THE DEVELOPMENT AND UTILIZATION OF ASSAYS TO CHARACTERIZE POPULATIONS OF *GAEUMANNOMYCES GRAMINIS*:

DISSERTATION

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By

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ABSTRACT

Take-all, caused by *Gaeumannomyces graminis*, is a devastating root disease on cereal crops and turfgrasses worldwide. Three varieties of the fungus have been described and are distinguished from one another based on hyphopodium type and ascospore length. *G. graminis* var. *avenae* (Gga) produces simple hyphopodia and generally longer ascospores as compared with *G. graminis* var. *tritici* (Ggt) and var. *graminis* (Ggg). *G. graminis* isolates were subjected to morphological, molecular and pathogenicity assays in an effort to determine the most reliable method for differentiating varieties. A host pathogenicity test tube assay was developed to induce the formation of perithecia and ascospores and to determine the pathogenicity of isolates on wheat and oats. Gga isolates were distinguished from Ggt and Ggg based on pathogenicity on oats and the production of amplicons with an avenacinase-specific primer set, Gga:AV3. Ggt and Ggg were not distinguished due to overlaps among all characters.

Gga is also the causal agent of take-all on creeping bentgrass. A rapid assay was developed and used to determine the effect of root zone mix composition on take-all incidence of creeping bentgrass. Sand type and organic matter amendment were shown to influence disease incidence. Plants grown in mason sands amended with 20% compost (v/v) had significantly less root disease incidence as compared with all other mixes.

Gga is the only variety to produce avenacinase, detoxify avenacin and to infect oats. Avenacin production in creeping bentgrass was determined using fluorimetry, TLC and HPLC. Avenacin was not detected in creeping bentgrass and Ggt isolates were shown to be able to infect creeping bentgrass indicating Gga or Ggt may incite take-all patch.

The development of a real-time PCR assay for Gga was unsuccessful due non-specific amplification from other varieties. However, avenacinase sequence information obtained indicated Gga and Ggt are more closely related to each other than to Ggg. Although Ggt does not express avenacinase activity, the aspartic acid residues which are believed to be the active sites of the avenacinase enzyme are conserved within the Ggt avenacinase-like gene sequence.

Dedicated to Alex and Wade. Merci, mes petits cheris.

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TABLE OF CONTENTS

Abstract.....ii

Dedication.....iv

Acknowledgments.....v

Vita.....viii

List of Tables.....xvi

List of Figures.....xvii

Chapters:

1. INTRODUCTION.....1

1.1 History of the taxonomy of *Gaeumannomyces graminis*.....1

1.2 Disease cycle of take-all.....3

1.3 Control of take-all.....4

1.4 Oat root defense and *G. graminis*.....9

1.5 Literature Cited.....12

2. MORPHOLOGICAL, MOLECULAR, AND PATHOGENIC IDENTIFICATION OF *GAEUMANNOMYCES GRAMINIS*.....17

2.1 INTRODUCTION.....17
2.2 MATERIALS AND METHODS.....19
2.2.1 Maintenance and cultivation of fungal isolates.....19
2.2.2 Pathogenicity of isolates on wheat, oats, and creeping bentgrass.....20
2.2.3 Induction of hyphopodia.....21
2.2.4 Production of perithecia.....21
2.2.5 Ascospore measurements.....22
2.2.6 Preparation of genomic DNA.....22
2.2.7 Amplification with *G. graminis*-specific primer sets.....23
2.2.8 Statistical analysis.....25
2.3 RESULTS.....25
2.3.1 Morphological characterization.....25
2.3.2 Pathogenicity of isolates on wheat, oats, and creeping bentgrass.....26
2.3.3 Molecular characterization.....27
2.4 DISCUSSION.....29
2.5 LITERATURE CITED.....36
3. THE EFFECT OF ROOT ZONE MIX COMPOSITION ON TAKE-ALL INCIDENCE OF CREEPING BENTGRASS.....51
3.1 INTRODUCTION.....51
3.2 MATERIALS AND METHODS.....53

3.2.1 Maintenance and cultivation of fungal isolates..... 53

3.2.2 Production of millet seed inoculum..... 53

3.2.3 Effect of isolate and inoculum dose on take-all incidence and severity..... 54

3.2.4 Effect of root zone mix composition on take-all incidence..... 56

3.3 RESULTS..... 57

3.3.1 Effect of inoculum dose on take-all incidence and severity..... 57

3.3.2 Effect of root zone mix composition..... 58

3.4 DISCUSSION..... 59

3.5 LITERATURE CITED..... 65

4. PATHOGENICITY OF *GAEUMANNOMYCES GRAMINIS* ON CREEPING BENTGRASS..... 78

4.1 INTRODUCTION..... 78

4.2 MATERIALS AND METHODS..... 80

4.2.1 Root extracts..... 80

4.2.2 TLC analysis and avenacin bioassay..... 81

4.2.3 HPLC analysis of root extracts..... 82

4.2.4 Maintenance and cultivation of fungal isolates..... 83

4.2.5 Pathogenicity assays..... 83

4.2.6 Statistical analysis..... 84

4.3 RESULTS..... 85

4.3.1 Avenacin production in creeping bentgrass..... 85

4.3.2 Pathogenicity of *G. graminis* on creeping bentgrass..... 86

4.4 DISCUSSION..... 87

4.5 LITERATURE CITED..... 90

5. FOLLOWING THE DISEASE PROGRESSION OF AN ECTOTROPHIC ROOT-INFECTING FUNGUS..... 99

5.1 INTRODUCTION..... 99

5.1.1 Objective..... 101

5.2 MATERIALS AND METHODS..... 101

5.2.1 For instructor or laboratory preparers..... 101

5.2.1.1 List of materials needed for instructors or laboratory preparers..... 101

5.2.1.2 Preparation of materials for students by instructors..... 102

5.2.2 For students..... 102

5.2.2.1 List of materials needed by students..... 102

5.2.2.2 Preparation of assay tubes by students..... 103

5.3 RESULTS..... 105

5.4 Follow-up questions..... 107

5.5 Lab instructor's notes..... 108

5.6 LITERATURE CITED..... 110

6.THE DEVELOPMENT OF A REAL-TIME QUANTITATIVE PCR ASSAY FOR THE DETECTION OF *GAEUMANNOMYCES GRAMINIS* 115

6.1 INTRODUCTION 115

6.2 MATERIALS AND METHODS 117

6.2.1 Maintenance and cultivation of fungal isolates 117

6.2.2 Preparation of fungal genomic DNA 117

6.2.3 Cloning and sequencing avenacinase and avenacinase-like fragments 117

6.2.4 Design of real-time PCR primers and probe 119

6.2.5 Amplifications with TaqMan primer sets 120

6.2.6 Real-time PCR amplifications with SYBR green 120

6.2.7 Real-time PCR amplifications with probe hydrolysis 121

6.3 RESULTS 121

6.3.1 Sequences of avenacinase and avenacinase-like fragments 121

6.3.2 Development of a Gga-specific real-time PCR assay 122

6.4 DISCUSSION 124

6.4.1 Avenacinase and avenacinase-like sequences 124

6.4.2 Development of a real-time PCR assay for Gga 126

6.5 LITERATURE CITED 130

7. SUMMARY OF RESULTS 161

7.1 Morphological, molecular, and pathogenic identification of *Gaeumannomyces graminis*. 161

7.2 The effect of root zone mix composition on take-all incidence of creeping bentgrass..... 162

7.3 Pathogenicity of *Gaeumannomyces graminis* on creeping bentgrass..... 162

7.4 The development of a real-time quantitative PCR assay for the detection of *Gaeumannomyces graminis* 163

APPENDIX A..... 164

BIBLIOGRAPHY..... 191

LIST OF TABLES

Table Page

1.1 Morphological characteristics and typical host range of *Gaeumannomyces graminis* varieties.....16

2.1 Fungal isolates and their morphological, molecular, and pathogenic characteristics as assessed in this study.....39

3.1 *Gaeumannomyces graminis* var. *avenae* isolates used in this study.....68

3.2 Chemical and elemental analyses of the compost-amended root zone mixes prior to use in take-all assays.....69

3.3 The effect of root zone mix composition and inoculum dose creeping bentgrass root disease incidence and foliar dry weight.....71

4.1 List of *Gaeumannomyces graminis* isolates used in this study.....92

4.2 Fluorimetric analysis of root extracts from wheat, creeping bentgrass, and oats.. 94

4.3 Take-all incidence and severity on graminaceous hosts.....95

5.1 Observations of host growth and disease progression.....106

6.1 Amplicon melting temperatures from real-time PCR reactions performed with the primer set Gga 723F:Gga 809R.....133

LIST OF FIGURES

Figure Page

2.1 Amplicons typical of PCR reactions with the avenacinase primer sets..... 49

2.2 Characteristic amplicons produced with the 18S rDNA primer sets..... 50

3.1 The effect of isolate and inoculum dose on root disease incidence of creeping bentgrass and disease severity of oats..... 73

3.2 Regression analyses of the observed symptomatic area versus root disease incidence for Gga isolates MB 013 and MB 0114..... 76

