. Its applications could result in a better means for prediction and prevention of the disease.

3.6 ACKNOWLEDGMENT

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CHAPTER 4

STUDY OF MANGANESE OXIDIZING ABILITY OF *Gaeumannomyces graminis* VARIETIES

4.1 ABSTRACT

Isolates of *G. graminis* var. *tritici* (Ggt), var. *avenae* (Gga) and var. *graminis* (Ggg) were tested for the ability to oxidize manganese in medium containing 50 and 100 mg $MnSO_4$ per liter. All Virginia isolates of Ggt were able to oxidize manganese but rates of oxidation varied among isolates. The color of manganese oxides observed in the agar media differed among Ggt, Ggg, and Gga isolates. Based on manganese oxidation scores, isolates were divided into four groups. The first group included the strong manganese oxidizers, Ggt isolates CD1, CE2, CK1a, CS1, and Ggg ATCC 12761. The second group contained the moderate manganese oxidizers, Ggt isolates M1, CB1, CK1b and CO1. The third group contained all but one of the Gga isolates and was considered weak manganese oxidizers. Group four, the non-manganese oxidizers, contained Ggt isolates ATCC 28230 and CE1 and the remaining Gga isolate, W4W-W. Concentrations of $MnSO_4$ used in this study did not affect growth of Ggt and Ggg but higher concentration of $MnSO_4$ (750 mg/L) reduced Gga growth. Microscopic examination of the manganese oxide precipitates in the agar of Ggt and Ggg showed that they were more abundant along the hyphae rather than at a distance from hyphae. With Gga, manganese oxides precipitates were scattered and not correlated with hyphae. Auto-oxidation of $MnSO_4$ was not observed in the control plates.

4.2 INTRODUCTION

Micronutrients such as manganese, iron, and zinc play an important role in host-pathogen interaction and have significant impact in the etiology of a wide range of economically important bacterial and fungal plant diseases (Graham, 1983; Schulz et al., 1995). Manganese is required as an activator for a wide variety of biochemical reactions in plant cells, especially in the shikimic pathway whose intermediates and products such as phenylalanine ammonia lyase, flavonoids, and lignin are associated with disease resistance mechanisms (Huber and McCay-Buis, 1993). Soils deficient in manganese result in reduced photosynthesis, inefficient nitrogen metabolism, and restricted defense responses by plants. Biological oxidation and reduction of manganese in soil by microorganisms dynamically transforms availability of manganese for plants. In soils, manganese can exist in several oxidation states; Mn^{2+}, Mn^{3+}, or Mn^{4+}. The Mn^{2+} ion is soluble and can be taken up from soil by plants while Mn^{4+} is insoluble and precipitates as various oxide and hydroxide minerals (Marscher et al., 1991; Schulze et al., 1995). On the other hand, Mn^{3+} serves as a redox mediator in the oxidation of a large number of phenolic substrates (Glenn and Gold, 1985).

Availability of manganese in soil influences the susceptibility of wheat (*Triticum aestivum* L.) to take-all, which is caused by *Gaeumannomyces graminis* (Sacc.) Arx & Olivier var. *tritici* Walker (Graham and Rovira, 1984; Wilhelm et al., 1990). Ability to oxidize manganese of *G. graminis* has been studied in culture and soil. Assessment of the manganese oxidizing ability was confirmed by the presence of brown manganese oxide precipitates in the agar medium containing $MnSO_4$. Graham and Rovira (1984) found that isolates of *G. graminis* var. *tritici* (Ggt) that oxidize manganese were highly virulent and isolates that failed to oxidize manganese were weakly virulent or non-pathogenic on wheat. *G. graminis* var. *graminis* (Ggg) is capable of oxidizing manganese but the pattern of oxidation differed from that of Ggt (Huber and McCay-Buis, 1993). Manganese oxidation by Ggg mainly occurred around lobed hyphopodia and related infection structures while highly virulent isolates of Ggt oxidized manganese at a distance from the mycelium. In diseased plants, manganese oxide precipitates were

observed in cortical cells and root hairs of plants as well as along runner hyphae of Ggt. Since manganese is oxidized in advance of Ggt hyphae rather than locally, manganese oxidation is thought to be a component of virulence of Ggt (Huber and McCay-Buis, 1993). By using X-ray absorption near edge structure (XANES) spectroscopy, Schulze et al. (1995) confirmed that Ggt oxidizes Mn^{2+} to insoluble Mn^{4+} which was localized around dark colonized wheat roots. In addition, a decrease capacity to oxidize manganese is correlated with a decrease in Ggt virulence and may result in take-all decline (Rengel, 1997).

Studies on the ability of Ggt to oxidize manganese are only at a preliminary stage. We know that Ggt and Ggg can oxidize manganese and it can be correlated with virulence of these fungi on wheat. However, the role for manganese oxidizing ability of *G. graminis* during pathogenesis is still hypothetical. In order to have better understanding on the role of manganese in host-pathogen interaction, studies on the molecular aspects of manganese oxidation need to be conducted. In this chapter, the ability of Ggt, Ggg, and Gga to oxidize manganese in culture media was investigated. This is the first study on manganese oxidation ability by Gga. Differences in manganese oxidation behaviors among these fungi were observed. However, the oxidation states of manganese have not been determined to confirm the differences. Although the experiments performed shared some similarities with previous work, the objectives of the study were not the same. This study was a preliminary step in an attempt to isolate genes involved in manganese oxidation by *G. graminis*. Information gained about manganese oxidation at the nucleic acid level may be combined with the information about the avenacinase gene for further development of a better PCR-based diagnostic test. Moreover, molecular biological studies on the regulation of gene expression will provide insight into pathogen virulence as well as the interaction of host and pathogens.

4.3 MATERIALS AND METHODS

4.3.1 Fungal isolates and maintenance

All fungi used in this study were listed in Table 4.1. All isolates were grown on ¼ x potato dextrose agar (¼ x PDA; 6 g of potato dextrose broth and 1.5% w/v agar per liter) at 25°C and stored at 4°C. No more than three successive transfers were made to preserve pathogenicity. Isolates other than those from American Type Culture Collection (ATCC, Manassas, VA 20110-2209) were purified by single hyphal tip isolation (Appendix A) prior to the experiment. For long-term storage, mycelial plugs (8-mm dia.) were taken from the colony margin using a no. 3 cork borer and stored in 15% glycerol at -80°C.

4.3.2 Sensitivity of *G. graminis* varieties to $MnSO_4$

Isolates of *G. graminis* used in this study were Gga ATCC 15419, Ggg ATCC 12761, and Ggt M1. Effects of $MnSO_4$ on growth of *G. graminis* was determined by growing of the fungus on ¼ x PDA amended with a series of $MnSO_4$ solutions (1g/ml $MnSO_4.H_2O$ stock solution) which were added to final concentrations ranging from 0 to 3000 mg /L. Each plate (30-mm dia.) was inoculated at a center with a 5-mm dia. mycelial plug (no. 2 cork borer) taken from the edge of the fungal colony. Plates were incubated in the dark for 10 days at 25°C. Colony growth was determined after 5 days by calculating colony area using the average of two colony diameter measurements taken at right angles to each other. The optimum concentration of $MnSO_4$ to be used in study of manganese oxidizing ability of the fungi will be determined based on the largest colony size. Each experiment was repeated twice with three replicates done for each isolate and concentration.

4.3.3 Manganese oxidizing ability of *G. graminis*

To determine the manganese oxidizing ability of *G. graminis*, all isolates were grown on ¼ x PDA amended with $MnSO_4$ solution to obtain a final concentration of 50 and 100 mg

of $MnSO_4$/L. Each plate (90-mm dia.) was inoculated at a center with an 8 mm mycelial plug (no. 3 cork borer) taken from the edge of a fungal colony. Plates were incubated in the dark for 10 days at 25°C. Colony growth and manganese oxidation were observed every other day during the incubation period. Colony areas were determined as described earlier. Manganese oxidizing ability was assessed visually by the presence and intensity of brown precipitates (of manganese oxides) formed in the agar. Each experiment was repeated three times with duplicate plates done for each isolate and concentration. Manganese oxidizing ability of the isolates was scored on a scale of 0-5 (0 = no oxidation and 5 = greatest oxidation) based on the intensity of brown precipitates formed in the agar. Inoculated control plates were obtained from ¼ x PDA without $MnSO_4$ amendment. To eliminate the possibility of autooxidation of $MnSO_4$, uninoculated ¼ x PDA plates amended with the same concentrations of $MnSO_4$ as described earlier were incubated under the same condition as of the test plates. These plates were observed at the end of the experiment.

Table 4.1. Origin of the isolates of *Gaeumannomyces graminis* used.

Isolate	Host	Source location	Source/reference
G. graminis var. *tritici*			
CB1	*Triticum aestivum* L.	Bedford Co., VA, USA	J.B. Crozier[1]
CD1	*T. aestivum*	Dinwiddie Co., VA, USA	J.B. Crozier
CE1	*T. aestivum*	Essex Co., VA, USA	J.B. Crozier
CE2	*T. aestivum*.	Essex Co., VA, USA	J.B. Crozier
CK1a	*T. aestivum*	King and Queen Co., VA, USA	J.B. Crozier
CK1b	*T. aestivum*	King and Queen Co., VA, USA	J.B. Crozier
CO1	*T. aestivum*.	Orange Co., VA, USA	J.B. Crozier
CS1	*T. aestivum*	Southampton Co., VA, USA	J.B. Crozier
M1	*T. aestivum*.	Montana, USA	D. Mathre[2]
ATCC 28230	*T. aestivum*	United Kingdom	ATCC[3]
G. graminis var. *avenae*			
ATCC 15419	*Avena sativa* L.	United Kingdom	ATCC
BM-W	*Agrostis stolonifera* L.	Maryland, USA	H.C. Wetzel III[4]
FR-W	*A. stolonifera*	France	H.C. WetzelIII[4]
PG-W	*A. stolonifera*	Pinegrove, Canada	H.C. WetzelIII
RB-W	*A. stolonifera*	Delaware, USA	H.C. WetzelIII
RI-W	*A. stolonifera*	Rhode Island, USA	H.C. WetzelIII
W4W-W	*A. stolonifera*	Washington, USA	H.C. WetzelIII
G. graminis var. *graminis*			
ATCC 12761	*T. aestivum*	United Kingdom	ATCC

[1] Mr. James Brooks Crozier, Department of Plant Pathology, Physiology and Weed Science, Virginia Polytechnic Institute and State University, Blacksburg, VA 24061-0331

[2] Dr. Don E. Mathre, Department of Plant Sciences, Montana State University-Bozeman, Bozeman, MT 59717-3150.

[3] American Type Culture Collection, 10801 University Boulevard, Manassas, VA 20110-2209

[4] Mr. Henry C. Wetzel III, Department of Plant Pathology, Kansas State University, Manhattan, KS66506-5502.

4.4 RESULTS

4.4.1 Sensitivity of *G. graminis* varieties to $MnSO_4$

As shown in Fig. 4.1, increasing concentrations of $MnSO_4$ did not affect growth of Ggt and Ggg until the medium was amended with more than 2000 mg of $MnSO_4$/L. Gga grew well at lower concentrations of $MnSO_4$. After the concentration of $MnSO_4$ was increased above 500 mg/L, significant inhibition of Gga growth was observed. There was no significant difference between colony diameters of Ggt and Ggg grown on the media-lacking and $MnSO_4$-amended agar media (data not shown).

4.4.2 Manganese oxidizing ability of *G. graminis* varieties

All Virginia Ggt and Ggg isolates except Ggt isolate CE1 and ATCC 28230 showed manganese-oxidizing ability (Table 4.2) by formation of brown precipitates in the agar and manganese oxidation scores were within the same range. Six of seven isolates of Gga demonstrated the ability to oxidize manganese. The Gga isolate that did not oxidize manganese (W4W-W) was originally from Washington State and its growth rate was much slower than that of the others. The color of manganese oxides produced varied from yellow or red to black brown (Figs. 4.2 and 4.3). Most of Ggt and Ggg isolates produced red-brown precipitates while Gga isolates produced yellow-brown precipitates. Differences in a pattern of manganese oxidation between Ggt and Gga were observed. Manganese oxidation by Ggt occurred simultaneously with colony growth. On the other hand, weak manganese oxidation by Gga initially occurred in areas of mature mycelia and the color of manganese precipitates was lighter than that of Ggt and Ggg. No brown precipitates were observed from uninoculated control plates amended with the same concentrations of $MnSO_4$.

As shown in Fig. 4.4, Gga weakly oxidized manganese and clumps of manganese oxides were dispersed randomly although a few clumps were observed along the hyphae. Microscopic examination of manganese oxides in the agar of Ggt, Ggg, and Gga showed

clumps of precipitates along the hyphal length as illustrated in Figs. 4.5 and 4.6. Aggregates of manganese oxides were the most abundant in the culture of Ggt and a little less in Ggg. Microscopic photographs taken from agar without $MnSO_4$ amendment showed no precipitates of manganese oxides (Fig. 4.7).

Table 4.2. Manganese oxidizing scores in the agar medium and virulence ratings for *Gaeumannomyces graminis* varieties on greenhouse-grown wheat[1].

Isolate	Manganese oxidizing ability[2]	Virulence rating[3]
G. graminis var. *tritici*		
CB1	3.5	4.1-4.4
CD1	5.0	4.1-5.0
CE1	0.0	0.4-2.6
CE2	4.5	3.4-4.4
CK1a	4.5	1.3
CK1b	4.0	4.1-4.4
CO1	4.0	3.9-4.7
CS1	5.0	3.9-4.7
M1	3.5	3.3-4.6
ATCC 28230	0.0	NT
G. graminis var. *avenae*		
ATCC 15419	2.0	0.1-0.9
BM-W	3.0	NT
FR-W	3.0	NT
PG-W	2.0	NT
RB-W	4.5	NT
RI-W	2.5	NT
W4W-W	0.0	NT
G. graminis var. *graminis*		
ATCC 12761	5.0	0.4-0.7

[1] From Crozier, J.B. 1990. Ph.D. Dissertation. Department of Plant Pathology, Physiology and Weed Science, Virginia Polytechnic Institute and State University, Blacksburg, VA 24061.

[2] Manganese oxidizing scores were visually determined from the intensity of brown precipitates of manganese oxides in the agar medium amended with 100mg/L $MnSO_4$ (0 = no brown precipitate and 5 = dark brown precipitates). Readings were taken after 10-day incubation in the dark at 25°C and from the average of two plates for each experiment. Experiment was repeated three times.

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

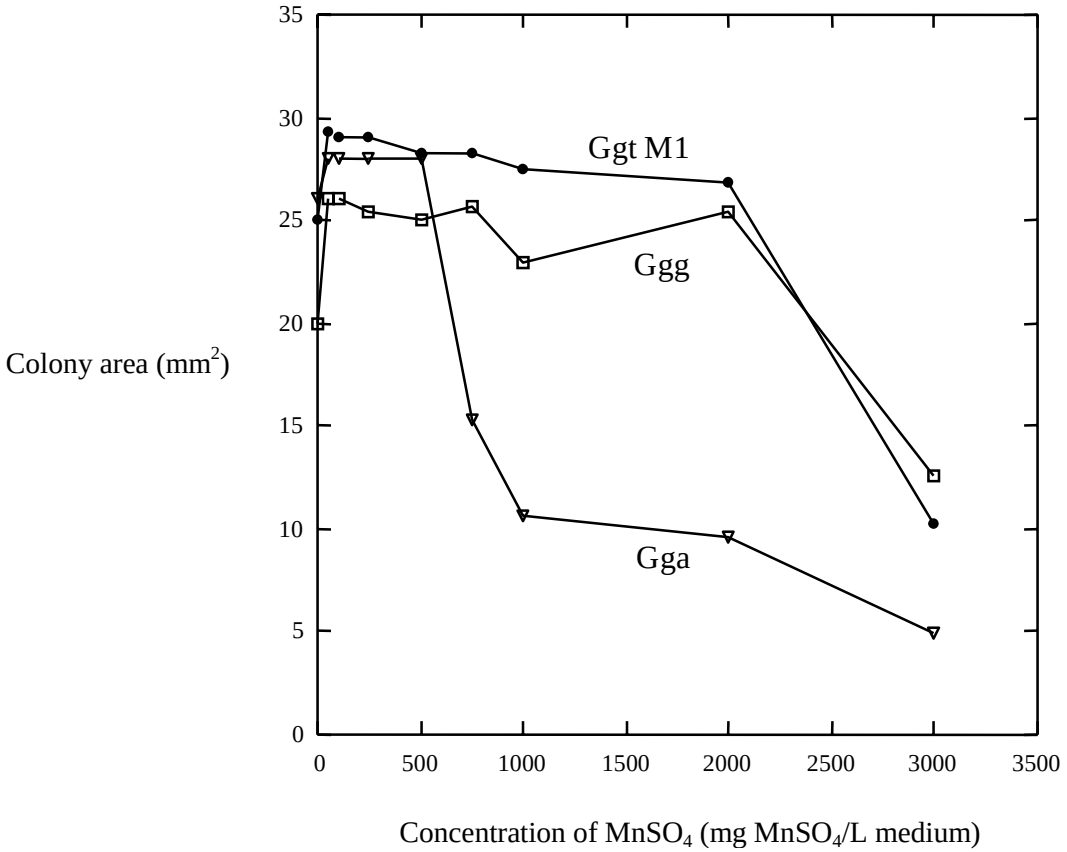


Fig. 4.1. $MnSO_4$ sensitivity of growth of *G. graminis* var. *tritici* (Ggt) M1, var. *avenae* (Gga) ATCC 15419, and var. *graminis* (Ggg) ATCC 12761. *G. graminis* was grown on 1/4 x PDA amended with a series of $MnSO_4$ solutions to obtain a final concentration ranging from 0 to 3000 mg/L. Plates were incubated in the dark for 10 days at 25°C. Colony growth was determined after incubation for 5 days.

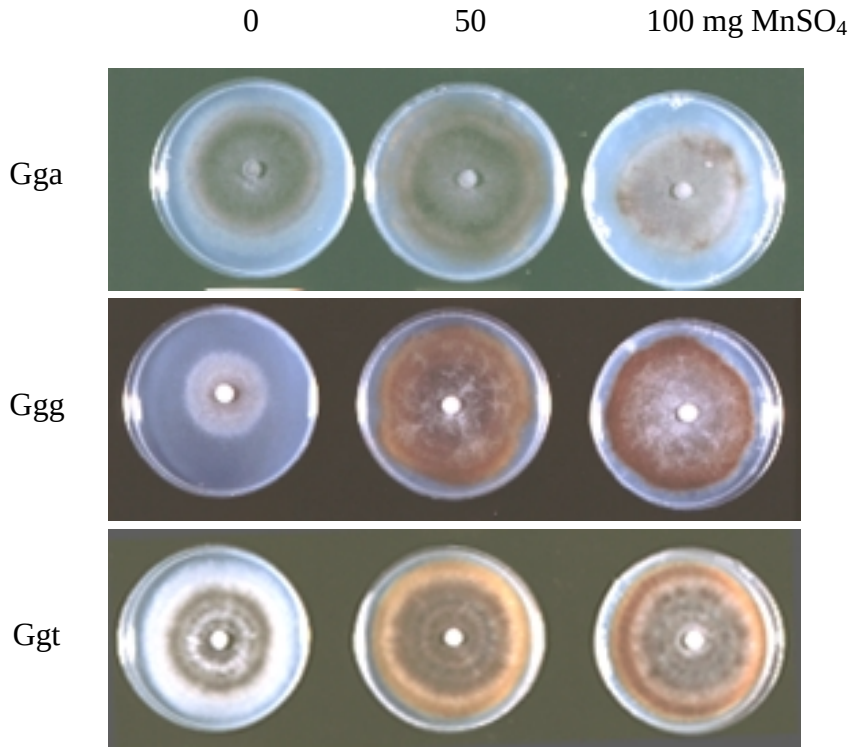


Fig. 4.2. Manganese oxidation by *Gaeumannomyces graminis* var. *avenae* (Gga) ATCC 15419, var. *graminis* (Ggg) ATCC 12761, and var. *tritici* (Ggt) M1 is indicated by the presence of brown precipitates of manganese oxides in the agar. All isolates were grown on 1/4 x PDA amended with 0, 50, and 100 mg $MnSO_4$ /L, respectively, incubated in the dark for 10 days at 25°C.

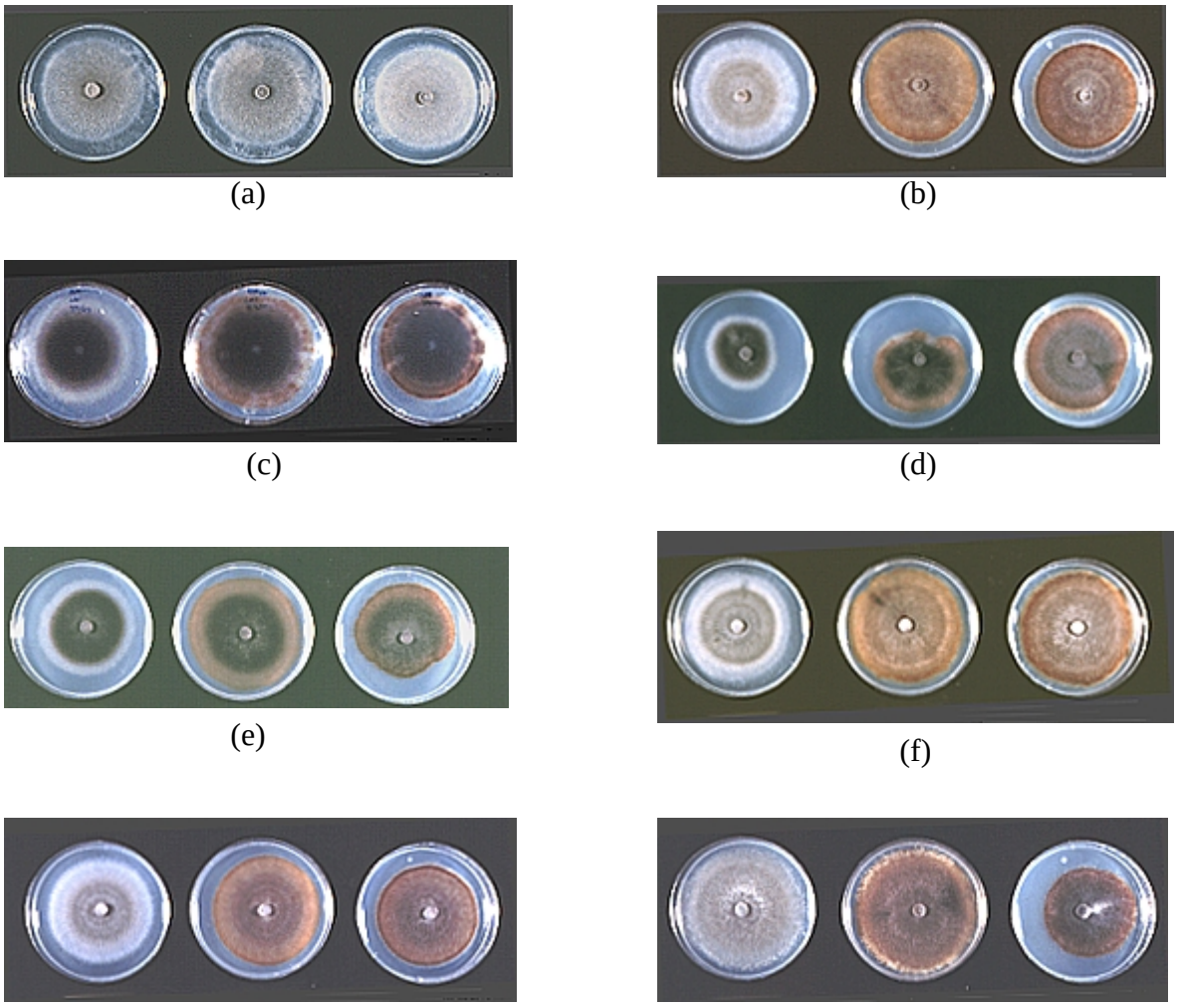


Fig. 4.3. Manganese oxidation by Virginia and British isolates of *Gaeumannomyces graminis* var. *tritici* (Ggt). Manganese oxidizing ability was shown as the presence of brown precipitates of manganese oxides in the agar. All isolates were grown on 1/4 x PDA amended with 0, 50, and 100 mg $MnSO_4$ /L, respectively, incubated in the dark for 10 days at 25°C. Panels a to h are Ggt isolates ATCC 28230, CK1a, CB1, CK1b, CD1, CO1, CE2, and CS1, respectively.

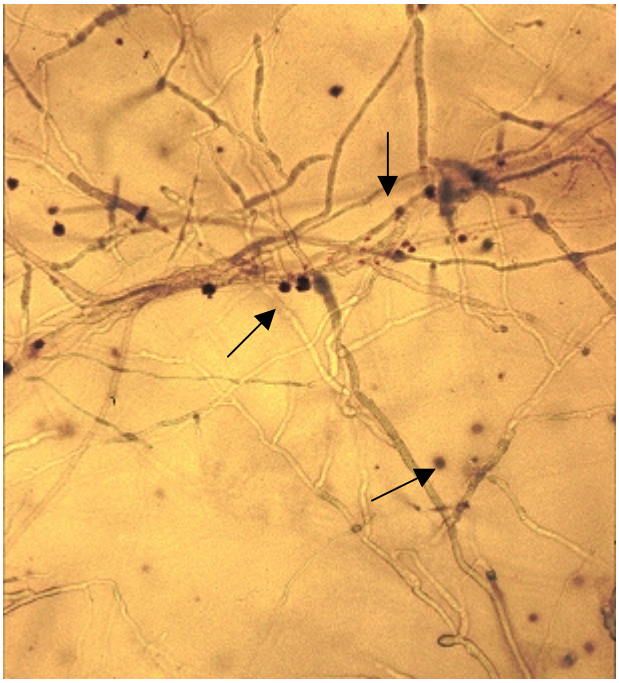


Fig. 4.4. *Gaeumannomyces graminis* var. *avenae* (Gga) manganese oxide precipitates (x 40) on a medium containing 1/4 x PDA amended with 100 mg $MnSO_4$. The plate was incubated in the dark for 10 days at 25°C. Clumps of precipitates are indicated by the arrows.