3.3 The effect of isolate and inoculum dose on the observed symptomatic oat area... 77

4.1 TLC analysis and fungal bioassay of oat, wheat, and creeping bentgrass root extracts 97

4.2 HPLC analysis of oat root extract and the fluorescent eluate from the TLC plate..... 98

5.1 Disease cycle for take-all patch on graminaceous hosts..... 111

5.2 Vascular discoloration and runner hyphae typical of *G. graminis* root infection on creeping bentgrass..... 112

5.3 Mature perithecium of *G. graminis* embedded in creeping bentgrass tissues..... 113

5.4 Set-up of creeping bentgrass pathogenicity test tube assay..... 114

6.1 Partial nucleotide sequences of the avenacinase and avenacinase-like genes from *Gaeumannomyces graminis*..... 134

6.2 Deduced amino acid sequence corresponding to the avenacinase gene of Gga and avenacinase-like genes of Ggg and Ggt..... 152

6.3 Primer sequences for the Gga-specific real-time PCR assay.....156

6.4 Amplicons produced in PCR reactions with primer set Gga723F:Gga809R with DNA from Gga, Ggt, and Ggg..... 157

6.5 Amplification curves generated from probe hydrolysis during amplification of genomic DNA from Gga, Ggt, and Ggg with the Gga-specific primer set, Gga723F:Gga809R..... 158

6.6 Curves generated with SYBR green under probe hydrolysis conditions to monitor dsDNA accumulation amplification of TE and genomic DNA from Gga isolate MB 013 and Ggt isolate MB 0126 with the Gga-specific primer set, Gga723F:Gga809R..... 159

6.7 Melting curve analysis of amplicons generated with the Gga-specific primer set and Gga, Ggt and TE samples.....160

A.1 Amplicons generated from PCR reactions with genomic DNA from Gga isolates containing the primer set NS5:GgtRP.....164

A.2 Amplicons generated from PCR reactions containing the primer set NS5:GgtRP and genomic DNA from Gga isolates..... 165

A.3 Amplicons generated from PCR reactions containing the primer set NS5:GgtRP with genomic DNA from Gga and Ggt isolates..... 166

A.4 Amplicons generated from PCR reactions containing the primer set NS5:GgtRP with genomic DNA from Gga and *G. graminis*-like isolates..... 167

A.5 Amplicons generated from PCR reactions containing the primer set NS5:GgtRP with genomic DNA from Gga, Ggt, and Ggg isolates..... 168

A.6 Amplicons generated from PCR reactions containing the primer set NS5:GgtRP with genomic DNA from Ggg and *G. graminis*-like isolates..... 169

A.7 Amplicons generated from PCR reactions containing the primer set NS5:GgtRP with genomic DNA from *Magnaporthe grisea* and *Microdochium nivale*..... 170

A.8 Amplicons generated from PCR reactions containing the primer set NS5:GgtRP with genomic DNA from Gga and Ggt isolates..... 171

A.9 Amplicons generated from PCR reactions containing the primer set NS5:GgtRP with genomic DNA from Ggt isolate MB 0315 and Ggg isolates MB 024 and MB 035 172

A.10 Amplicons generated from PCR reactions containing the primer set NS5:GgaRP with genomic DNA from Gga and *G. graminis*-like isolates..... 173

A.11 Amplicons generated from PCR reactions containing the primer set NS5:GgaRP with genomic DNA from Gga and *G. graminis*-like isolates..... 174

A.12 Amplicons generated from PCR reactions containing the primer set NS5:GgaRP with genomic DNA from G. graminis-like and Gga isolates.....175
A.13 Amplicons generated from PCR reactions containing the primer set NS5:GgaRP with genomic DNA from Gga and Ggt isolates.....176
A.14 Amplicons generated from PCR reactions containing the primer set NS5:GgaRP with genomic DNA from Ggt and Ggg isolates.....177
A.15 Amplicons generated from PCR reactions containing the primer set NS5:GgaRP with genomic DNA from Ggg, *G. graminis*-like and known fungal isolates..... 178
A.16 Amplicons generated from PCR reactions containing the primer set NS5:GgaRP with genomic DNA from Ggt and Ggg isolates.....179
A.17 Amplicons generated from PCR reactions containing the primer set Gga:AV3 with genomic DNA from Gga, Ggt and Ggg isolates..... 180
A.18 Amplicons generated from PCR reactions containing the primer set Gga:AV3 with genomic DNA from Gga, Ggt and *G. graminis*-like isolates.....181
A.19 Amplicons generated from PCR reactions containing the primer set Gga:AV3 with genomic DNA from Gga, Ggt and *G. graminis*-like isolates.....182
A.20 Amplicons generated from PCR reactions containing the primer set Ggg:AV3 with genomic DNA from Gga and Ggg isolates..... 183
A.21 Amplicons generated from PCR reactions containing the primer set Ggg:AV3 with genomic DNA from Gga, Ggg, Ggt and *G. graminis*-like isolates.....184

A.22 Amplicons generated from PCR reactions containing the primer set Ggg:AV3 with genomic DNA from Gga, Ggt and *G. graminis*-like isolates.....185
A.23 Amplicons generated from PCR reactions containing the primer set Ggt:AV3 with genomic DNA from Gga and Ggg isolates..... 186
A.24 Amplicons generated from PCR reactions containing the primer set Ggt:AV3 with genomic DNA from Gga, Ggt, Ggg and *G. graminis*-like isolates.....187
A.25 Amplicons generated from PCR reactions containing the primer set Ggt:AV3 with genomic DNA from Gga, Ggt and *G. graminis*-like isolates.....188
A.26 Amplicons generated from PCR reactions containing the primer set AV1:AV3 with genomic DNA from Ggt isolates..... 189
A.27 Amplicons generated from PCR reactions containing the primer set AV1:AV3 with genomic DNA from Gga, Ggt, and Ggg isolates..... 190

CHAPTER 1

INTRODUCTION

1.1 History of the taxonomy of *Gaeumannomyces graminis*

Gaeumannomyces graminis is a soil-borne ascomycete fungus that infects the roots of susceptible graminaceous hosts and produces hyphopodia, dark ectotrophic runner hyphae, and ostiolate perithecia. Among the diseases incited by *G. graminis* is take-all of wheat, oats, creeping bentgrass and St. Augustinegrass and brown sheath rot of rice. Identifications and descriptions of the causal agent were first reported in Europe in the 1800's (Walker, 1981). In 1875, Saccardo described a fungus causing the rotting of the base of a grass host (*Cynodon* or *Agropyron* spp.) and named it *Rhaphidophora graminis* (Walker, 1981). He transferred the fungus to the genus *Ophiobolus* in 1881. Nine years later in France, Prilliex and Delacroix associated *O. graminis* with a root disease of wheat (Piétin) based on comparisons of Saccardo's descriptions with infected plants. Similar results were reported by Masser in 1912 for take-all of wheat and oats in England, Waters in 1920 for take-all of wheat in New Zealand, and McKinney in 1925 for take-all of wheat in America (Walker, 1981).

Turner (1940) differentiated oat take-all isolates from wheat take-all isolates based on generally longer mean ascospore lengths and pathogenicity on oats. She re-named the causal agent of take-all on oats *O. graminis* var. *avenae* (*Oga*). When Turner described

Gga the original type specimen described by Saccardo became *O. graminis* var. *graminis* based on the rules of nomenclature. However, this name would never be used.

In 1952, von Arx and Olivier renamed *Ophiobolus graminis* (the wheat take-all pathogen) to a new genus, *Gaeumannomyces*, based on differences in asci and pathogenicity on plant hosts. Isolates within the *Ophiobolus* genus produced bitunicate asci and none of the species exhibited pathogenicity towards plant hosts whereas isolates placed in the genus *Gaeumannomyces* produced unitunicate asci and infected roots of cereals and grasses. Dennis (1960) accepted the descriptions by von Arx and Olivier and moved *Oga* to the genus *Gaeumannomyces*, establishing the name *G. graminis* var. *avenae* (*Gga*) for the oat take-all fungus.

Throughout the late 1800's and early 1900's, the causal agent of take-all on wheat was referred to as *O. graminis*. In 1972, Walker published a review of the morphological characters of Saccardo's type specimen and isolates inciting take-all of wheat and oats. He found that the type specimen described by Saccardo had perithecia and ascospores similar to the wheat take-all isolates, but it also produced lobed hyphopodia which were not seen in conjunction with any of the wheat- or oat-infecting isolates. Lobed hyphopodia were also characteristic of *O. oryzinus*, which caused brown sheath rot of rice. The type specimen described by Saccardo and *O. oryzinus* were concluded to be the same fungus and renamed *G. graminis* var. *graminis* (*Ggg*). Wheat take-all isolates formed simple hyphopodia similar to oat take-all isolates, but generally shorter ascospores as compared to *Gga*. Based on the differences in mean ascospore lengths, Walker (1972) identified the wheat take-all pathogen as *G. graminis* var. *tritici* (*Ggt*). Currently, three varieties of *G.*

graminis have been described which are differentiated based on a combination of hyphopodium type and mean ascospore length (Table 1.1).