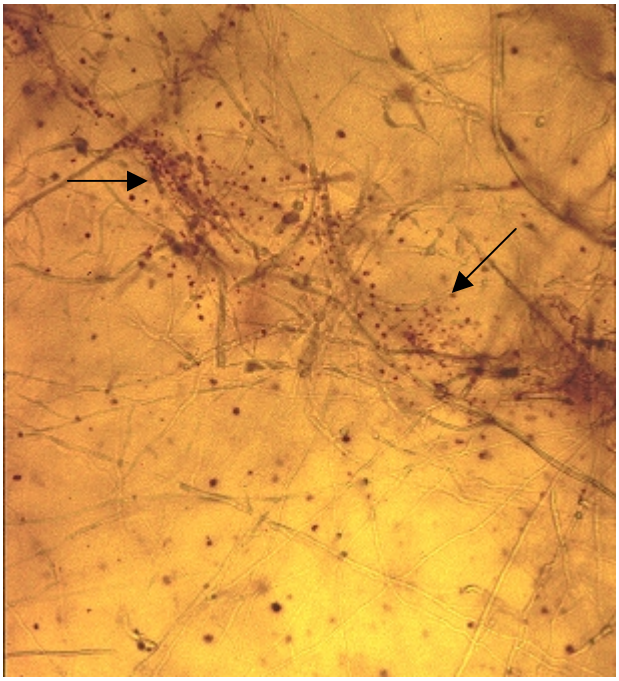


Fig. 4.5. *Gaeumannomyces graminis* var. *graminis* (Ggg) manganese oxide precipitates (x 40) on a medium containing 1/4 x PDA amended with 100 mg $MnSO_4$. The plate was incubated in the dark for 10 days at 25°C. Clumps of precipitates along the hyphae are indicated by arrows.

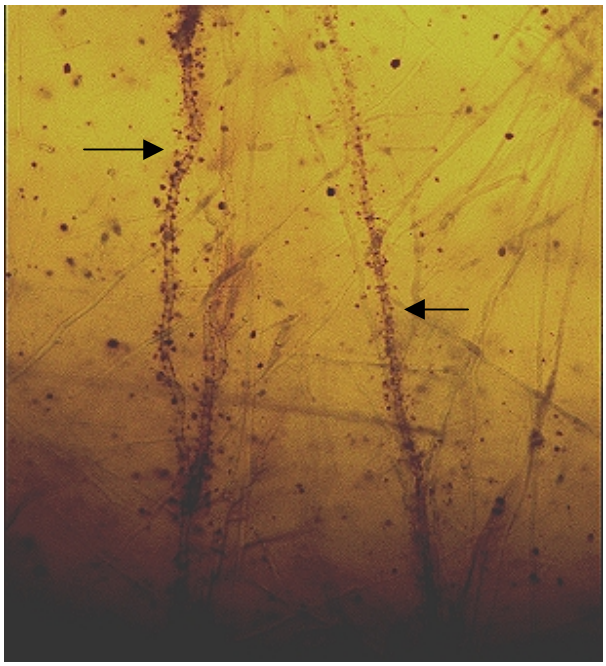
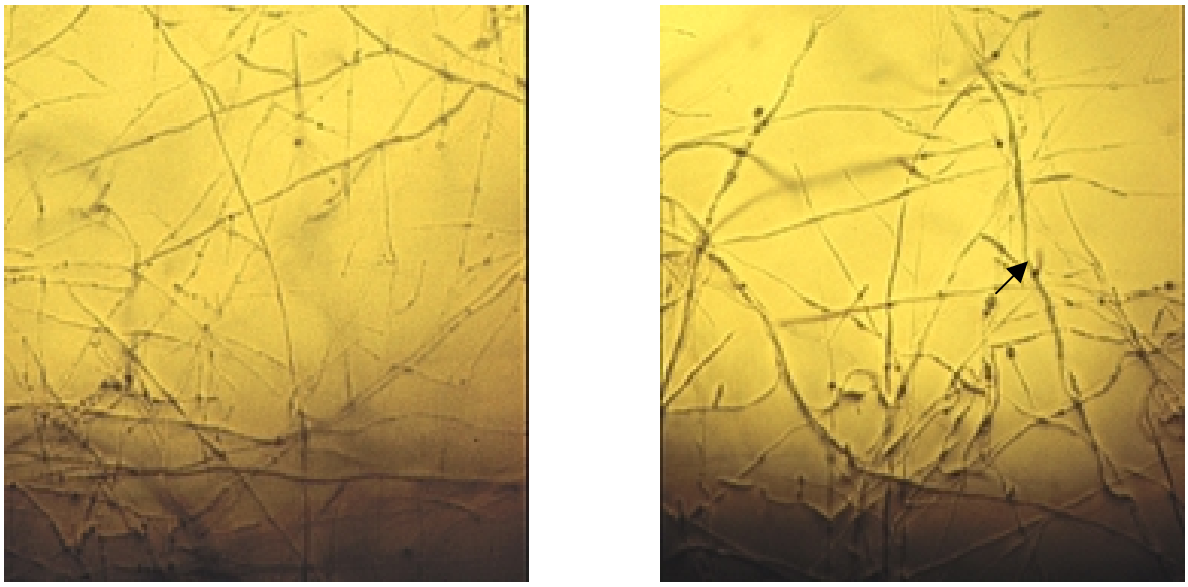


Fig. 4.6. *Gaeumannomyces graminis* var. *tritici* (Ggt) manganese oxide precipitates (x 40) on a medium containing 1/4 x PDA amended with 100 mg $MnSO_4$. Plate was incubated in the dark for 10 days at 25°C. Clumps of precipitates along the hyphae are indicated by arrows.



(a) (b)

Fig. 4.7. Control plates of 1/4 x PDA not amended with $MnSO_4$ (x 40). Plates were inoculated with *Gaeumannomyces graminis* var. *tritici* (Ggt); (panel a) and var. *graminis* (Ggg); (panel b) and incubated in the dark for 10 days at 25°C. Arrow in panel b indicates an hyphopodium of Ggg (40x).

4.5 DISCUSSION

Study of manganese oxidizing ability of *G. graminis* varieties was carried out. The ability of Ggt and Ggg isolates to oxidize manganese has been confirmed. It is also apparent that Gga has the ability to oxidize manganese as well although to a much lesser degree than Ggt or Ggg. All isolates tested can be divided into four groups based on scores of manganese oxidation (Table 2). Strong manganese oxidizers (score 4.5-5.0) were Ggt isolates CD1, CS1, CE2, CK1a, and Ggg ATCC 12761. Those isolates whose score was between 3.5 and 4.0 were placed in an intermediate group consisting of Ggt isolate M1, CB1, CK1b, and CO1. The weak manganese oxidizers were mainly Gga isolates (score 2.0-3.0). Lastly, isolates considered non-manganese oxidizers (score 0.0) were Ggt isolate ATCC 28230 and CE1 and Gga W4W-W. It is interesting to note that Gga RB-W was scored at 4.5 on manganese oxidation and may be placed as a strong oxidizer. However, this isolate has traits of both Ggt and Gga and may be a mixed culture or a heterokaryon containing nuclei of both varieties (see Chapter 3). Therefore, the significance of its strong manganese oxidation ability is not known at this point.

The ability of Ggt to oxidize manganese is correlated positively with virulence of the fungus on wheat (Graham and Rovira, 1984; Huber and McCay-Buis, 1993, Pedler et al., 1996; Rengel, 1997). Previous studies were carried out on wheat in soil culture pot tests and visually scored for manganese oxidizing capacity on agar plates. This corresponded well with other pathogenicity assays conducted in our laboratory (Crozier, 1999). Pathogenicity of Virginia isolates of Ggt were assayed on wheat seedlings and all Ggt isolates, that were strong manganese oxidizers except, isolate CK1a, were also highly virulent on wheat. Ggt isolates ATCC 28230 and CE1, non-manganese oxidizers, were not virulent while those isolates placed in an intermediate group only caused mild damage to wheat seedlings (Crozier, 1999). On the other hand, Ggg ATCC 12761 was considered a strong manganese oxidizer but it did not cause take-all of wheat. This may be due to the difference in manganese oxidation patterns between Ggt and Ggg. Manganese oxidation in Ggg occurred around pathogenesis-related fungal structures and is more localized than that of the virulent Ggt isolates where oxidation of manganese

occurred in advance of the infective hyphae. Although more studies must be conducted to confirm this hypothesis, it is quite possible that the ability of Ggt to oxidize manganese is correlated with pathogenicity of the fungus.

In this study, addition of 50 and 100 mg/L of $MnSO_4$ to the medium did not affect growth of Ggt. This confirms the experiment carried out earlier by Pedler et al. (1996) and showed that there were differences in the growth rate among isolates but it was not due to the presence of $MnSO_4$ in the medium. However, Rengel (1997) demonstrated that $MnSO_4$ severely reduced growth of Ggt isolates when high concentration of $MnSO_4$ (200-400 mg/L) was added to the medium. In our case, reduction of growth of Ggt and Ggg was noticeable at 2000 mg/L $MnSO_4$.

Interesting results were obtained from microscopic examination of the agar containing manganese oxides precipitates of Ggt, Ggg and Gga. Differences in amount and color of precipitates were obvious among the three varieties (Figs. 4.4, 4.5, and 4.6). Manganese oxides formed intensively along the hyphal length of the fungi. In Ggt and Ggg, it was clear that manganese oxides occurred more sparsely at a distance from hyphae. We did not confirm, in terms of physiological immobilization of manganese oxides, whether it was more localized in Ggg than in Ggt. Dispersive X-ray microanalysis with a scanning electron microscope could be used to determine the oxidation states of manganese oxides deposited in the agar medium (Huber and McCay-Buis, 1993). Brown precipitates in the agar can be stained dark blue with 0.1% (v/v) benzidine in 1% (v/v) ethanol. This will indicate the presence of Mn^{3+} and Mn^{4+} oxides in unspecified proportions (Huber and Graham, 1992; Rengel, 1997). However, more detailed information on manganese oxidation can be obtained with dispersive X-ray microanalysis or X-ray absorption near edge structure (XANES) spectroscopy (Huber and McCay-Buis, 1993).

Biological study of manganese oxidation of *G. graminis* in culture provides a basis for molecular study of the genes involved in manganese oxidation in these fungi. The genes encoding enzymes responsible for manganese oxidation, *i.e.*, manganese peroxidase, from *G. graminis* could be isolated via reverse genetic approach. Currently, only the

genes encoding for manganese peroxidases from the white rot fungi have been identified (Pribnow et al., 1989; Mayfield et al., 1994). Manganese peroxidases oxidize Mn^{2+} to Mn^{3+} which, in turn, oxidizes a variety of phenolic substances including lignin (Glenn and Gold, 1985; Mayfield et al., 1994). Another approach for isolation of the genes from *G. graminis* is to use amino acid and nucleic acid sequences information from the isolated genes to design degenerate oligonucleotide primers to identify the gene of interest from *G. graminis*. Characterization of the genes encoding for manganese oxidation enzymes in *G. graminis* will be valuable for understanding role of manganese oxidation of *G. graminis* during pathogenesis as well as defense mechanisms of the host involving manganese-catalyzed lignin biosynthesis.

4.6 ACKNOWLEDGMENT

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CHAPTER 5

CONCLUSIONS AND FUTURE DIRECTION

5.1 CONCLUSION

Two distinctive biochemical characteristics associated with pathogenicity of *G. graminis* have been investigated. The first one is the production of avenacinase in Gga to detoxify avenacin, a chemical barrier present in oat roots, vital for the fungus to be pathogenic on oat. On the other hand, the avenacinase-like enzymes present in Ggt and Ggg are not active, thus, these fungi are sensitive to the toxic effects of avenacin and can not cause disease in oats. The ability to oxidize manganese of *G. graminis* is another distinguishing characteristic among the varieties of this fungus. Ability to oxidize manganese of Ggt has been correlated with its pathogenicity on wheat. Furthermore, Ggt isolates that are strong manganese oxidizers are more virulent than those that are weak oxidizers. However, in-depth information on molecular aspects of these characteristics is limited and it could be an interesting area to be investigated. In this study, I have exploited these distinctions as a basis for development of a simple and specific differentiation test for the varieties of *G. graminis*. When compared with other tests, this test provides several advantages over previously published methods. Comparisons of the advantages and disadvantages among conventional assay, PCR-based using rDNA as a template and the PCR-based test reported in this study are summarized as in Table 1.

5.2 AVENACINASE AND DIFFERENTIAL PCR TESTS.

Sequence differences among the avenacinase genes of Ggt, Gga, and Ggg allowed development of a specific identification and differentiation test for these fungi. The test demonstrated high specificity towards Ggt, Gga, and Ggg in both single and mixed populations of DNA templates. In its current form, the test may be used for isolate identification. For example, isolates, which were thought to be Ggt turned out to be Ggg isolates which was impossible to judge from cultural characteristics. The test detects picogram amounts of DNA and is rapid and simple to perform.

Table 5.1 Comparisons of conventional diagnosis, PCR-based test with rDNA and the PCR-based test reported here.

Conventional test[1]	PCR-based using rDNA as a template[2]	PCR-based test reported here
1. Time-consuming, take weeks to complete	1. Rapid assay, complete in a day	1. Rapid assay, complete in a day
2. Laborious due to a number of tests performed.	2. Simple and straightforward	2. Simple and straightforward
3. Requiring experienced personnel to perform the test	3. Easy to perform and no need for experienced personnel	3. Easy to perform and no need for experienced personnel
4. Results usually based on laboratory, microscopic examinations and pathogenicity assay in the greenhouse.	4. Results based on the presence/absence of a band.	4. Results based on size difference of PCR products
5. Loss of virulence is common due to long term storage.	5. No need for long term storage of the culture.	5. No need for long term storage of the culture.
6. Direct detection from field samples is impossible.	6. Direct detection of the pathogens is possible.	6. Direct detection of the pathogens is possible.
7. Inconclusive. Difficult to differentiate among the varieties and other closely related fungi	7. Use of ITS region as a target sequence, provide specific identification among varieties of *G. graminis* and other closely related fungi.	7. Specific identification among varieties of *G. graminis* and other closely related fungi
8. Diagnosis based on characteristic field symptoms is too late for proper disease control.	8. Early detection of the pathogen in the field is possible.	8. Early detection of the pathogen in the field is possible.

Table 5.1. Comparisons of conventional diagnosis, PCR-based with rDNA and the PCR-based test reported here. (continued).

Conventional Test[1]	PCR-based using rDNA as template[2]	the PCR-based test reported here
	9. Requires nested PCR	9. Basic PCR uses
	10. Specific primers available for Ggt and Gga	10. Specific primers available for Ggt, Gga, and Ggg
	11. Individual PCR requires for detection of each variety	11. Differentiate all three varieties in a single PCR tube
9. Assay is informative for pathogenicity of the pathogens.	12. Assay is not informative for pathogenicity of the pathogens.	12. Assay may be informative and correlated with pathogenicity of the pathogens.
	13. Applicable for ecological study of the pathogen	13. Applicable for ecological study of the pathogen
10. Inconvenient for routine test	14. Applicable for routine test	14. Applicable for routine test.

[1]Juhnke, M.E., Mathre, D.M. and Sands, D.C. 1984. A selective medium for *Gaeumannomyces graminis* var. *tritici*. Phytopathology 68: 233-236.

[2]Bryan, G.T., Daniels, M.J. and Osbourn, A.E. 1995. Comparison of fungi within the *Gaeumannomyces-Phialophora* complex by analysis of ribosomal DNA sequences. Appl. Environ. Microbiol. 61:681-689.

Although my PCR-based identification and differentiation test provides several advantages over previous tests, it is still premature to be used as a routine diagnostic test. Additional experiments need to be conducted to confirm the accuracy and specificity of the test.

5.2.1 More strains need to be tested.

First of all, more isolates of Ggt, Gga, Ggg, *Phialophora* spp., other *Gaeumannomyces* species from diverse locations around the world as well as other soilborne pathogens, such as *Magnaporthe* spp., *Rhizoctonia* spp., must be tested in order to establish significance for the specificity of the test. DNA sequencing of PCR products produced from those isolates, as well as the isolates tested in this study should be carried out simultaneously to confirm identities of PCR fragments and to gain confidence for the specificity of the test. More attention should be paid to Ggg isolates since those isolated from hosts other than wheat produced non-specific PCR products apart from Ggg-specific product and efficiency of amplification was not satisfactory (see Chapter 3). A number of Ggg isolates from wheat and other hosts should be tested and further characterized.

5.2.2 Internal control needed.

A second consideration is based on the fact that one of the main problems with PCR-based test is the possibility of false negatives due to reaction failure (Ward, 1995). Addition of an internal control in every reaction will ensure that each PCR reaction is functional. Internal control fragments should be a different size from all of the diagnostic fragments but should be amplified with primers having the same annealing temperature as the variety-specific primers. In fungi, rDNA can be as a good choice since primers designed from fungal rDNA can amplify DNA from all fungal species (Ward, 1995). Others have constructed a plasmid containing a specific part of rDNA so that same pair of primers could be used in PCR assay (Hu et al., 1993). An internal control must be included in the test to differentiate reaction failure due to the absence of *G. graminis*

DNA from reaction failure due to poor DNA quality or the presence of inhibitors. This criterion is essential if the test will be performed routinely as a diagnostic test with field samples. However, justification has to be made to find a proper internal control for this particular test. Currently, confirmation was made by repetition of PCR experiments.

5.2.3 Detection of *G. graminis* from soil and plant tissues.

One priority for the application of the test will be to optimize it for direct identification of the pathogens from infested soil and the crowns and roots of diseased wheat plants. I demonstrated earlier that my test is more sensitive than those using DNA probe hybridization alone. Direct detection of the take-all fungi with PCR-based method has been reported by several groups. Methods developed by both Henson et al. (1993) and Bryan et al. (1995) were successfully used to identify Ggt and Gga from colonized plant samples without culturing the fungi. Both PCR assays required nested PCR to produce visible PCR products. My test has some advantages over these methods as described earlier. For example, this test can differentiate Ggt, Gga, and Ggg in one reaction and PCR protocol is simple. This is important since colonized host plants usually harbor a mixed population of plant pathogens and saprophytes and misidentification can be easily made by conventional culturing method. Currently, I am optimizing test conditions for direct detection of the varieties of *G. graminis* using a procedure modified from the method of Klimyuk et al. (1993). I hope to be able to identify the take-all pathogens in both fresh and dried wheat plants. Early detection of the fungus in young wheat plants may lead to a prompt cultural practices to minimize yield loss. In addition, detection of any of *G. graminis* varieties in wheat straw left in the field will provide valuable information on ecological behavior of these fungi in their natural habitats as well as greater understanding of survival mechanisms. In this case, preliminary experiments can be carried out in the greenhouse by artificially inoculating Ggt on wheat seeds and directly using colonized green seedlings as templates for PCR. Optimization of PCR conditions generated from this study will be easily applied to field samples. Quantification of the amount of *G. graminis* in the soil taken from infested wheat fields is another key area for further investigation of the test. In this case, DNA isolation of soil

organic matter will be required prior to PCR to get rid of humic substances that can inhibit *Taq* DNA polymerase (Tebbe and Vahjen, 1993). I am also in the process of optimizing DNA isolation procedure for this purpose. In combination with the presence of the fungi in wheat straw, prediction of disease severity by determining levels of inoculum in the soil may possibly lead to improved disease management to prevent severe damage to the future crops.

5.2.4 Molecular mechanisms for avenacinase-like genes.

Identification of the avenacinase-like genes in Ggt and Ggg is a preliminary step for investigation of the mechanism of host-pathogen interaction. The presence of avenacinase-like gene in Ggt and Ggg also indicates close phylogenetic relationships among these fungi. It is possible that they were the same fungi and, through time, host exposure, and evolution, they were able to adapt to changes in their environment resulting in small differences among their genomes. This leads to a speculation on the differences among avenacinase gene in Gga and avenacinase-like genes in Ggt and Ggg may mirror these differences. However, the avenacinase-like genes in Ggt and Ggg may be important for nutrition or for detoxification of unknown saponins in wheat or other grasses (Osbourn, 1994). It will be interesting to isolate and characterize the complete avenacinase-like genes in Ggt and Ggg and compare the structure of the gene with that of Gga. Investigation of the promoter regions of the genes may provide an insight into the expression of these genes. Generation of specific mutants of these fungi through targeted disruption of the gene and comparison of the pathogenicity of the mutants with that of the wild type will allow us to investigate the function of the gene in pathogenesis (Oliver and Osbourn, 1995). This strategy was used to study the function of the avenacinase gene in Gga and confirmed that the gene is associated with pathogenicity of Gga on oats but not important for its colonization on wheat (Bowyer et al., 1995). It may also be interesting to identify any saponins in wheat roots and use it to characterize the protein encoded by the avenacinase-like gene of Ggt. If this could be done, it will be a real breakthrough in understanding pathogenesis of the take-all pathogens. Moreover, an ideal experiment

would be to produce transgenic wheat containing an analog of a saponin which can not be detoxified by the enzyme(s) presents in Ggt, thus, preventing pathogenesis on wheat.

5.3 MANGANESE OXIDATION AND PATHOGENICITY.

5.3.1 More isolates need to be tested and oxidation states of Mn^{2+} precipitates should be determined.

The ability to oxidize manganese by Ggt has been correlated with virulence of the fungus on wheat (Huber and McCay-Buis, 1993; Pedler et al., 1996; Rengel, 1997). I have confirmed that all Ggt isolates except ATCC isolate have the ability to oxidize manganese although not all of them were strong manganese oxidizers. The majority of Gga isolates were weak oxidizers. In this study, only a single isolate of Ggg was tested and found to be a strong oxidizer. Therefore, more isolates are needed before any conclusion can be drawn. Although microscopic examination revealed that deposition of manganese oxide precipitates in the agar from Ggt were more intense than those of Ggg and Gga, determination of oxidation states of those precipitates is essential. My speculation on the oxidation states of manganese is that they should be different among the varieties due to color of the precipitates observed and the pattern of oxidation (Huber and McCay-Buis, 1993). However, this procedure requires sophisticated equipment such as micro X-ray analysis and scanning electron microscope (Schulze et al., 1996).

5.3.2 Sequencing of the genes involved in manganese oxidation of *G. graminis*.

My PCR-based identification test could be more informative for pathogenicity of *G. graminis* if the ability to oxidize manganese could be included in the test as another marker. This can be accomplished by isolation of the gene involved in manganese oxidation of these fungi. Availability of DNA sequence information of manganese peroxidase genes from *Phanerochaete chrysosporium*, *Trametes versicolor*, and *Pleurotus ostreatus* was used to design degenerate oligonucleotide primers for isolation of the genes from *G. graminis* (Appendix E). However, all attempts had failed. It will be

more reliable to purify and characterize the enzymes responsible for manganese oxidation from all three varieties. Degenerate oligonucleotide primers designed from the actual amino acid sequence of these enzymes will be more specific than those used earlier. Gene isolation through library screening may also be possible. Once DNA sequence information of these genes is available, similar strategies could be used to design variety-specific primers, which could be included in the diagnostic procedure.

5.3.3 Additional information possible for the test.

Other markers may be used in combination with avenacinase gene to generate an ideal diagnostic test for G. graminis. One possibility may be to exploit of the availability of sequence information of the ITS regions of *G. graminis* (Goodwin et al., 1995; Bryan et al., 1995). Since identification tests based on these regions are also specific, introduction of this marker into my test may extend areas of its applications. For example, it can be used to study population dynamics of the take-all pathogen, as well as, determination of the proportion of the virulent pathogen in that population. This may provide valuable information in understanding ecological behavior of the pathogens, which could lead to prediction of disease severity and effective disease management programs.

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APPENDICES

APPENDIX A

MYCOLOGICAL METHODS

1. Hyphal tipping of isolates of *G. graminis*

G. graminis isolates were grown on ¼ x PDA for 7-10 days. Mycelial plugs (8-mm diameter) were taken from colony margin and inoculated in a 250 ml flask containing 7 g of sterile millet (*Setaria italica* L.) seeds. The inoculum was incubated statically in the dark at 25°C until millet seeds were covered with mycelia. At the end of this period, four colonized millet seeds were transferred to a water agar plate (15 g Noble agar in 1 L distilled water) and the fungus was allowed to grow for a few days or until single hypha spread from the seed on to the agar. Single hyphal tips were cut under a dissecting scope and inoculated separately on ¼ x PDA. Cultures from single hyphal tips were incubated for 7-10 days statically in the dark at 25°C.

2. Preparation of genomic DNA of *Gaeumannomyces graminis* for Southern hybridization analysis.

The procedure modified by Picknett et al. (1987) was used for large scale preparation in which approximately 2-5 g of dried mycelia were ground in liquid nitrogen using sterile mortar and pestle. Mycelial powder was resuspended in 10 ml of extraction buffer (25 mM Tris-HCl [pH 8], 25 mM EDTA, 50mM NaCl, 1% SDS) and incubated in an ice-water slurry (0°C) for 1 hour. This was followed by addition of 5 M NaCl to a final concentration of 1 M and incubation in ice-water slurry for 1 hour. The mixture was centrifuged for 30 min (2,500 x g, 4°C). The supernatant, following separation from cell debris, was treated with proteinase K (20 mg/ml) to obtain a final concentration of 50 µg/ml at 37°C for 20 min. The supernatant was extracted with an equal vol. of phenol:chloroform:isoamyl alcohol (25:24:1), centrifuged for 15 min (2,500 x g, room temp.), treated with RNase A (50 µg/ml) at 37°C for 3hours and extracted again with phenol:chloroform:isoamyl alcohol. If needed, additional phenol:chloroform extractions

were performed until a clear aqueous phase was obtained. A clear aqueous phase was extracted once with an equal vol. of chloroform:isoamyl alcohol (24:1) and centrifuged to separate the phases for 15 min. (14,000 x g, room temp.). DNA was precipitated by adding 0.03 vol of 3 M sodium acetate and 2.5 vol of cold ethanol (-20°C) and incubating at -20°C overnight. At the end of this period, the mixture was centrifuged for 30 min. (14,000 x g, 4°C), and the pellet was washed twice with 70% ethanol and air-dried. DNA was dissolved in either 1x TE (10mM Tris-HCl [pH8] and 1mM EDTA) or deionized water and stored at 4°C until used.