1.2 Disease cycle of take-all

All varieties exhibit a similar disease cycle on susceptible hosts (Figure 5.1). *G. graminis* generally survives from host to host as mycelium in colonized plant tissues. Fungal growth and emergence from infected crop residues has been shown to be stimulated by host roots (Brown and Hornby, 1971). *G. graminis* produces dark ectotrophic hyphae that grow along the outside of the host roots (Walker, 1981). These dark ectotrophic hyphae may grow from plant to plant disseminating the pathogen or give rise to a finer hyaline hypha that form hyphopodia. Hyphopodia serve two functions: 1) attach the fungus to the host plant and 2) penetrate the root. Hyphopodia may be deeply lobed or simple (unlobed) and simple hyphopodia are typically formed in aggregates (Walker, 1981) or in infection cushions of hyphae. Once the fungus has penetrated the root, infection begins and the fungus grows through the cortex cell layers to the endodermis and stele (Weste, 1972) where it disrupts the water and nutrient translocation abilities of the host plant as the xylem and phloem are colonized (Clarkson et al., 1975). In cereal crops, infected plants generally mature sooner than uninfected plants producing characteristic whiteheads among a group of healthy plants. Whiteheads are straw-colored, stunted, and have poorly filled spikes. In amenity turfgrasses, infected plants generally express symptoms during periods of drought or heat stress. Tip dieback is noticeable on individual grass blades within thinning, bleached patches of turfgrass.

G. graminis is homothallic (Padwick, 1939) which means one ascospore can germinate, grow and undergo sexual reproduction to produce perithecia containing asci and ascospores. As host plants begin to senesce, *G. graminis* may form perithecia embedded in the base of leaf sheaths or stems. The importance of ascospores in disseminating *G. graminis* is unknown as ascospores have been shown to be unable to generate significant levels of infection when used as the sole source of inoculum for Ggt due to their inability to compete with the established soil microbial community for nutrients (Garrett, 1939; Padwick, 1939).

Similarly, *G. graminis* isolates have limited competitive and saprophytic abilities (Cunningham, 1981). Hyphae or perithecia with ascospores remain viable only as long as the crop residues in which these structures are formed remain intact. However, *G. graminis* populations can be maintained asymptomatically on the roots of weedy grass species in the absence of economically important susceptible hosts (Brooks, 1965; Garrett, 1941; Nilsson and Smith, 1981). Over 300 grass species have been identified to be hosts for *G. graminis* leading several researchers to postulate that these are the true hosts for *G. graminis* (Nilsson and Smith, 1981).

1.3 Control of take-all

Controlling take-all can be difficult as there is no known resistance in any of the hosts infected by Gga, Ggt, or Ggg. Crop rotations are recommended for cereal crops to prevent the build-up of inoculum within the field (Yarham, 1988). In addition, cultural practices which remove or promote the degradation of infected crop residues will result in less viable inoculum and fewer infections. Weedy grass species should be removed to reduce

the chances of *G. graminis* surviving from crop to crop on living hosts. For established aesthetic areas of turfgrass in which these cultural practices cannot be used, fungicide or fertilizer applications may alleviate turf symptoms and halt the progression of the disease (Smiley et al., 1992; Smith et al., 1989).

Biological control may also be used to control or minimize take-all outbreaks in fields or areas monocultured to susceptible cereals and grasses. Two forms of suppression have been recognized, general and specific, which differ in their microbial attributes (Cook and Rovira, 1976; Rovira and Wildermuth, 1981; Weller et al., 2002). General suppression is a property of all soils and increases in suppression have been correlated to increases in microbial activity. Amending soils with organic matter and higher soil temperatures (i.e., >20^{o}C; Rovira and Wildermuth, 1981) have been shown to decrease take-all incidence or severity. General suppression arises due to the non-specific activity of microbes and it is characterized as being nontransferable between soils, able to survive pasteurization, and unrelated to the cropping history of a given site (Rovira and Wildermuth, 1981).

Alternatively, specific suppression has been shown to be transferable between soils, sensitive to pasteurization and dependent upon the monoculture of wheat or barley in fields in which take-all occurs (Smiley, 1979). Specific suppression has been shown to occur due to qualitative and quantitative shifts in microbial populations that are selected for by monocultures of wheat or established areas of creeping bentgrass in which *G. graminis* incites disease (Rovira and Wildermuth, 1981; Cook and Rovira, 1976; Sarniguet and Lucas, 1992). As specific antagonistic microbial populations build-up in the soil, take-all incidence and severity declines, hence the name take-all decline for this phenomenon. Take-all decline was defined by Rovira and Wildermuth (1981) as the

spontaneous reduction in take-all and increase in yield with the continuous cropping of wheat or barley. It has been an intensively studied example of biological control and numerous review articles have been written describing the organisms and mechanisms involved with disease control (Rovira and Wildermuth, 1981; Weller, 1988; Weller et al., 2002).

Decreases in take-all incidence were reported as early as the 1930s in fields continuously cropped to wheat, however, it was not until the 1970s that researchers began to investigate the mechanisms responsible for the sudden decrease in take-all severity. Although a variety of microorganisms have been linked to take-all suppression or shown to reduce take-all incidence including vampyrellid amoebae (Rovira and Wildermuth, 1981), avirulent *G. graminis* var. *graminis* isolates (Wong et al., 1996), and microbial populations of *Bacillus* (Kim et al., 1997; Ryder et al., 1999), *Streptomyces* (Smiley, 1978; Andrade et al., 1994), and *Trichoderma* (Ghisalberti et al., 1990), fluorescent pseudomonads have been linked to the decline phenomenon more than any other group (Andrade et al., 2994, Cook and Rovira, 1976; Smiley, 1979; Sarniguet et al., 1992a; Sarniguet et al., 1992b). Cook and Rovira (1976) concluded, based on their own research and the results of others, that antagonistic rhizoplane bacterial populations, in particular the fluorescent pseudomonads, were responsible for suppression of take-all. Pseudomonads recovered from suppressive soils were shown to be antagonistic to Ggt in *in vitro* assays (Ridge, 1976) and suppressive to take-all development in pot bioassays (Cook and Rovira, 1976). In addition, higher *Pseudomonas* spp. populations were recovered from suppressive soils as compared with conducive soils (Cook and Rovira, 1976) and from infected roots as compared with uninfected roots (Rovira and Cook,

1975). Gram negative rod-shaped bacteria were observed to colonize take-all lesions on wheat roots and lyse the infecting hyphae of Ggt (Vojinovic, 1972; Rovira and Campbell, 1975). The soil suppressive quality was found to be sensitive to pasteurization and fumigation but later studies showed the suppressive ability could be re-established through small additions (1-10%; w/w) of untreated suppressive soil into the sterilized soil (Smiley, 1979). Smiley (1979) also showed that the build-up of fluorescent pseudomonads did not occur without the presence of *G. graminis*. Rovira and Wildermuth (1981) showed TEM and SEM micrographs in which the lesions developed by the take-all fungus were colonized by fluorescent pseudomonads. No colonization and establishment of bacterial populations were observed on healthy roots.

Fluorescent pseudomonads have been shown to be rhizosphere competent, extremely competitive and to produce a variety of antifungal compounds including antibiotics and siderophores (Thomashow and Weller, 1988; Thomashow and Weller, 1990; Brisbane and Rovira, 1988; Raajimakers et al., 1999). Take-all decline has been linked to the breakdown of infested crop residues, hyphal colonization and lysis by fluorescent pseudomonads, and the inhibition of the trophic response in Ggt to wheat roots (Brown and Hornby, 1971). In addition, antibiosis has been shown to be one of the most prevalent forms of specific antagonism. Fluorescent pseudomonads have been shown to produce a variety of compounds inhibitory to Ggt including phenazine-1-carboxylic acid (PCA; Brisbane and Rovira, 1988; Ownley et al., 1992; Thomashow and Weller, 1988; Thomashow and Weller, 1990), 2,4 diacetylphloroglucinol (2,4-DAPG; Keel et al., 1996; Raajimakers et al., 1999), and pyoluteorin (Thomashow and Weller, 1990).

Numerous studies have also shown the ability of *Pseudomonas* spp. to suppress take-all when reintroduced as wheat seed treatments or bacterial drenches (Capper and Higgins, 1993; Pierson and Weller, 1994; Weller and Cook, 1983; Weller et al., 1985). Weller(1984) showed the introduction of a strain of *Pseudomonas fluorescens* colonized the roots of wheat when applied as a seed treatment and was able to maintain high populations for up to 7 weeks after planting indicating the rhizosphere fitness of this pseudomonad.

Fluorescent pseudomonads have been shown to produce iron-chelating compounds called siderophores which inhibit the growth of other microorganisms through a reduction in the available iron. Initially, siderophores were suspected to be the primary antifungal compounds responsible for inhibiting Ggt growth . Brisbane and Rovira (1988) showed the Ggt inhibition ability of a *Pseudomonas fluorescens* strain was not affected by the addition of iron to the soil used in infectivity assays illustrating that iron availability and siderophore production are not responsible for the control of take-all. They also isolated a compound from broth cultures of *P. fluorescens* identified as phenazine-1-carboxylic acid which was toxic to Ggt in vitro. Thomashow and Weller (1988) generated phenazine mutants (*Phn*$^-$) of the *P. fluorescens* strains and found greater take-all severity on plants inoculated with the *Phn*$^-$ mutant strains as compared with the wild type strain. However, *Phn*$^-$ mutants were able to colonize roots as equally as well as the wild type strain and control efficacy could be restored through complementation of phenazine biosynthesis pathway. These results indicate that the antibiotic phenazine was primarily responsible for take-all control. Later studies showed phenazine, pyoluteorin, and anthranilic acid all had some antifungal activity and could contribute to take-all control (Thomashow and

Weller, 1990). These studies illustrate the complexity of the nature of biological control as one organism can hinder Ggt with three different compounds.

Phloroglucinol has also been shown to be an important antibiotic associated with *Pseudomonas* populations present in take-all decline soils. Keel et al. (1996) found the phloroglucinol (phl) probes hybridized to 45 known diacetylphloroglucinol (DAPG) producing *Pseudomonas* strains from Europe, Africa, and the United States. These results showed that genotypically similar bacterial strains could be isolated from geographically diverse locations. Raajimakers et al. (1999) investigated the level of phloroglucinol in the rhizosphere and correlated concentrations to bacterial populations. They found the amount of phloroglucinol present in the rhizosphere of wheat is proportional to the population of phloroglucinol-producing *Pseudomonas* spp. Further studies by McSpadden-Gardener et al. (2000) correlated genetically similar *Pseudomonas* populations to take-all decline in 8 soils from 4 different locations and found DAPG producers were prevalent in take-all decline soils. These studies show a similar mechanism, an increase in the populations of antibiotic-produced Pseudomonads as the most common mechanism for establishing take-all suppressive soils.

1.4 Oat root defense and *G. graminis*

Saponins are fungitoxic plant defense compounds produced by several genera of plants including *Avena*, *Lycopersicon*, and *Solanum* spp. (Osbourn, 1996). In their general form they contain a sugar residue connected to a steroid or terpenoid residue resulting in a compound that can integrate into fungal cell membranes, bind to sterols forming pores or channels which lead to leaky membranes and eventual fungal cell death (Morrissey and

Osbourn, 1999). The fungal-like *Pythium* spp. have a different cell membrane composition and are unaffected by this compound. Plant pathogenic fungi that are sensitive to the saponins must detoxify them in order to infect.

Avena spp. have been shown to produce avenacin which is a triterpenoid saponin containing a trisaccharide residue (Crombie and Crombie, 1986; Osbourn et al., 1994; Turner, 1960). Avenacin is localized in the epidermal cell layer of root tips with the greatest concentration in the terminal 0.5-cm section of the root tip (Osbourn et al., 1994; Turner, 1960). Four avenacin compounds have been described and are referred to as avenacin A-1, A-2, B-1, and B-2 (Crombie and Crombie, 1986). Avenacins A-1 and B-1 contain *N*-methylanthranilate residues which fluoresce bright blue under ultraviolet (UV) light (Crombie and Crombie, 1986). Avenacin compounds have been shown to be produced in similar proportions in different *Avena* spp. and *A. sativa* cultivars (Crombie and Crombie, 1986). Avenacin A-1 is the most prevalent compound, comprising up to 75% of the total avenacin concentration which explains the blue fluorescence of oat root tips under UV light (Crombie and Crombie, 1986; Turner, 1960).

Turner (1961) identified a glucosidase enzyme produced in oat-attacking take-all isolates that detoxified avenacin and named the enzyme avenacinase. Avenacinase detoxifies all four of the avenacin compounds through the cleaving of the β,1-2 glucose molecules (Osbourn et al., 1995). Wheat-infecting take-all isolates (i.e., Ggt) lack avenacinase activity and fungal growth is inhibited if isolates are exposed to either purified avenacin compounds (Crombie et al., 1986) or oat root extract (Turner, 1960; Yeates and Parker, 1986).

The avenacin-avenacinase interaction has been shown to mediate the oat-*G. graminis* interaction. Ggt isolates are unable to infect oat species that produce avenacin, however, they can infect *Avena* spp. that do not produce avenacin (Osbourn et al., 1994; Papadopoulou et al., 1999). Similarly, Gga isolates with disrupted avenacinase genes lose the ability to infect oats but the ability to infect wheat is unchanged (Bowyer et al., 1995). These results clearly show avenacinase activity is the key determinant of pathogenicity for *G. graminis* on oats.

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Variety	Asci[y] (l x w µm)	Ascospores (l x w µm)	Hyphopodium Type	Host Range[z]
G. graminis var. *avenae*	110-150 X 12-16	100-130 X 2.5-3.5	Simple	Oats, Cool season grasses (particularly *Agrostis* spp.)
G. graminis var. *tritici*	80-130 X 10-15	70-105 X 2.5-3	Simple	Wheat, Barley
G. graminis var. *graminis*	100-135 X 10-15	80-105 X 2.5-3	Lobed or Simple	Rice, Warm season grasses

[y] Morphological characteristics listed are as described by Walker (1981) for the varieties.

[z] Host range is not comprehensive and species listed are the prevalent economically impacted crops.

Table 1.1. Morphological characteristics and typical host range of *Gaeumannomyces graminis* varieties.

CHAPTER 2

MORPHOLOGICAL, MOLECULAR AND PATHOGENIC IDENTIFICATION OF *GAEUMANNOMYCES GRAMINIS*

2.1 INTRODUCTION

Take-all, caused by *Gaeumannomyces graminis* (Sacc.) Arx & Olivier, is a devastating root disease of cereal crops and turfgrasses worldwide. Three varieties of *G. graminis* have been described based on differences in hyphopodium type and ascospore length (23). *G. graminis* var. *graminis* (Ggg) is typically differentiated from the other varieties based on the production of lobed hyphopodia. Ggg also infects warm season cereals and grasses such as rice (*Oryza sativa* L.) and St. Augustinegrass (*Stenotaphrum secundatum* (Walt.) Kuntze). Both *G. graminis* var. *avenae* (Turner) Dennis (Gga) and *G. graminis* var. *tritici* Walker (Ggt) produce simple hyphopodia and are distinguished from one another based on mean ascospore lengths (22). Gga generally produces longer ascospores as compared with Ggt. These two varieties can also be separated based on their ability to infect on oats (*Avena sativa* L.). Although both Gga and Ggt infect cool season graminaceous hosts such as wheat (*Triticum aestivum* L.) and barley (*Hordeum vulgare* L.), only Gga is able to infect oats (1, 16). Gga is also considered the causal agent of take-all patch of creeping bentgrass (*Agrostis stolonifera* L. syn. *A. palustris* Huds.) (19, 20).

Clinical diagnosis of take-all patch of creeping bentgrass is typically made based on the observation of foliar symptoms, the presence of dark ectotrophic runner hyphae on the

roots, and vascular discoloration (21). However, other *Gaeumannomyces*, *Magnaporthe*, *Ophiosphaerella*, and *Phialophora* spp. have been shown to exhibit similar ectotrophic growth along roots of cereals and grasses. Collectively, the pathogenic species of these genera are referred to as ectotrophic root-infecting fungi (ERIs). The identification of isolates recovered from symptomatic plant tissues is rarely made and is confounded by similar colony morphologies. In addition, non-pathogenic *Phialophora* spp. anamorphs have been described for each *G. graminis* variety but these fungi exhibit similar growth habits on roots and in culture as *G. graminis* (24). Perithecia and ascospores can be used to distinguish *G. graminis* from their anamorphs and other ERIs, but perithecia are rarely produced in a timely manner in culture, if produced at all (14). Numerous reports of ascospore lengths for *G. graminis* varieties have also shown that overlaps in ascospore lengths between Gga and Ggt limit the ability to identify isolates to the variety level based on morphology (4, 24, 30, 31).

Due to the difficulty in identifying isolates based on morphology, researchers have developed molecular assays to detect or identify *G. graminis* and its varieties. Molecular tools for fungal identification often provide speed and specificity that other identification methods lack. Amplification of mitochondrial DNA (12, 13, 18), RFLP analysis of rDNA sequences (15, 25, 26) and RAPD banding patterns (11, 28) have been used with some success in distinguishing *G. graminis* varieties from other genera and each other. More recently, researchers have focused on the sequence differences in the rRNA genes and the internal transcribed spacers (ITS) regions surrounding them to differentiate *Gaeumannomyces*, *Magnaporthe*, and *Phialophora* spp. (3, 8, 10). Fouly and Wilkinson (8) found the 18S rRNA genes of *G. graminis* contained insertions of different sizes

which were consistent among varieties *avenae* and *tritici*. Based on sequence differences among the insertions, Fouly and Wilkinson (9) developed variety-specific primers for Gga and Ggt. Rachdawong et al. (17) took a different approach by sequencing the avenacinase gene in Gga and the avenacinase-like genes in Ggt and Ggg. Variety-specific primer sets were developed based on nucleotide differences among the varieties. Comparisons of the primer sets developed for the *G. graminis* varieties by Fouly and Wilkinson (9) and Rachdawong et al. (17) to identify and differentiate *G. graminis* isolates have not yet been made using the same collection of fungal isolates. In addition, morphological and pathogenic data have not been correlated with the results of either variety-specific primer set. The objective of this study was to assess the utility of morphological, molecular, and pathogenic characteristics to identify *G. graminis* isolates to the variety level.

2.2 MATERIALS AND METHODS

2.2.1 Maintenance and cultivation of fungal isolates.

Fungal isolates and purified DNA samples (Table 2.1) used in this study were obtained from infected plant tissues and acquired from other researchers. Isolates originated from England, France, Canada, and 16 states within the United States. Rifampicin-amended (100 μg ml^{-1} ; Sigma Chemical Corp., St. Louis, MO) 1/5 potato dextrose agar (1/5 PDA_{RIF}; Becton Dickinson, Cockeysville, MD) was used in the recovery of isolates from infected plant material and to characterize isolates received from other researchers. When grown on 1/5 PDA_{RIF}, *G. graminis* isolates induce a color change from orange to purple in the area immediately surrounding mycelial growth (7). Isolates growing from infected

roots that caused the expected color change and exhibited the curling back of hyphae at the colony margin typical of *G. graminis* (24) were selected and used in this study. There were 27 Gga, 11 Ggt and 12 Ggg isolates and 7 purified DNA samples (1 Gga, 5 Ggt and 1 Ggg) provided by Erik Stromberg. There were also 15 *G. graminis*-like isolates which were collected from bentgrass roots. In addition, there was 1 isolate of *Phialophora graminicola*, *G. cylindrosporus*, *Magnaporthe grisea*, and *Microdochium nivale*.