Reference:
Picknett, T.M., Saunders, G., Ford, R. and Holt, G. 1987. Development of a gene transfer system for *Penicillium chrysogenum*. Curr. Genet. 12:449-455.

3. Rapid isolation of genomic DNA from *Gaeumannomyces graminis* .

This protocol was modified by Lee and Taylor (1990) which was used for small scale DNA preparation (≤ 1g dry mycelium). After grinding mycelial mass in liquid nitrogen, 5-800 µl of lysis buffer (50 mM Tris-HCl [pH, 7.2], 50mM EDTA, 3% v/v SDS, 1% v/v β-mercaptoethanol) was added to the powdered mycelia. The mixture was stirred with a 5 ml pipet tip and vortexed vigorously until it became homogeneous. The mixture was incubated for 1-2 hours at 65°C, extracted with an equal vol. of phenol:chloroform and centrifuged for 15 min. (14,000 x g, room temp.) repeatedly until the aqueous phase was clear. The aqueous phase was extracted once with an equal vol. of chloroform:isoamyl alcohol (24:1) and centrifuged to separate the phases for 5 min. (14,000 x g, room temp.). RNA was digested with RNase A (10 mg/ml) to obtain a final concentration of 50 µg/ml at 37°C for 3 hours, re-extracted once with phenol:chloroform and the DNA was precipitated with 0.03 vol of 3 M sodium acetate and 0.6 vol of 99% isopropanol at 4°C for at least 30 min. The DNA pellet was subsequently obtained by centrifugation for 20 min (14,000 x g, 25°C). The pellet was rinsed with 70% ethanol, dried, and dissolved in either 1 x TE or deionized water. The DNA was stored at 4°C.

Reference:
Lee, S.B. and Taylor, J.W. 1990. Isolation of DNA from fungal mycelia and single spores. In: PCR Protocols: A Guide to Methods and Applications. Pages 282-287. M.A. Innis, D.H. Gelfand, J.J. Sninsky, and T.J. White (Eds.). Academic Press, San Diego.

4 Preparation of plasmid DNA.

An overnight culture (37°C with shaking at 250 rpm) of *E. coli* containing pA3G2 grown in 25 ml of Luria-Bertani (LB) broth (Difco Laboratories, Detroit, MI 48232-7058) was harvested by centrifugation at 8000 x g for 5 min. The cells were resuspended in 5 ml 50mM glucose, 25 mM Tris-HCl, pH 8 and 10 mM EDTA and incubated for 5 min at room temperature. Cell lysis was achieved by addition of 10 ml freshly made 0.2N sodium hydroxide with 1% SDS and incubation of the mixture for 5 min in an ice-water slurry (0^{o}C). After addition of 8 ml 5M potassium acetate and glacial acetic acid solution, pH 4.8 to precipitate high molecular weight DNA, the mixture was centrifuged for 5 min at 13,000 x g. The supernatant was transferred to a new tube, extracted once with an equal volume of phenol:chloroform, and once with an equal volume of chloroform. Plasmid DNA was precipitated by addition of 0.6 to 0.7 volume of 99% isopropanol, incubation for 2 min at room temperature, and centrifugation for 5 min at 13,000 x g. The DNA pellet was rinsed with 70% ethanol and dried at 37°C. Plasmid DNA was dissolved in 50 µl of 0.1x TE and stored at –20°C until used.

APPENDIX B

EARLIER PRIMERS FOR AVENACINASE-LIKE GENES

Design of variety-specific olignonucleotide primers for differentiation of *G. graminis* varieties from DNA sequences of avenacinase genes (developmental process)

The first attempt in designing a specific test for *G. graminis* varieties.

1. 5' Gga specific primer (Gga-2) 5' ACGGCGGTGGATGGCAAGAC 3' (position in sequence: 769-788, see Chapter 2, Fig. 2.7).
2. 5' Ggg specific primer (Ggg-1) 5' ATGAACATGCCCGGACCGCC 3' (position in sequence: 1110-1129).
3. 5' Ggt specific primer (Ggt-2) 5' ACATCACCACTGCGGTCAAT 3' (position in sequence: 1171-1190).
4. 3' Universal primer 5' TGCTCATGGTGGTTCCTGCG 3' (position in sequence: 1369-1388).

Polymerase Chain Reaction (PCR) conditions

For PCR assays, each 50 µl reaction volume contained 50 pmol of each primer, 2.5 units of *Taq* DNA polymerase (Qiagen Inc. Valencia, CA 91355), 200 µm of each deoxynucleotide triphosphates, 1 x Qiagen reaction buffer, 3 mM $MgCl_2$ and 50 ng of fungal DNA. PCR was 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 30 cycles of 94°C for 45 sec, 68°C for 30 sec, and 72°C for 1:30 min. At the end of this incubation, additional incubation for 10 min at 72°C was always included to ensure complete extension of PCR products. For PCR product analyses, see Materials and Methods in Chapter 3.

Results

As shown in Fig. B.1, specificity of these varieties-specific primers was poor. Only the Gga-specific primer showed high specificity to Gga DNA template. Thus, this primer was used in the final identification assay. PCR products of the same size were produced when both Ggg and Ggt DNA were amplified with primer Ggg-2. Primer Ggt-2 was highly non-specific because it generated products of the same size from all three varieties although it showed higher specificity towards Ggt. Due to the non-specificity of Ggg and Ggt specific primers, they can not be used for identification and differentiation assay. These primers gave inconclusive identification and did not differentiate the presence of Ggt and Ggg from each other (Fig. B.2). However, the advantage of this set of primers are that PCR products generated from Ggg- and Ggt-specific primers are small fragments of 277 and 216 bp, respectively, which are of optimum size for PCR products.

The second attempt in designing a specific test for *G. graminis* varieties.

1. 5' Gga primer (Gga-2) is the same as above.
2. 5' Ggg primer (Ggg-3) 5' CACCCCCGGTCCCTGCGTAA 3' (position in sequence: 301-320)
3. 5' Ggt primer (Ggt-2) is the same as above.
4. 3' Universal primer is the same as above.

PCR conditions are the same as above.

Results

New Ggg-specific primer produced 1,086 bp amplified product and very specific to Ggg DNA (data not shown). However, when DNA of Ggg and Ggt were amplified with all of the 5' primers added in each PCR, a doublet was produced from Ggg sample (Fig. B.3). This meant that although primer Ggg-3 was specific to Ggg but primer Ggt-2 still gave non-specific product with Ggg. Although distinctive PCR patterns were generated

form Gga, Ggg, and Ggt at this point, it was still inconclusive especially when applied to a mixed DNA population. Examples of ambiguity of the results was shown in Figs. B.4 and B.5 when all Virginia isolates of Ggt were amplified with primer Ggt-2 alone and with all of the 5' primers in each reaction. All samples gave positive result with primer Ggt-2 alone but it could not be concluded that all of them were Ggt. In Fig. B.5, some of the isolates produced PCR pattern of Ggg, however, conclusive identification could not be made if those isolates were true Ggg isolates or a mixed population of Ggg and Ggt isolates. Therefore, specific primer for Ggt was essential for conclusive identification and differentiation of *G. graminis* varieties.

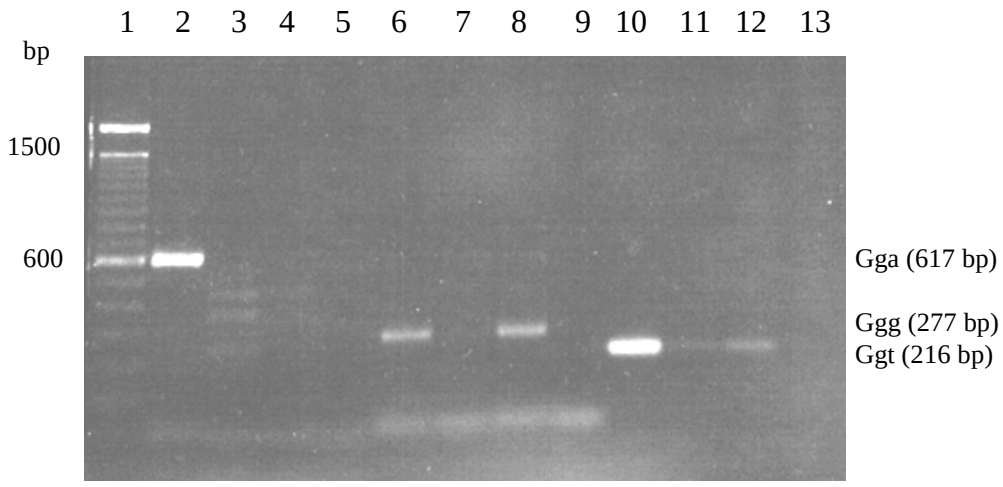

Fig. B.1. First attempt: Specificity of *G. graminis* var. *tritici* (Ggt), var. *avenae* (Gga), and var. *graminis* (Ggg) varieties-specific primers. Each lane contains a single template with a variety-specific 5' primer and the universal 3' primer. All templates were amplified with all types of primers. PCR products were electrophoresed on 1.8% agarose gel, stained with ethidium bromide, visualized and photographed with 302 nm UV light using a Wratten 22A filter. Lane 1 contains 100 bp DNA ladder as molecular weight standards. Lanes 2-5 are products from Gga, Ggg, Ggt, and no DNA control with Gga-specific primer. Lanes 6-9 are products from Ggg, Gga, Ggt, and no DNA control with Ggg-specific primer. Lanes 10-13 are products from Ggt, Gga, Ggg, and no DNA control with Ggt-specific primer. Note that Ggg and Ggt primers produce same size of products when amplified with non-template DNA.

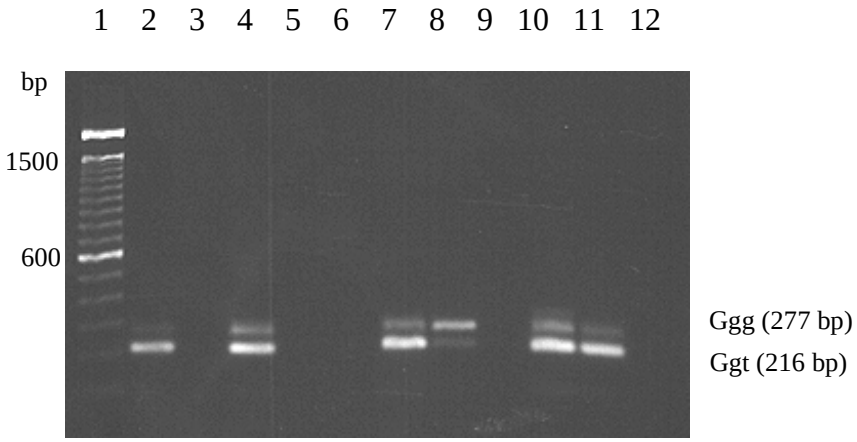

Fig. B.2. First attempt: Differential PCR of Virginia isolates of *G. graminis* var. *tritici* with a combination of Gga-2, Ggg-1 and Ggt-2 primers. PCR products were electrophoresed on 1.8% agarose gel, stained with ethidium bromide, visualized and photographed with 302 nm UV light using a Wratten 22A filter. Lane 1 contains 100 bp DNA ladder as molecular weight standards. Lanes 2-12 contain PCR products from Ggt isolates M1, ATCC 28230, CB1, CD1, CE1, CH1, CHe1, CK1a, CK1b, CS1 and *Cercospora zea-maydis* as a negative control, respectively.