All isolates were maintained on 1/5 potato dextrose agar amended with 100 μg ml^{-1} penicillin and streptomycin sulfate (1/5 PDA; Sigma Chemical Corp., St. Louis, MO). Isolates were stored as colonized agar disks (5-mm diam.) at -80°C in 1/5 strength potato dextrose broth (PDB; Becton Dickinson, Cockeysville, MD) amended with 15% glycerol and as colonized agar plates at 4°C covered with sterilized mineral oil (27). Isolates were transferred monthly to freshly prepared media and maintained in culture for a maximum of six months (six generations) then re-isolated from stored cultures.

2.2.2 Pathogenicity of isolates on wheat, oats, and creeping bentgrass.

The ability of each isolate to infect wheat (cv. Norm), oats (cv. Armor) and creeping bentgrass (cv. Penneagle) was assessed using the test tube pathogenicity assays described in Chapter 5. Pathogenicity assays were incubated in a growth chamber set to provide an 18 h photoperiod (18 h 18°C; 6 h 12°C) for 5 to 10 weeks. Microscopic observations of root colonization and perithecial development were made weekly. All isolates were tested twice on each host.

2.2.3 Induction of hyphopodia.

Hyphopodia were observed using clarified V8 juice agar as described by Crozier (5). V8 juice was centrifuged (8,000 rpm, 10 min) and the supernatant collected. Clarified V8 juice agar was produced by mixing 200 ml of supernatant with 800 ml water, 4 g $CaCO_3$ and 17.5 g agar. Prior to inoculation, the agar in half of the petri dish was removed exposing a portion of the petri dish bottom. An 8-mm diameter agar plug was taken from the margin of an actively growing culture of *G. graminis* and placed on the clarified V8 juice agar 2 to 3 mm from the cut edge. Plates were incubated in the dark at room temperature (25 to 27ºC). Microscopic observations were made every 2 days for 14 days following inoculation. Hyphopodia were produced against the exposed plastic petri dish bottom. The type of hyphopodia produced by each isolate was determined annually throughout the study.

2.2.4 Production of perithecia.

Perithecia were induced in culture using the aforementioned pathogenicity test tube assays with wheat, oats and creeping bentgrass and on post-harvest soybean litter based on the protocol described by Crozier (5). Dried pods and stems were collected from a soybean field. Stem pieces were cut into 2.5 cm sections and pods were separated into halves. Stem and pod pieces were soaked overnight in distilled water and excess water was decanted. For each isolate, two pod and stem pieces were placed in a glass petri dish containing two pieces of Whatman no. 1 filter paper (7-cm diam.). Prior to autoclaving, the filter paper was moistened with distilled water. Upon cooling, two agar plugs (8-mm diam.) taken from the margin of actively growing cultures were placed on the filter paper

adjacent to the stem and pod pieces. Dishes were sealed with Parafilm (Pechiney Plastic Packaging, Neenah, WI) and incubated for 6 to 10 weeks in a growth chamber set to provide conditions identical to those previously described. All isolates were tested twice on soybean litter.

2.2.5 Ascospore measurements.

Ascospore measurements were made from ascospores released from asci in perithecia upon placement in a drop of water on a microscope slide. Spore measurements were made using an ocular micrometer on a Nikon Eclipse E400 compound microscope (Nikon Instruments Inc., Melville, NY) under the 40X objective (total magnification was 400X). Three mature perithecia were examined for each isolate in each assay in which perithecia were produced. Thirty spore measurements were taken from each perithecia examined and the maximum number of spore measurements made for any isolate was 360. The ascospore length range reported for each variety was calculated as the mean spore length plus or minus two standard deviations.

2.2.6 Preparation of genomic DNA.

Five mycelial plugs (8-mm diam.) from the margins of actively growing cultures were used to inoculate 125 ml of 1/5 PDB in 250-ml Erlenmeyer flasks. Cultures were grown in an orbital shaker for 5 to 7 days at 25^{o}C, 120 rpm. Mycelia were harvested on sterile Whatman no. 1 filter paper by vacuum filtration. Agar plugs were excised from the mycelial mats and discarded. Mycelial mats were transferred to sterile 1.6-ml microcentrifuge tubes and stored at -20^{o}C until DNA extraction.

Harvested mycelia was ground in liquid N_2 with a mortar and pestle. Ground mycelia was placed in 1.6-ml centrifuge tubes containing 500 µl of lysis buffer (50 mM Tris-HCl, pH 7.2; 50 mM EDTA, pH 8.0; 3% [v/v] SDS; and 1% [v/v] 2-mercaptoethanol added just before use) and incubated at 65°C for 1 hr. DNA was extracted with 700 µl chloroform and 700 µl chloroform:isoamyl alcohol (24:1; v/v). DNA was pelleted by adding 50 µl of 3 M sodium acetate and an equal volume of isopropanol, and centrifuging at 10,000 rpm for 20 min. The pellet was washed with 1 ml of 70% ethanol, allowed to dry, then resuspended in 100 µl TE (10 mM Tris-HCl, pH 8.0 and 1 mM EDTA, pH 8.0). RNAse A was added to a final concentration of 0.5 µg μl^{-1}. The solution was incubated at 4°C overnight, repelleted by centrifugation (5,000 rpm for 5 min) then the supernatant was transferred to a new tube and used for DNA analyses. DNA concentrations were determined with a spectrophotometer (GeneQuant *pro*, Amersham Biosciences, Piscataway, NJ) and diluted to 10 ηg μl^{-1} for use in PCR reactions.

2.2.7 Amplification with *G. graminis*-specific primer sets.

Varietal-specific PCR primer sets for the avenacinase and avenacinase-like genes (17) and the 18S rRNA gene (9) were evaluated for specificity against fungal isolates (Table 2.1). All reactions were carried out in 15-µl reaction volumes containing 1x reaction buffer, 200 µM dNTPs, 0.375 U Platinum *Taq* DNA polymerase (Invitrogen Corp., Carlsbad, CA) and 10-20 ηg fungal DNA. Amplification reactions were performed using a thermal cycler (PTC-200, Peltier Thermal Cycler, M.J. Research, Inc, Reno, NV). Each isolate was tested twice against all primer sets.

The avenacinase and avenacinase-like primer sets AV1:AV3, Gga:AV3, Ggt:AV3, and Ggg:AV3 were used with PCR conditions described by Rachdawong et al. (17). Primers were synthesized by Invitrogen (Carlsbad, CA). PCR was carried out with primer set AV1:AV3 using amplification mixtures containing 4.5 mM $MgCl_2$ and 50 ηg of each primer with the following cycle: an initial denaturation at 95°C for 3 min; then 35 cycles of denaturation at 94°C for 45 sec, annealing at 52°C for 1 min and extension at 72°C for 2.5 min; followed by a final extension period at 72°C for 7 min.

Amplification mixtures with primer sets Gga:AV3, Ggt:AV3, and Ggg:AV3 contained 3 mM $MgCl_2$ and 50 ηg of each primer. PCR was performed with the following cycle: an initial denaturation at 95°C for 3 min; then 35 cycles of denaturation at 94°C for 45 sec and annealing and extension at 72°C for 2.5 min; followed by a final extension period at 72°C for 10 min.

The 18S rDNA primer sets NS5:NS6, NS5:GgtRP, and NS5:GgaRP were used under PCR conditions described by Fouly and Wilkinson (9). Primers were synthesized by Integrated DNA Technologies (Coralville, IA). Reaction mixtures contained 2 mM $MgCl_2$ and 0.4 μM of each primer. DNA was amplified using the following cycle: an initial denaturation at 93°C for 3 min; then 35 cycles of denaturation at 93°C for 1 min, annealing at 52°C for 1 min and extension at 72°C for 1 min; followed by a final extension period at 72°C for 5 min.

PCR products were separated by electrophoresis on 1.2 to 1.4% agarose gels, visualized with ethidium bromide, and photographed with the Gel DOC-IT DNA imaging system (Ultra-Violet Products, Inc., Upland, CA). All reactions were performed twice.

2.2.8 Statistical Analysis.

Two sample t-tests were used to assess the effect of host material and perithecial maturity on measured ascospore lengths. Analyses were performed using Minitab Statistical Software (Minitab Inc., College Sta., PA).

2.3 RESULTS

2.3.1 Morphological characterization.

All *G. graminis* and *G. graminis*-like isolates exhibited curling back of hyphae at the colony margin and most (60/65) induced the color change from orange to purple on 1/5 PDA_{RIF} (data not shown). Most isolates (18/27 Gga; 8/11 Ggt; 10/12 Ggg; and 8/15 *G. graminis*-like) produced hyphopodia when grown on clarified V8 agar (Table 2.1). Isolates consistently produced the same type of hyphopodia when retested. Simple hyphopodia were produced by Gga, Ggt, and *G. graminis*-like isolates. Of the Ggg isolates producing hyphopodia, nine produced lobed hyphopodia whereas Ggg isolate MB 036 produced simple hyphopodia.

The median number of ascospore measurements made per isolate was 180 due to differences in isolates' abilities to produce perithecia on wheat, oat, creeping bentgrass, and soybean litter. Most Gga isolates (18/27) produced perithecia and ascospores (Table 2.1). Of the 18 Gga isolates producing perithecia, 13 produced them on soybean litter, 16 in pathogenicity test tube assays and 11 with both methods. The mean ascospore length calculated for Gga was 110.9 μm with a range (95% confidence interval) of 85.1 to 135.3 μm. Perithecia and ascospores were produced by four Ggt isolates. Isolates MB 025, MB 026, and MB 029 produced perithecia on soybean litter whereas isolates MB 0126 and

MB 029 produced perithecia in the pathogenicity test tube assays. The mean ascospore length calculated for Ggt was 79.2 µm with a range of 68.2 to 90.1 µm. These measurements were shorter than those for Gga and Ggg. Of the five Ggg isolates producing perithecia, only isolate MB 0220 produced perithecia on the soybean litter and the remaining four isolates (MB 035, MB 037, MB 038, and MB 039) produced perithecia in the wheat pathogenicity test tube assays. The mean ascospore length calculated for Ggg was 88.1 µm with a range of 81.8 to 94.4 µm. Isolates producing perithecia on more than one type of host material (i.e., soybean litter, wheat, oat, or bentgrass) had ascospore lengths not significantly different (P=0.05) from one another. Perithecia which had to be broken up to release asci and ascospores were shown to have significantly (P=0.05) shorter ascospore mean lengths and ranges as compared with measurements made from perithecia in which asci and ascospores were ejected of the same isolate. None of the *G. graminis*-like isolates produced perithecia. Less than half (22/50) of the Gga, Ggt, and Ggg isolates produced both hyphopodia and ascospores.

2.3.2 Pathogenicity of isolates on wheat, oats, and creeping bentgrass.

All Gga and Ggt isolates infected and colonized wheat roots producing vascular discoloration within 14 days of assay initiation. Ggg isolates MB 0220 and MB 039 both exhibited pathogenicity on wheat, however, isolate MB 039 was deemed weakly aggressive as infection and colonization of the roots was observed only after 21 days.

All Gga isolates were pathogenic on oats and creeping bentgrass. None of the Ggt isolates infected oats but several Ggt isolates (MB 029, MB 025, and MB 0311) produced vascular discoloration in, the roots of creeping bentgrass. None of the Ggg isolates

infected oats or creeping bentgrass. Isolates recovered from symptomatic creeping bentgrass that failed to exhibit pathogenicity on any host were identified as *G. graminis*-like due to the similar culture morphology.

2.3.3 Molecular characterization.

Each isolate was tested twice against all primer sets with similar results and gel pictures are presented in Appendix A. The AV1:AV3 primer set amplified a 1.4 kb fragment within the avenacinase gene in Gga and avenacinase-like genes in Ggt and Ggg. All Gga and Ggt isolates, with the exception of Gga isolates MB 0319 and MB 0317, produced 1.4 kb amplicons when genomic DNA was amplified (Table 2.1). Only 10/13 Ggg isolates (12 Ggg isolates in culture and 1 Ggg DNA sample) in this study produced the expected 1.4 kb amplicon. No amplicons were produced when AV1:AV3 was tested using DNA from *G. graminis*-like isolates.

All Gga isolates produced 617 bp fragments when genomic DNAs were amplified with the Gga-specific primer set, Gga:AV3 (Table 2.1, Figure 2.1). No amplification occurred in PCR reactions using DNA from Ggt, Ggg, or *G. graminis*-like isolates with this primer set.

Primer set Ggt:AV3 was designed to be specific to variety *tritici* isolates which produced amplicons of 870 bp (Figure 2.1). Only 10/16 Ggt isolates produced the expected amplicon when tested against Ggt:AV3. Twenty-five percent of Gga isolates (7/28) also produced amplicons of 870 bp in length when genomic DNA was amplified with Ggt:AV3. None of the Ggg or *G. graminis*-like isolates produced amplicons with this Ggt-specific primer set.

Only one of the Ggg isolates (MB 024) produced the expected 1086 bp amplicon when tested using the primer set Ggg:AV3 (Figure 2.1). Several of the Gga (8/28) and Ggt (5/16) generated 1086 bp products when amplified with the Ggg:AV3 primer set. No amplicons were observed when DNA from *G. graminis*-like isolates was amplified with Ggg:AV3.

The universal 18S rDNA primer set NS5:NS6 was expected to produce amplicons of 607 bp from Gga isolates, 627 bp from Ggt isolates, and 310 bp from other fungi. Most Gga isolates (18/28) generated fragments of 607 bp when genomic DNA was amplified (Table 2.1, Figure 2.2). Several Gga isolates (7/28) produced 950 bp amplicons. The remaining Gga isolates produced amplicons of different lengths. All of the Ggt isolates (16/16) generated fragments of 627 bp when amplified with the NS5:NS6 primer set. Ten Ggg isolates produced 310 bp fragments. Of the remaining three Ggg isolates, isolates MB 024 and MB 016 produced 627 bp amplicons and isolate MB 034 produced a 950-bp amplicon. Most of the *G. graminis*-like isolates generated 310 bp amplicons and several isolates produced additional amplicons of larger sizes.

The Ggt-specific 18S rDNA primer set NS5:Ggt-RP was designed to produce unique amplicons of 300 and 410 bp from Gga and Ggt isolates, respectively (Figure 2.2). Amplicons generated from reactions with Gga genomic DNA were measured to be 225 bp in this study and 21/28 Gga isolates produced fragments of this size. Gga isolate MB 033 produced a fragment of 410 bp and four Gga isolates failed to produce any amplicon when tested against the NS5:Ggt-RP primer set. All of the Ggt isolates produced amplicons of 410 bp. Most Ggg isolates (7/13) did not produce any amplicons when amplified with this primer set, however, isolates MB 015, MB 016, MB 0220, and MB

024 produced amplicons equivalent in length (i.e., 410 bp) to those produced by Ggt isolates. The remaining Ggg isolates produced fragments of differing sizes. Similar to the results for Ggg, 10/15 *G. graminis*-like isolates did not produce an amplicon when genomic DNA was amplified with the NS5:Ggt-RP primer set and several isolates produced fragments of 225 or 410 bp. The *G. graminis*-like isolate MB 0120 produced an amplicon of 520 bp which was not observed for any other isolate.

A 400 bp amplicon was expected when genomic DNA from Gga and Ggt was amplified with the NS5:Gga-RP primer set (9). Most (24/28) of the Gga isolates gave the expected amplicon (Figure 2.2). Gga isolates MB 0319, MB 018, MB 003, and MB 0217 did not produce any amplicons. All of the Ggt isolates produced the expected 400 bp amplicon. About half (6/13) of the Ggg isolates did not produce an amplicon when genomic DNA was amplified with NS5:Gga-RP, however, six isolates produced 400-bp fragments. Isolate MB 034 produced an amplicon of 750 bp which was not observed for any other isolates. The *G. graminis*-like isolates either produced 400 bp amplicons (8/15) or no amplicons (7/15).

2.4 DISCUSSION

G. graminis is currently subdivided into three varieties based on hyphopodium type and mean ascospore length (23). However, as shown by previous authors (4, 20, 30, 31) and to some extent in this study (Table 2.1), the morphological characters of the varieties overlap and are not definitive. All three varieties may produce simple hyphopodia and reported ascospore ranges typically overlap by 5-10 µm or more (24). In addition, many factors may influence the mean ascospore length determined for a given perithecium

including laboratory and environmental conditions, culture or host material, and perithecial maturity. Isolates recovered from symptomatic plant tissues often cannot be readily identified as var. *tritici* or *avenae* and remain labeled as indeterminate or intermediate (24).

Ascospores produced by Gga isolates used in this study were generally longer than those produced by Ggt and Ggg (Table 2.1) with a mean of 111 +/- 25 µm (95% confidence interval). When compared with the mean ascospore length ranges given by Walker (24) for Gga, Ggt, and Ggg, seven isolates (e.g., MB 0216, MB 0113, etc.) produced ascospores with mean lengths that fell solely in the range described for Gga (i.e., > 105 µm). However, Turner (22) differentiated oat take-all isolates (var. *avenae*) from wheat take-all isolates based on longer mean ascospore lengths and pathogenicity on oats. Twenty-seven isolates were clearly identified as variety *avenae* when pathogenicity on oats was used as a definitive character for Gga including those isolates producing ascospores with mean lengths greater than 105 µm (i.e., isolates morphologically identified as Gga), intermediate lengths (i.e., 100-105 µm) and lengths just short of 100 µm. In addition, nine isolates that failed to produce perithecia were also identified as Gga based on pathogenicity to oats illustrating that this was a reliable characteristic to distinguish Gga isolates.

The ascospore lengths observed in this study (Table 2.1) are similar to those previously reported for Gga (2, 22, 24, 30, 31), Ggt (6, 24, 31) and Ggg (24). Similar to previous results (22, 31), host did not have a significant (P=0.05) effect on ascospore length when ascospore measurements were made for the same isolate on different hosts, however, perithecial maturity significantly (P<0.001) influenced both the range and mean of

ascospore lengths. Given this observation, all ascospore measurements reported and used for isolate identification were those made only on asci and ascospores ejected from perithecia.