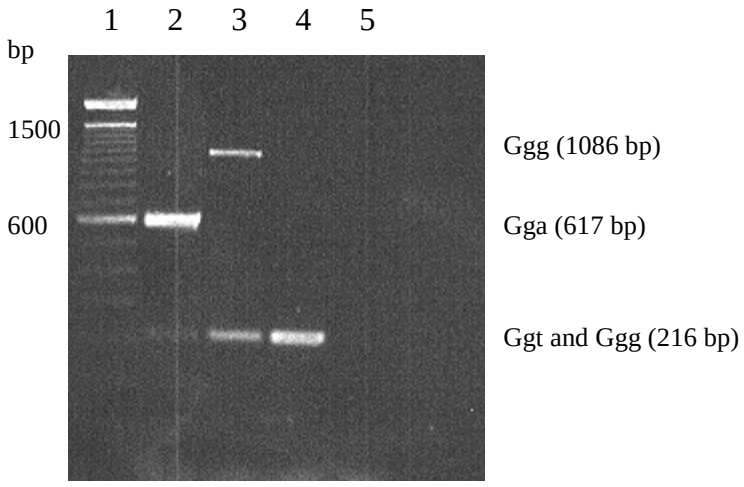

Fig. B.3. Second attempt: Amplification of *G. graminis* var. *tritici* (Ggt), var. *avenae* (Gga), and, var. *graminis* (Ggg) genomic DNA with all three variety-specific primers (Gga-2, Ggg-3, and Ggt-2). PCR products were electrophoresed on 1.8% agarose gel, stained with ethidium bromide, visualized and photographed with 302 nm UV light using a Wratten 22A filter. Lane 1 contains 100 bp DNA ladder as molecular weight standards. Lanes 2-4 contain PCR products from Gga, Ggg, Ggt, and no DNA control, respectively. Lane 5 is a no DNA control. Note that Ggt specific primer produced non-specific product from Ggg DNA template.

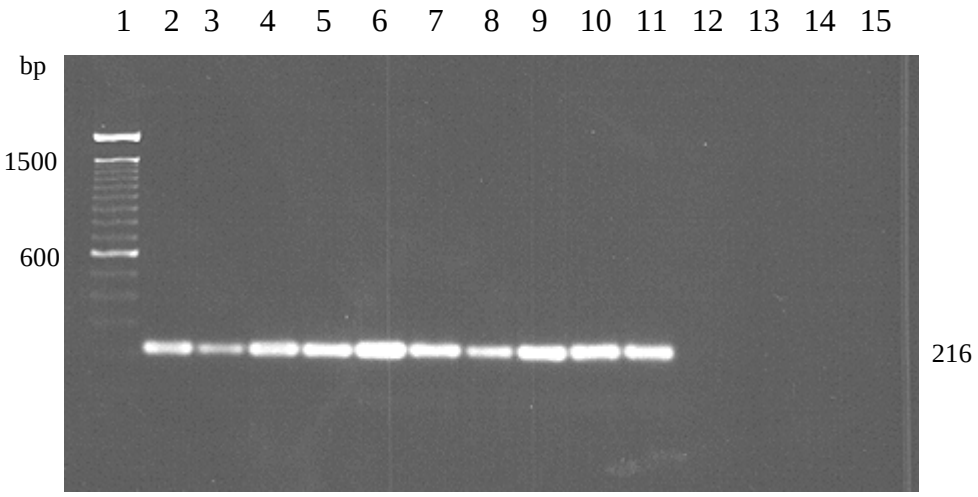

Fig. B.4. Second attempt: Differential PCR of Virginia isolates of *G. graminis* var. *tritici* (Ggt) from wheat with Ggt-2 primer. PCR products were electrophoresed on 1.8% agarose gel, stained with ethidium bromide, visualized and photographed with 302 nm UV light using a Wratten 22A filter. Lane 1 contains 100 bp DNA ladder as molecular weight standards. Lanes 2-13 contain PCR products from isolates M1, ATCC 28230, CB1, CD1, CE1, CH1, CHe1, CK1a, CK1b, CS1 and *Cercospora zea-maydis* as a negative control, and no DNA control, respectively.

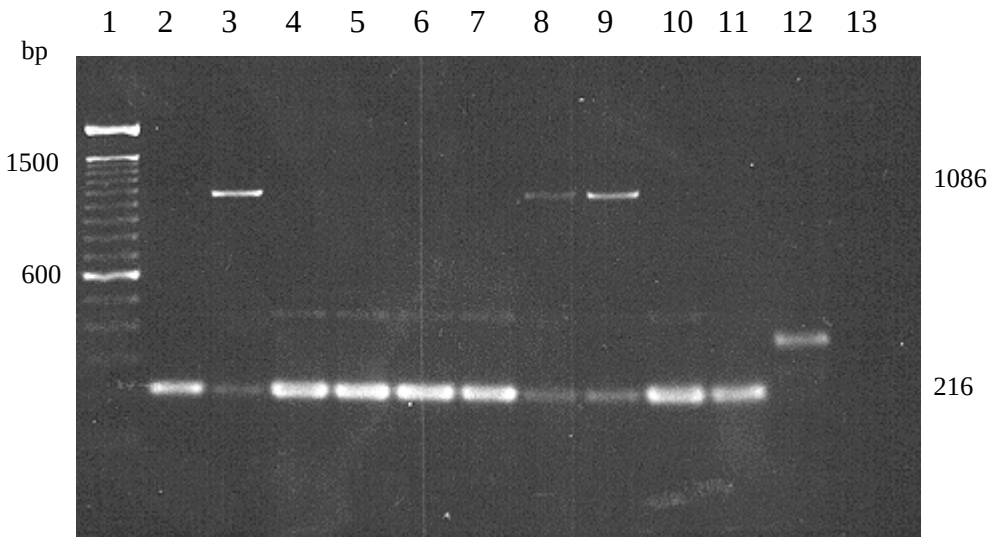

Fig. B.5. Second attempt: Differential PCR of Virginia isolates of *G. graminis* var. *tritici* (Ggt) from wheat with a combination of Gga-2, Ggg-3 and Ggt-2 primers. PCR products were electrophoresed on 1.8% agarose gel, stained with ethidium bromide, visualized and photographed with 302 nm UV light using a Wratten 22A filter. Lane 1 contains 100 bp DNA ladder as molecular weight standards. Lanes 2-13 contain PCR products from isolates M1, ATCC 28230, CB1, CD1, CE1, CH1, CHe1, CK1a, CK1b, CS1, *Gaeumannomyces cylindrosporus*, *Cercospora zea-maydis* as a negative control, respectively.

APPENDIX C

DNA ISOLATION FROM SOIL AND PLANT TISSUES

1. Isolation of DNA from soil organic matters.

Procedure (adapted from Ernst et al. , 1996)

1. Grind 10 g of field soil with liquid nitrogen until it becomes powder.
2. Mix soil powders with 20 ml of 120 mM Na_2HPO_4 (pH 6.8) and immediately add 4 ml of 5% (w/v) SDS and 0.4 g of polyvinylpolypyrollidone (PVPP). Incubate the mixture for 30 min with shaking at room temperature.
3. Precipitate humic acids by addition of $CaCl_2$ to obtain a final concentration of 2% (w/v). Incubate for another 30 min with gentle shaking at room temperature.
4. Transfer the mixture to a 65°C water bath and incubate for 1 hr with occasional stirring.
5. Centrifuge at 8,000 x g for 20 min at 10°C.
6. Transfer the supernatant to a new tube. Precipitate DNA by addition of 5 M NaCl to obtain a final concentration of 500mM, followed by 0.5 vol. of 50% (w/v) PEG 8000.
7. Incubate the mixture overnight at 4°C.
8. Centrifuge the mixture at 6,000 g for 30 min at 4°C. Carefully pour off the supernatant.
9. Dissolve the resulting pellet in 0.5 ml TE (10mM Tris-HCl, 1mM EDTA, pH 8.0).
10. Extract once with 0.5 vol of Tris-saturated phenol. Then extract once with 1 vol of chloroform:isoamyl alcohol.
11. Precipitate DNA with 0.1 vol of 5M sodium acetate and 2 vol of 95% ice-cold ethanol (-20°C).
12. Incubate at –20°C for at least 1 hr to overnight.
13. Centrifuge at 16,000 g for 30 min at 4°C. Discard the supernatant.
14. Wash the pellet with 70% ethanol. Centrifuge at 10,000 g for 10 min at 4°C. Repeat this step.

15. Dissolve the DNA pellet in 10-50 µl of deionized water.

Reference:
Ernst, E., Kiefer, E., Drouet, A., Sandermann Jr., H. 1996. A simple method of DNA extraction from soil for detection composite transgenic plants by PCR. Plant Mol. Biol. Rep. 14:143-148.

2. Isolation of DNA from diseased wheat samples using CTAB procedure

Procedure

(adapted from a procedure used in Dr. Saghai Maroof's laboratory, Department of Crop, Soil and Environmental Science, VPI & SU).

1. Finely chop the darkened area of the samples, *i.e.*, roots, crowns, culms, leaf sheaths, into small pieces (≤1mm) and dry at 60°C overnight.
2. Grind 0.4 g of dried sample in liquid nitrogen to fine powders and immediately transfer to a 40ml centrifuge tube containing 10 ml of preheated CTAB 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 before use). Thoroughly mix and stir the content with a pipet tip or by inversion.
3. Incubate at 65°C for 1 hr. Mix the content every 10-15 min.
4. Add an equal vol. of choloroform:isoamyl alcohol (24:1) to the content. Mix by shaking the tube horizontally on a shaker for 30 min.
5. Centrifuge at 5,000 g at 4°C for 10 min.
6. Transfer the clear aqueous phase to a new tube. If debris are still present, repeat step 4 and 5. Add RNase A (10 mg/ml) to obtain a final concentration of 100µg/ml. Mix and incubate at room temperature for 30 min.
7. Add 0.6 vol. of ice-cold (-20°C) isopropanol to the tube. Incubate at –20°C for 30 min.
8. Centrifuge at 2,000 g for 10 min at 4°C. Pour off the supernatant.

9. Wash the pellet with 10 ml of 70% ethanol/10 mM ammonium acetate, mix gently and centrifuge at 5,000 g for 10 min at 4°C to remove CTAB residue. Air-dry the pellet.
10. Dissolve in an appropriate volume of sterile deionized water at 4°C overnight without agitation.

PCR amplification of DNA isolates from wheat tissue samples.

Two of the 5'oligonucleotide primers and a single 3' universal primer (see Chapter 3) were used which were primer Ggt-4 and primer Ggt-2. Primer Ggt-4 is a Ggt-specific primer used for identification and differentiation assay (see Chapter 3) and primer Ggt-2 is less specific and can amplify from either Ggt and Ggg template (see Appendix B). Sequences of the primers are shown in Chapter 3 and Appendix B. For PCR condition, each 50 µl reaction volume contained 50 pmol of each primer, 2.5 U of *Taq* DNA polymerase (Qiagen Inc. Valencia, CA 91355), 200 µM of each deoxynucleotide triphosphates, 1 x reaction buffer, 4.5 mM $MgCl_2$ and 0.5-1 µg of template DNA. PCR was performed in a thermal cycler (Mastercycler Gradient, Eppendorf Scientific Inc., Westbury, NY 11590) programmed for an initial denaturation of 5 min at 95°C, followed by 35 cycles of 94°C for 1 min, 65°C for 2 min, and 72°C for 2:30 min. At the end of this incubation, additional incubation for 10 min at 72°C was always included to ensure complete extension of PCR products. For analyses of PCR products see Chapter 3.

Results

Yield of total DNA extracted was higher in the crown and culm than in the leaf sheaths (Fig. C.1) but purity of the DNA sample was higher in the leaf sheaths than the former parts. However, DNA solution obtained from all plant parts had light brown color.

For PCR assay, genomic DNA of Ggt M1 was added as a control in order to detect inhibitory substances present in the DNA samples. For first PCR trial, primer Ggt-2 which has lower specificity than primer Ggt-4 was used for 5' primer with a 3' universal

primer. PCR results are shown in Fig. C.2. No band was observed from samples containing DNA extracted from wheat. However, a right size PCR product was produced from sample containing 500 μg of DNA from wheat + 100 μg of Ggt M1 DNA. This indicated that this DNA sample contained less inhibitory substances than other samples.

Primer Ggt-4 was later used for 5' primer and concentration of template DNA was raised to 1 μg. No PCR product was observed from all samples (Fig. C.3).

Discussion and comments

The objective of these experiments was to prove that our identification and differentiation test could be applied directly to field samples. However, attempts with soil samples failed since yield of DNA extracted from soil organic matter was very low and contaminated with high levels of phenolic substances. Therefore, direction was changed towards diseased wheat samples. Higher yield of total DNA was obtained from wheat but most of them were wheat DNA. Although a mass of runner hyphae of *G. graminis* was observed microscopically on the culms of these samples, we had no clue how much fungal DNA was presented in the total DNA sample. In addition, dead wheat tissues contain high levels of tannins, which are potent inhibitors of *Taq* DNA polymerase in PCR. All PCR amplifications were fail regardless of the specificity of 5' primers. It is interesting to note that when DNA of Ggt M1 was increased to 100 ng in a sample containing 500 ng of DNA extracted from leaf sheath, a PCR product was produced. This suggested a possibility of overcoming the effect of inhibitory substances in the reaction. Since some inhibitors may form complexes with DNA template, increasing the concentration of specific DNA template will increase a number of free DNA molecules available for efficient amplification. However, too much of an increase in DNA template extracted from wheat tissues may have a reverse effect on PCR amplification.

In conclusion, the quality of DNA extracted from dead wheat tissues using this method was not good for PCR. Improvement of the protocol is needed in order to remove all phenolic substance from DNA samples. Optimization of PCR condition such as $MgCl_2$

and primer concentrations may be important. Moreover, direct method for amplification of DNA from plant tissues sample may be useful due to no requirement for DNA isolation (see Chapter 5).

1 2 3 4 5 6

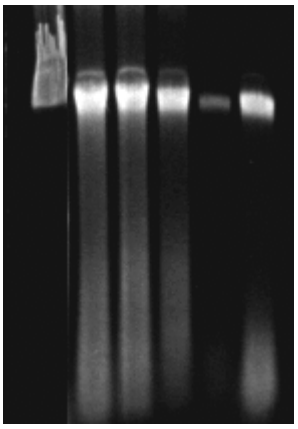

Fig. C.1. Genomic DNA isolated from wheat samples with take-all disease. Total DNA was isolated as using modification of CTAB method and 5 µl of each sample was loaded on 0.8% agarose gel in 0.5 x TBE. The gel was stained with ethidium bromide (0.5 µg/ml final concentration, visualized under UV light at 302 nm and photographed using a Polaroid camera with a Wratten. 22A filter. Lane 1 contains 500 ng of lambda DNA as a control DNA. Lanes 2-4 contain DNA isolated from culms, crowns and roots of wheat. Lanes 5 and 6 contain DNA isolated from lower leaf sheaths of wheat.

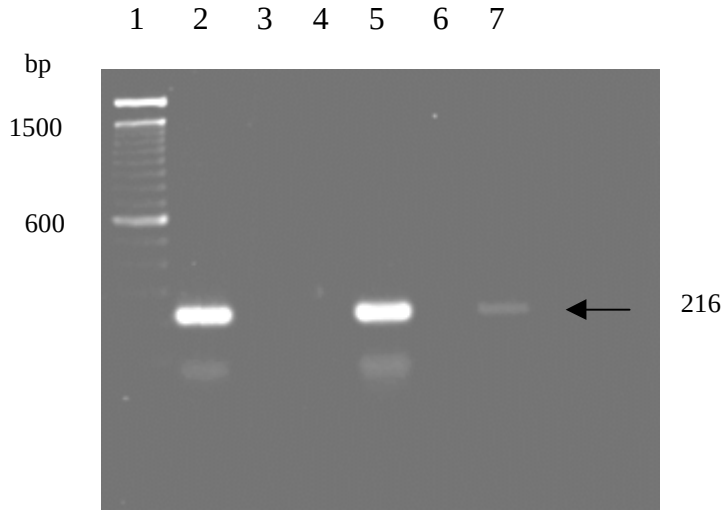

Fig. C.2. PCR amplification of DNA extracted from diseases wheat using Ggt-specific primer (Ggt-2) and 500 ng of total DNA template. Genomic DNA from Ggt M1 was added as an internal control for detection of inhibitory substances present in DNA samples prepared from wheat tissues. PCR products (15 µl each) were electrophoresed on 1.8% agarose gel, stained with ethidium bromide, visualized and photographed with 302 nm UV light using Polaroid camera with a Wratten 22A filter. Lane 1 contains 100 bp DNA ladder as molecular weight standards. Lane 2 contains 50 ng/µl of Ggt M1 DNA only. Lane 3 contains DNA from leaf sheaths only. Lane 4 contains DNA from leaf sheath and 50 ng/µl of Ggt M1 DNA. Lane 5 contains 100 ng/µl of Ggt M1 DNA only. Lane 6 contains DNA from leaf sheaths only. Lane 7 contains DNA from leaf sheath and 50 ng/µl of DNA of Ggt.

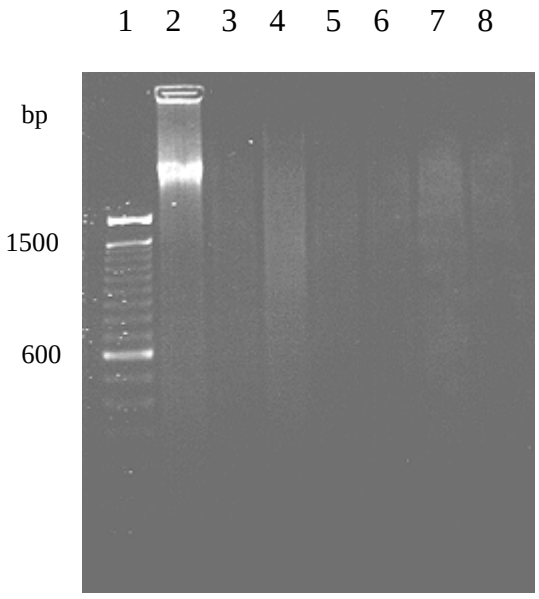

Fig. C.3. PCR Amplification of DNA extracted from PCR amplification of DNA extracted from diseases wheat using Ggt specific primer (Ggt-4). Genomic DNA from Ggt M1 was added as an internal control for detection of inhibitory substances present in DNA samples prepared from wheat tissues. PCR products (15 µl each) were electrophoresed on 1.8% agarose gel, stained with ethidium bromide, visualized and photographed with 302 nm UV light using Polaroid camera with a Wratten 22A filter. Lane 1 contains 100 bp DNA ladder as molecular weight standards. Lane 2 contains 5 µl of pure DNA extracted from wheat as a control. Lanes 3 and 4 contain PCR products from 1 and 2 µg of total DNA extracted from wheat. Lanes 5-8 contain PCR products from 1 µg of total DNA extracted from wheat and 100, 50, 20, and 10 ng, respectively, of Ggt M1genomic DNA.