Yeates (31) identified take-all isolates recovered from the roots of cereals and pasture grasses based solely on morphological characters as described by Turner (22) in which isolates producing simple hyphopodia and mean ascospore lengths greater than 101 μm were classified as Gga and those producing shorter ascospores were identified Ggt. When pathogenic data was combined with the morphological data, Yeates concluded that there were two oat-infecting populations of *G. graminis*: Gga and oat-infecting isolates of Ggt. We and other researchers (4, 30) detected several isolates exhibiting pathogenicity on oats but failing to produce ascospores with means greater than 101 μm (Table 2.1). Given that a variety is a morphological variant within a species, it seems inappropriate to use pathogenicity on a host as a definitive character for *G. graminis*. However, studies by Ward and Akrofi (25) and Bryan et al. (3) have shown that the oat-attacking Ggt isolates found by Yeates (31) were more closely related to Gga rather than Ggt based on molecular analyses of rDNA regions. These results indicate that isolates with similar pathogenic abilities are genetically more alike and support differentiating varieties based on pathogenicity and morphology.

When genomic DNA was amplified with the avenacinase primer set Gga:AV3, all oat-infecting isolates produced 617 bp fragments. No amplicons were produced by any other isolates resulting in a 100% correlation between infectivity of oats and the production of amplicons with Gga:AV3 (Table 2.1). This high correlation is not surprising as avenacinase activity has been shown to be the key pathogenic determinant of *G. graminis*

on oats (1, 16). These results clearly show that the avenacinase-specific primer set Gga:AV3 can be used to identify isolates that exhibit characteristics described by Turner (22) to be typical of var. *avenae* (i.e., isolates producing generally longer ascospores and exhibiting pathogenicity on oats).

All *G. graminis* isolates recovered from symptomatic bentgrass were identified as Gga based on morphology, pathogenicity on oats, and molecular traits (Table 2.1) illustrating the prevalence of this variety as the causal agent of take-all patch on creeping bentgrass. Similar to Yeates and Parker (32), all *G. graminis* isolates recovered from *Agrostis* spp. in this study were highly pathogenic on oats. Previous work in our lad has demonstrated that creeping bentgrass does not produce avenacin, yet all isolates recovered from *Agrostis* spp. detoxified avenacin. Based on these observations, the avenacinase primer set, Gga:AV3, should give consistent results in determining Gga isolates from Ggg and Ggt.

The range of ascospore lengths measured in this study for Ggt (i.e., 68 to 90 µm; Table 2.1) and Ggg (i.e., 81 to 94 µm) overlapped illustrating that these varieties can not be differentiated based on ascospore length. Further comparisons of morphological features and pathogenic abilities revealed both Ggt and Ggg can produce simple hyphopodia and infect wheat. However, our results indicate that most Ggg isolates produce lobed hyphopodia and do not infect wheat.

Fouly and Wilkinson (9) showed DNA from Ggt isolates generated amplicons of 627 and 410 bp when amplified with the NS5:NS6 (29) and NS5:Ggt-RP primer sets, respectively. All Ggt isolates evaluated in this study generated amplicons of the expected lengths when DNA was amplified with the primer sets used and developed by Fouly and

Wilkinson (9). Although similarly-sized amplicons were produced by several Ggg isolates, the NS5 primer sets accurately distinguished 90% of the Ggt and Ggg isolates evaluated (Table 2.1).

Previously developed avenacinase-specific primer sets for Ggt and Ggg (17) were inconsistent in their abilities to classify isolates. Only 67% of the Ggt isolates evaluated in this study produced the expected 870 bp amplicon when amplified with avenacinase-like primer set Ggt:AV3, whereas, only Ggg isolate MB 024 generated the 1086 bp fragment when tested against the primer set Ggg:AV3. Cross reactivity among Gga isolates was high for both primer sets (Table 2.1). Interestingly, five Ggt isolates, which did not produce any fragments with the Ggt-specific primer set, produced 1,086 bp amplicons when amplified with Ggg:AV3.

Rachdawong et al. (17) indicated further testing was needed against a larger collection of *G. graminis* isolates to ensure specificity of their varietal-specific primer sets. They cited two examples of non-specific amplification and explained that these results may be due to the presence of mixed cultures or heterokaryons. As shown in this study (Table 2.1) cross reactivity is more prevalent than originally assessed and the utilization of these primer sets in differentiating varieties can lead to misidentifications or indeterminate identifications. This cross reactivity may be explained by a lack of specificity in primer sequences as the avenacinase primer sets were designed to incorporate nucleotide differences present among varieties, but only six isolates (1 Gga, 2 Ggg and 3 Ggt) were sequenced and the polymorphisms detected may not have been predominant throughout the varietal population.

Additionally, the misidentification of a culture from the American Type Culture

Collection (ATCC, Manassas, VA) may have led to the production of two variety-specific primer sets for Ggt and no Ggg-specific primer set. Isolate MB 024 was the only Ggg isolate to produce amplicons with Ggg:AV3 (Figure 2.1) and one of the Ggg isolates that produced amplicons of similar lengths to those produced by Ggt with the 18S rRNA primer sets (Figure 2.2). According to the ATCC, this isolate was recovered from winter wheat and deposited by Turner with the identification of *G. graminis*. Rachdawong et al. (17) utilized the isolate as Ggg, probably based on the dissimilarities in the avenacinase sequence as compared with Ggt isolates. However, molecular reactions performed in this study indicate isolate MB 024 is most likely Ggt as several of the Ggt isolates reacted only with the Ggg-specific primer set developed based on sequence information from this isolate and MB 024 reacts with the NS5:NS6 and NS5:Ggt-RP primer sets identically to other Ggt isolates. Unfortunately only a DNA sample was received of this isolate and further morphological or pathogenic characterization could not be performed. Based on these results, there could be two sub-populations of Ggt which differ slightly in their avenacinase-like gene sequence.

Molecular analyses with the avenacinase primer set AV1:AV3 facilitated identifications of isolates as most (> 90%) isolates of Gga, Ggg, and Ggt produced 1.4-kb amplicons illustrating the usefulness of this primer set in distinguishing *G. graminis* from other soil-borne fungi. When morphology was used exclusively, isolates were identified as *G. graminis* only when they produced hyphopodia, perithecia, and ascospores as described by Walker (24). Less than half (22/50) of *G. graminis* isolates used in these study met these criteria.

When reviewing the taxonomic history of *G. graminis*, one finds the conclusion by Turner (22) that oat take-all isolates differed from wheat take-all isolates was based on mean ascospore lengths made from only six oat-infecting isolates and four wheat-infecting isolates which is a very small collection. Although all of the oat-infecting isolates had mean ascospore lengths greater than 101 µm, there was a 15 µm gap in the ranges observed for wheat take-all isolates as compared with oat take-all isolates. Walker (23, 24) reviewed the taxonomy through examination of herbaria samples and take-all isolates and expanded the expected mean ascospore lengths for each variety. He concluded that Gga generally produced longer ascospore lengths as compared with Ggt and Ggg, however, overlaps do exist and in his opinion "these physiological and pathogenic differences between isolates [Gga and Ggt] do not necessarily reflect varietal differences". This study and others (4, 22, 30, 31) have shown that the mean ascospore lengths ranges for *G. graminis* varieties form a continuum and that pathogenicity on oats is the easiest distinction to make between Gga and Ggt, therefore these varieties perhaps may be better separated as forma specialis within *G. graminis*.

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Table 2.1. Fungal isolates and their morphological, molecular, and pathogenic characteristics as assessed in this study.

Isolate	Host	Source	Morphological		Molecular Primer Sets[n]							Pathogenicity	
					Avenacinase gene				18 S rRNA gene				
			Hyphopodium	Mean Ascospore Size (µm)[o]	AV1: AV3	Gga: AV3	Ggt: AV3	Ggg: AV3	NS5: NS6	NS5: GgtRP	NS5: GgaRP	Wheat	Oats
Gaeumannomyces graminis var. *avenae*													
Expectations[p]			**Simple**	**100-130 X 2.5-3.5**	**1.4 kb**	**617**	**--**	**--**	**607**	**300 (225)[q]**	**400**	**Yes**	**Yes**
MB 001	*Agrostis stolonifera*	OH	Simple	103.1 X 3.4	+[r]	+	--	--	+	+	+	Yes	Yes
MB 013	*A. stolonifera*	NJ/B. Clarke[s]	Simple	99.2 X 3.2	+	+	--	--	+	+	+	Yes	Yes
MB 0114	*A. stolonifera*	MD/N. Tisserat[t]	Simple	99.2 X 3.5	+	+	--	--	+	+	+	Yes	Yes
MB 0216	*A. stolonifera*	OH	Simple	106.4 X 2.8	+	+	--	--	+	+	+	Yes	Yes
MB 032	*A. stolonifera*	OH	Simple	99.4 X 2.9	+	+	--	--	+	+	+	Yes	Yes
MB 014	*A. stolonifera*	PA	Simple	123.4 X 3.6	+	+	870[u]	--	+	+	+	Yes	Yes
MB 0113	*A. stolonifera*	MD/N. Tisserat	Simple	119.6 X 3.0	+	+	870	--	950	--	+	Yes	Yes
MB 0116	*A. stolonifera*	Canada/N. Tisserat	Simple	113.9 X 3.7	+	+	--	1086	+	+	+	Yes	Yes