APPENDIX D

DNA SEQUENCES OF Gga-, Ggg-, AND Ggt-DIAGNOSTIC FRAGMENTS

1. Alignment of Gga-specific fragment, the published avenacinase gene of Gga (Genbank accession number U35463), and the avenacinase gene fragment of Gga ATCC 15419. Alignments were performed using Clustal method with Weighted residue weight table.

```
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1     A G A T G T T G C G C T C A A G T G C T T T C G C T C T C C T C G C C T G G G C    Gga ATCC 15419
1     ----------------------------------------------------------------------------    diagnostic Gga

41    C T C C C T C T C G G A A G C C C A G T T C G G G C A T C A A G C A T A C T C A G    Gga U35463
41    C T C C C T C T C G G A A G C C C A G T T C G G G C A T C A A G C A T A C T C A G    Gga ATCC 15419.
1     ----------------------------------------------------------------------------    diagnostic Gga

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1     ----------------------------------------------------------------------------    diagnostic Gga

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1     ----------------------------------------------------------------------------    diagnostic Gga

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1     ----------------------------------------------------------------------------    diagnostic Gga

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1     ----------------------------------------------------------------------------    diagnostic Gga
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361  C C T A C A G G A C G G G C C G G C C A C C C T C C G C C A G G C C A C T T A C   Gga ATCC 15419.
1    ----------------------------------------------------------------------------------------------------   diagnostic Gga

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1    ----------------------------------------------------------------------------------------------------   diagnostic Gga

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1    ----------------------------------------------------------------------------------------------------   diagnostic Gga

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484   T G T C G A C G G C T C C A C G G T G T C C G T C G G - T T C T G G C C G C C C    diagnostic Gga

1321  G G C T T C T G G A G C C A C G A A T T C C C C C T C G G C C C C A C G G T C G    Gga U35463
1321  G G C T T C T G G A A C C A C G A A T T C C C C C T C G G C C C C A T G G T T G    Gga ATCC 15419
523   G G C T T C T G G A A C C A C G A A T T C C G C C T C G G C C C C A - G A G G G    diagnostic Gga

1361  A C G T G C G C A G G A A C C A C C A T G A G C A                                Gga U35463
1361  A C G T G C G C A G G A A C C A C C A T G A G C A                                Gga ATCC 15419
562   A T - - T G C A A A N G N N G ---------- A T T N G C                            diagnostic Gga
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2. Alignment of Ggg-specific fragment and the avenacinase-like gene fragment of Ggg ATCC 12761. Alignment was performed using Clustal method with Weighted residue weight table.

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1      A G A T G T T G C G C T C A A G T G C T T T C G C T C T C C T C G C T T G G G C    Ggg ATCC
1      G N A C A T ------------------------------------------------------------------    Diagnostic Ggg

41     C T C C C T C T C G G A A G C C C A G T T C G G C A T C A A G C A T A C T C A G    Ggg ATCC
7      ------------------------------------------------------------------------------    Diagnostic Ggg

81     T A T G G C A C G A G C G A G C C T G T C T A C C C G T C G C G T A C G T T G T    Ggg ATCC
7      ------------------------------------------------------------------------------    Diagnostic Ggg

121    C A A C A A G C C A A A A G C C T T C C G C A G A C C A T C C C A C T T T T T T    Ggg ATCC
7      ------------------------------------------------------------------------------    Diagnostic Ggg

161    T T C T G T C T C G T A C T T G T G C T A A T C T G C T C G C A C C T C T A G C    Ggg ATCC
7      ------------------------------------------------------------------------------    Diagnostic Ggg

201    C G A A A T C T T G G G C T C T G G A G G A T G G G A A G C T G G C C T G G C C    Ggg ATCC
7      ------------------------------------------------------------------------------    Diagnostic Ggg

241    A A A G C C A A G G A C T T C G T C G C G C A G C T G A C G C C C G A G G A G A    Ggg ATCC
7      ------------------------------------------------------------------------------    Diagnostic Ggg

281    A G G C G A A C A T G G T C A C G G G C A C C C C C G G T C C C T G C G T A A G    Ggg ATCC
7      ------------------------------------------------------------------------------    Diagnostic Ggg

321    C A A C A T C G C C C C C G T G C C G C G C C T C A A C T T C A C C G G C C T G    Ggg ATCC
7      ------------------------ C C C C C T G C C G C G C C T C A A C T T C A C C G G C C T G    Diagnostic Ggg

361    T G C C T A C A G G A C G G C C C G G C C A C C C T C C G C C A G G C C A C T T    Ggg ATCC
38     T G C C T A C A G G A C G G C C C G G C C A C C C T C C G C C A G G C C A C T T    Diagnostic Ggg

401    A C G T C A C C G T C T T C C C G G G C G G T G T C A G C G C G G C T T C G T C    Ggg ATCC
78     A C G T C A C C G T C T T C C C G G G C G G T G T C A G C G C G G C T T C G T C    Diagnostic Ggg

441    G T G G G A C A A G G A C C T C A T C T A C A A G C A C G G C G T G C T G A T G    Ggg ATCC
118    G T G G G A C A A G G A C C T C A T C T A C A A G C A C G G C G T G C T G A T G    Diagnostic Ggg

481    G C C C G A G A G T T C C G T G A C A A G G G C T C T C A C A T C A T C C T C G    Ggg ATCC
158    G C C C G A G A G T T C C G T G A C A A G G G C T C T C A C A T C A T C C T C G    Diagnostic Ggg

521    G C C C T G T A A T T G G T C C C C T T G G A A G G T C C C C G T A C G C C G G    Ggg ATCC
198    G C C C T G T A A T T G G T C C C C T T G G A A G G T C C C C G T A C G C C G G    Diagnostic Ggg
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561	G C G C A A C T G G G A G G G A T T C T C C C C C G A C T C G T A C C T C G C G	Ggg ATCC
238	G C G C A A C T G G G A G G G A T T C T C C C C C G A C T C G T A C C T C G C G	Diagnostic Ggg
601	G G C G T C C T G G C A G A G C A G A C G G T C A A G G G G A T G C A G G T A A	Ggg ATCC
278	G G C G T C C T G G C A G A G C A G A C G G T C A A G G G G A T G C A G G T A A	Diagnostic Ggg
641	G G G G C C C T C T C C A G C A A C A T G T T G G C G C C G A G C C T A T T C C	Ggg ATCC
318	G G G G C C C T C T C C A G C A A C A T G T T G G C G C C G A G C C T A T T C C	Diagnostic Ggg
681	C T G T A A T A C T G A C A C T T T G A C A G T C G G T C G G C G T G C A A A C	Ggg ATCC
358	C T G T A A T A C T G A C A C T T T G A C A G T C G G T C G G C G T G C A A A C	Diagnostic Ggg
721	C T G C A C C A A G C A C T T C A T C G G C A A T G A G C A G G A G G A G C A G	Ggg ATCC
398	C T G C A C C A A G C A C T T C A T C G G C A A C G A G C A G G A G G A G C A G	Diagnostic Ggg
761	C G C A A C C C C A C G A C G G T G G A T G G C A A G G G G G T T G A G G C C A	Ggg ATCC
438	C G C A A C C C C A C G A C G G T G G A T G G C A A G G G G G T T G A G G C C A	Diagnostic Ggg
801	T C T C G T C C A A C A T T G A C G A C C G C A C A A T G C A C G A G A C C T A	Ggg ATCC
478	T C T C G T C C A A C A T T G A C G A C C G C A C A A T G C A C G A G A C C T A	Diagnostic Ggg
841	C C T G T G G C C C T T T T A C A A C G C C G T C A G G G C C G G C A C C A C G	Ggg ATCC
518	C C T G T G G C C C T T T T A C A A C G C C G T C A G G G C C G G C A C C A C G	Diagnostic Ggg
881	T C C A T A A T G T G C T C T T A C C A G A G G A T C A A C G G C A G C T A C G	Ggg ATCC
558	T C C A T A A T G T G C T C T T A C C A G A G G A T C A A C G G C A G C T A C G	Diagnostic Ggg
921	G C T G C C A G A A C A G C A A G A C C C T C A A C G G G C T T C T C A A G A C	Ggg ATCC
598	G C T G C C A G A A C A G C A A G A C C C T C A A C G G G C T T C T C A A G A C	Diagnostic Ggg
961	C G A G C T C G G C T T C C A G G G C T T C G T C G T G T C G G A C T G G T G C	Ggg ATCC
638	C G A G C T C G G C T T C C A G G G C T T C G T C G T G T C G G A C T G G T G C	Diagnostic Ggg
1001	G T G G C T A C C T C C C T C T A C C A G A T G A A A C A T G C A G T G C C T T	Ggg ATCC
678	G T G G C T A C C T C C C T C T A C C A G A T G A A A C A T G C A G T G C C T T	Diagnostic Ggg
1041	G T T T T T G C T A A T G G C C A T A A C A G G G C C G C T A C C C A T T C C G	Ggg ATCC
718	G T T T T T G C T A A T G G C C A T A A C A G G G C C G C T A C C C A T T C C G	Diagnostic Ggg
1081	G A G T C G C C T C C A T T G A G G C T G G T C T G G A C A T G A A C A T G C C	Ggg ATCC
758	G A G T C G C C T C C A T T G A G G C T G G T C T G G A C A T G A A C A T G C C	Diagnostic Ggg
1121	C G G A C C G C T C A A T T T T T T T G C C C C A A C C C T C G A G T C T T A C	Ggg ATCC
798	C G G A C C G C T C A A T T T T T T T G C C C C A A C C C T C G A G T C T T A C	Diagnostic Ggg
1161	T T T G G C A A G A A C A T C A C C A C T G C G G T C A A C A A C G G C A C A C	Ggg ATCC
838	T T T G G C A A G A A C A T C A C C A C T G C G G T C A A C A A C G G C A C A C	Diagnostic Ggg
1201	T C T C C T C C C G G A G G G T C G A C G A C A T G A T T G A G C G C A T C A T	Ggg ATCC
878	T C T C C T C C C G G A G G G T C G A C G A C A T G A T T G A G C G C A T C A T	Diagnostic Ggg

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1241  G A C T C C C T A C T T C G C C C T G G G T C A G G A C A A G G A C T A C C C C    Ggg ATCC
918   G A C T C C C T A C T T C G C C C T G G G T C A G G A C A A G G A C T A C C C C    Diagnostic Ggg

1281  C C T G T C G A C G G C T C C A C G G T G C C C A T C G G C T A C T T G C A G C    Ggg ATCC
958   C C T G T C G A C G G C T C C A C G G T G C C C A T C G G C T A C T T G C A G C    Diagnostic Ggg

1321  C C G A C G C C T G G A A C C A C G A A T T C C C C C T C G G C C C C A C G G T    Ggg ATCC
998   C C G A C G C C T G G A A C C A C G A A T T C C C C - T C G G C C C C A - G G T    Diagnostic Ggg

1361  C G A C G T G C G C A G G A A C C A C C A T G A G C A                            Ggg ATCC
1036  C G A C--- T G C N                                                             Diagnostic Ggg
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3. Alignment of Ggt-specific fragment and the avenacinase-like gene fragment of Ggt M1. Alignment was performed using Clustal method with Weighted residue weight table.

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1     A G A T G T T G C G C T C A A G T G C T T T C G C T C T C C T C G C T T G G G C    Ggt M1
1     A----------------------------------------------------------------------------    diagnostic Ggt

41    C T C C C T C T C G G A A G C C C A G T T C G G G C A T C A A G C A T A C T C A G    Ggt M1
2     ----------------------------------------------------------------------------    diagnostic Ggt

81    T A T G G C A C G A G C G A G C C T G T C T A C C C G T C G C G T A C G T T G T    Ggt M1
2     ----------------------------------------------------------------------------    diagnostic Ggt

121   C A A C A A G C C A A A A G C C T T C C G C A G A C C A T C C C A C T T T T T T    Ggt M1
2     ----------------------------------------------------------------------------    diagnostic Ggt

161   T T T C T G T C T C G T A C T T G T G C T A A T C T G C T C G C A C C T C T A G    Ggt M1
2     ----------------------------------------------------------------------------    diagnostic Ggt

201   C C G A A A T C T C G G G C T C T G G A G G A T G G G A A G C T G G C C T G G C    Ggt M1
2     ----------------------------------------------------------------------------    diagnostic Ggt

241   C A A A G C C A A G G A C T T C G T C G C G C A G C T G A C G C C C G A G G A G    Ggt M1
2     ----------------------------------------------------------------------------    diagnostic Ggt

281   A A G G C G A A C A T G G T C A C G G G C A C C C C C G G T C C C T G C G T G G    Ggt M1
2     ----------------------------------------------------------------------------    diagnostic Ggt

321   G C A A C A T C G C C C C C G T G C C G C G C C T C A A C T T C A C C G G G C T    Ggt M1
2     ----------------------------------------------------------------------------    diagnostic Ggt
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361	G T G C C T A C A G G A C G G C C C G G C C A C C C T C C G C C A G G C C A C T	Ggt M1
2	----------	diagnostic Ggt
401	T A C G T C A C C G T C T T C C C G G G C G G T G T C A G C G C G G C T T C G T	Ggt M1
2	----------	diagnostic Ggt
441	C G T G G G A C A A G G A C C T C A T C T A C A A G C A C G G C G T G C T G A T	Ggt M1
2	----------	diagnostic Ggt
481	G G C C C G A G A G T T C C G T G A C A A G G G C T C T C A C A T C A T C C T C	Ggt M1
2	----------	diagnostic Ggt
521	G G C C C C G T A A T T G G C C C C C T T G G A A G G T C C C C G T A C G C C G	Ggt M1
2	----------C G A T T G A C A N G T A C C A G T A C G C C G	diagnostic Ggt
561	G G C G C A A C T G G G A G G G A T T C T C C C C C G A C T C G T A C C T C G C	Ggt M1
26	G G C G C A A C T G G G A G G G A T T C T C C C C C G A C T C G T A C C T C G C	diagnostic Ggt
601	G G G C G T C C T G G C A G A G C A G A C G G T C A A G G G G A T G C A G G T A	Ggt M1
66	G G G C G T C C T G G C A G A G C A G A C G G T C A A G G G G A T G C A G G T A	diagnostic Ggt
641	A G G A G C C C T C T C C A G C A A C A T G T C G G C G C C G A G C C T A T T C	Ggt M1
106	A G G A G C C C T C T C C A G C A A C A T G T C G G C G C C G A G C C T A T T C	diagnostic Ggt
681	C C T G T A A T A C T G A C A C T T T G A C A G T C G G T C G G C G T G C A A A	Ggt M1
146	C C T G T A A T A C T G A C A C T T T G A C A G T C G G T C G G C G T G C A A A	diagnostic Ggt
721	C C T G C A C C A A G C A C T A C A T C G G C A A T G A G C A G G A G G A G C A	Ggt M1
186	C C T G C A C C A A G C A C T A C A T C G G C A A T G A G C A G G A G G A G C A	diagnostic Ggt
761	G C G C A A C C C C A C G A C G G T G G A T G G C A A G G G G G T T G A G G C C	Ggt M1
226	G C G C A A C C C C A C G A C G G T G G A T G G C A A G G G G G T T G A G G C C	diagnostic Ggt
801	A T C T C G T C C A A C A T T G A C G A C C G C A C A A T G C A C G A G A C C T	Ggt M1
266	A T C T C G T C C A A C A T T G A C G A C C G C A C A A T G C A C G A G A C C T	diagnostic Ggt
841	A C C T G T G G C C C T T T T A C A A C G C C G T C A G G G C C G G C A C C A C	Ggt M1
306	A C C T G T G G C C C T T T T A C A A C G C C G T C A G G G C C G G C A C C A C	diagnostic Ggt
881	G T C C A T A A T G T G C T C T T A C C A G A G G A T C A A C G G C A G C T A C	Ggt M1
346	G T C C A T A A T G T G C T C T T A C C A G A G G A T C A A C G G C A G C T A C	diagnostic Ggt
921	G G C T G C C A G A A C A G C A A G A C C C T C A A C G G G C T T C T C A A G A	Ggt M1
386	G G C T G C C A G A A C A G C A A G A C C C T C A A C G G G C T T C T C A A G A	diagnostic Ggt
961	C C G A G C T C G G C T T C C A G G G C T T C G T C G T G T C G G A C T G G T G	Ggt M1
426	C C G A G C T C G G C T T C C A G G G C T T C G T C G T G T C G G A C T G G T G	diagnostic Ggt
1001	C G T G G C T A C C T C C C T C T A C C A G A T G A A A C A T G C A G T G C C T	Ggt M1
466	C G T G G C T A C C T C C C T C T A C C A G A T G A A A C A T G C A G T G C C T	diagnostic Ggt

1041	T G T T T T T G C T A A T G G C C A T A A C A G G G C C G C T A C C C A T T C C	Ggt M1
506	T G T T T T T G C T A A T G G C C A T A A C A G G G C C G C T A C C C A T T C C	diagnostic Ggt
1081	G G A G - T C G C C T C C A T T G A G G C T G G T C T G G A C A T G A A C A T G	Ggt M1
546	G G A G G T C G C C T C C A T T G A G G C T G G T C T G G A C A T G A A C A T G	diagnostic Ggt
1120	C C C G G A C C G C T C A A T T T T T T T G C C C C A A C C C T C G A G T C T T	Ggt M1
586	C C C G G A C C G C T C A A T T T T T T T G C C C C A A C C C T C G A G T C T T	diagnostic Ggt
1160	A C T T T G G C A A G A A C A T C A C C A C T G C G G T C A A T A A C G G C A C	Ggt M1
626	A C T T T G G C A A G A A C A T C A C C A C T G C G G T C A A T A A C G G C A C	diagnostic Ggt
1200	A C T C T C C T C C C G G A G G G T C G A C G A C A T G A T T G A G C G C A T C	Ggt M1
666	A C T C T C C T C C C G G A G G G T C G A C G A C A T G A T T G A G C G C A T C	diagnostic Ggt
1240	A T G A C T C C C T A C T T C G C C C T G G G T C A G G A C A A G G A C T A C C	Ggt M1
706	A T G A C T C C C T A C T T C G C C C T G G G T C A G G A C A A G G A C T A C C	diagnostic Ggt
1280	C C C C T G T C G A C G G C T C C A C G G T G C C C A T C G G C T A C T T G C A	Ggt M1
746	C C C C T G T C G A C G G C T C C A C G G T G C C C A T C G G C T A C T T G C A	diagnostic Ggt
1320	G C C C G A C G C C T G G A A C C A C G A A T T C C C C C T C G G C C C C A C G	Ggt M1
786	G C C C G A C G C C T G G A A C C A C G A A T T C C C C -- T C G N C C C N A --	diagnostic Ggt
1360	G T C G A C G T G C G C A G G A A C C A C C A T G A G C A	Ggt M1
815	G T C G----------	diagnostic Ggt

APPENDIX E

DEGENERATE PRIMERS FOR MANGANESE PEROXIDASE GENE

Design of degenerate oligonucleotide primers for isolation of the genes involved in manganese oxidation in *Gaeumannomyces graminis* var. *tritici*.

Conserved regions of the deduced amino acid sequences alignment of manganese peroxidase genes (Mnp) of the following white rot fungi were used to design degenerate oligonucleotide primers.

1. *Phanerochaete chrysosporium* (Mnp-1) (Genbank accession no. PHAMNP1)
2. *Trametes versicolor* (MPGI) (Genbank accession no. TVMPGI)
3. *Pleurotus ostreatus* (Mnp) (Genbank accession no. POU 21878)

Sequences of basic and degenerate primers are:

1. Upstream primer (Mnp1): 5'-GAGACCATCTTCCAGAACGAG-3'
2. Upstream primer(Mnp2): 5'-CTGCTGGCCTCGCACTCTGT-3'
3. Downstream primer (Mnp3): 5'-CCTTGAGCAGCACCTCGAGG-3'
4. Downstream primer (Mnp4): 5'-AGTCGGACTGCAGCCGCATC-3'
5. Upstream degenerate primer (Mnpdg1): 5'-ATHMGNYTNACNTTYCAYGA-3'
6. Downstream degenerate primer (Mnpdg2): 5'-GCVACMSWGTGSGASRYSAG-3'

Mixed base code: **W**=A,T; **M**=A,C; **R**=A,G; **Y**=C,T; **K**=G,T; **S**=C,G; ; **H**=A,C,T; **B**=C,G,T; **V**=A,C,G; **D**=A,G,T; **N**=A,C,G,T.

PCR condition

PCR was carried out for 30 cycles of 95°C, 1min; 42-65°C, 1 min; 72°C, 2:30 min. and, at the end, 72°C for 7 min.

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