Table 2.1 (continued)

Table 2.1 (continued)

MB 021	*A. stolonifera*	Canada/E. Stromberg[v]	Simple	104.7 X 3.5	+	+	--	1086	+	+	+	Yes	Yes
MB 022	*A. stolonifera*	France/E. Stromberg	Simple	102.8 X 2.8	+	+	--	1086	+	+	+	Yes	Yes
MB 023	*A. stolonifera*	OH/E. Stromberg	Simple	108.9 X 3.0	+	+	--	1086	+	+	+	Yes	Yes
MB 0215	*A. stolonifera*	OH	Simple	109.0 X 3.3	+	+	--	1086	+	+	+	Yes	Yes
MB 0319	*A. stolonifera*	OH	Simple	131.2 X 3.4	--	+	870	--	950		--	Yes	Yes
MB 002	*A. stolonifera*	OH	NP	108.1 X 3.0	+	+	--	--	+	+	+	Yes	Yes
MB 004	*A. stolonifera*	OH	NP	98.8 X 3.2	+	+	--	--	+	+	+	Yes	Yes
MB 0317	*Agrostis* spp.	IN/R. Smiley[w]	NP	106.9 X 3.0	--	+	--	--	+	+	+	Yes	Yes
MB 0318	*A. stolonifera*	OH	NP	106.7 X 3.9	+	+	--	1086	+	+	+	Yes	Yes
MB 0321	*A. stolonifera*	MD/P. Dernoeden[x]	NP	123.6 X 3.0	+	+	870	1086	950	--	+	Yes	Yes
MB 011	*A. stolonifera*	IL/M. Elliott[y]	Simple	NP	+	+	--	--	950	+	+	Yes	Yes
MB 012	*A. stolonifera*	IL/B. Clarke	Simple	NP	+	+	--	--	950	+	+	Yes	Yes
MB 0214	*A. stolonifera*	OH	Simple	NP	+	+	--	1086	+	+	+	Yes	Yes

Table 2.1 (continued)

Table 2.1 (continued)

MB 019	*A. stolonifera*	OH/N. Tisserat	Simple	NP	+	+	--	--	+	+	+	Yes	Yes
MB 0115	*A. stolonifera*	RI/N. Tisserat	Simple	NP	+	+	--	--	+	+	+	Yes	Yes
MB 0110	*A. stolonifera*	DE/N. Tisserat	NP	NP	+	+	870	--	750	~700, 810	+	Yes	Yes
MB 018	*A. stolonifera*	WA/N. Tisserat	NP	NP	+	+	870	--	950	--	--	Yes	Yes
MB 033	*A. stolonifera*	MD/P. Dernoeden	NP	NP	+	+	870	--	310	410	+	Yes	Yes
MB 003	*A. stolonifera*	OH	NP	NP	+	+	--	--	--	+	--	Yes	Yes
MB 0217	*Avena sativa*	England/E. Stromberg ATCC 15419	DNA ONLY		+	+	--	--	1100	--	--	NT	NT
G. graminis var. *tritici*													
Expectations			**Simple**	**70-105 X 2.5-3**	**1.4 kb**	**--**	**870**	**--**	**627**	**410**	**400**	**Yes**	**No**
MB 0126	*Triticum aestivum*	OH	Simple	83.5 X 2.9	+	--	+	--	+	+	+	Yes	No

Table 2.1 (continued)

Table 2.1 (continued)

MB 025	*T. aestivum*	VA/E. Stromberg	Simple	80.3 X 3.3	+	--	+	--	+	+	+	Yes	No
MB 026	*T. aestivum*	MT/E. Stromberg	Simple	78.9 X 2.9	+	--	+	--	+	+	+	Yes	No
MB 029	*T. aestivum*	VA/E. Stromberg	Simple	84.7 X 3.6	+	--	+	--	+	+	+	Yes	No
MB 0313	*T. aestivum*	WA/R. Smiley	Simple	NP	+	--	--	1086	+	+	+	Yes	No
MB 0314	*T. aestivum*	WA/R. Smiley	Simple	NP	+	--	--	1086	+	+	+	Yes	No
MB 0315	*T. aestivum*	WA/R. Smiley	Simple	NP	+	--	--	1086	+	+	+	Yes	No
MB 0218	*T. aestivum*	VA/E. Stromberg	Simple	NP	+	--	--	1086	+	+	+	Yes	No
MB 0211	*T. aestivum*	England/E. Stromberg ATCC 28230	NP	NP	+	--	--	1086	+	+	+	Yes	No
MB 0311	*T. aestivum*	WA/R. Smiley	NP	NP	+	--	+	--	+	+	+	Yes	No

Table 2.1 (continued)

Table 2.1 (continued)

MB 0312	*T. aestivum*	WA/R. Smiley	NP	NP	+	--	--	--	+	+	+	Yes	No
MB 027	*T. aestivum*	VA/E. Stromberg	DNA ONLY		+	--	+	--	+	+	+	NT	NT
MB 028	*T. aestivum*	VA/E. Stromberg	DNA ONLY		+	--	+	--	+	+	+	NT	NT
MB 0210	*T. aestivum*	VA/E. Stromberg	DNA ONLY		+	--	+	--	+	+	+	NT	NT
MB 0212	*T. aestivum*	VA/E. Stromberg	DNA ONLY		+	--	+	--	+	+	+	NT	NT
MB 0213	*T. aestivum*	VA/E. Stromberg	DNA ONLY		+	--	+	--	+	+	+	NT	NT

G. graminis var. *graminis*

Expectations			**Lobed**	**80-105 X 2.5-3**	**1.4 kb**	**--**	**--**	**1086**	**310**	**--**	**--**	**Yes/No**	**No**
MB 0220	*Glycine max*	GA/M. Elliott	Lobed	85.1 X 3.5	+	--	--	--	+	410	400 600	Yes	No
MB 037	*Cynodon dactylon*	FL/C. Stiles[z]	Lobed	89.2 X 3.1	--	--	--	--	+	--	400	No	No

Table 2.1 (continued)

Table 2.1 (continued)

MB 038	*C. dactylon*	FL/C. Stiles	Lobed	85.8 X 3.0	--	--	--	--	+	--	--	No	No
MB 039	*Pennisetum clandestinum*	CA/N. Tisserat	Lobed	87.4 X 3.3	+	--	--	--	+	--	--	Yes	No
MB 035	*Oryza sativa*	KS/R. Smiley	Lobed	96.1 X 4.3	+	--	--	--	+	--	--	No	No
MB 015	*C. dactylon*	FL/M. Elliott	Lobed	NP	+	--	--	--	+	410	400	No	No
MB 031	*Paspalum vaginatum*	FL/C. Stiles ATCC MYA 2854	Lobed	NP	+	--	--	--	+	1000	--	No	No
MB 034	*C. dactylon*	MS	Lobed	NP	+	--	--	--	950	620	750	No	No
MB 0310	*P. clandestinum*	CA/N. Tisserat	Lobed	NP	--	--	--	--	+	--	--	No	No
MB 036	*C. dactylon*	FL/C. Stiles	Simple	NP	+	--	--	--	+	--	--	No	No
MB 016	*Stenotaphrum secundatum*	FL/M. Elliott	NP	NP	+	--	--	--	627	410	400 600	No	No
MB 0219	*O. sativa*	FL/M. Elliott	NP	NP	+	--	--	--	+	--	400	No	No
MB 024	*T. aestivum*	England/E. Stromberg ATCC 12761	DNA ONLY		+	--	--	+	627	410	400	NT	NT

Table 2.1 (continued)

Table 2.1 (continued)

G. graminis-like													
Expectations					**--**	**--**	**--**	**--**	**310**	**--**	**--**	**No**	**No**
MB 005	*A. stolonifera*	OH	NP	NP	--	--	--	--	1600, 310	410	--	No	No
MB 017	*A. stolonifera*	OH	NP	NP	--	--	--	--	650	225	400	No	No
MB 0111	*A. stolonifera*	N. Tisserat	NP	NP	--	--	--	--	1500, 627, 310	410	400	No	No
MB 0112	*Poa* spp.	WA/N. Tisserat	NP	NP	--	--	--	--	310	410	400	No	No
MB 0117	*A. stolonifera*	OH	Simple	NP	--	--	--	--	310	--	400	No	No
MB 0118	*A. stolonifera*	OH	Simple	NP	--	--	--	--	310	--	--	No	No
MB 0119	*A. stolonifera*	OH	Simple	NP	--	--	--	--	310	--	--	No	No
MB 0120	*A. stolonifera*	OH	Simple	NP	--	--	--	--	310	520	400	No	No
MB 0121	*A. stolonifera*	OH	Simple	NP	--	--	--	--	310	--	--	No	No
MB 0122	*A. stolonifera*	OH	Simple	NP	--	--	--	--	310	--	--	No	No
MB 0123	*A. stolonifera*	OH	NP	NP	--	--	--	--	310	--	400	No	No
MB 0124	*A. stolonifera*	OH	Simple	NP	--	--	--	--	310	--	400	No	No
MB 0125	*A. stolonifera*	OH	Simple	NP	--	--	--	--	310	--	400	No	No

Table 2.1 (continued)

Table 2.1 (continued)

MB 0223	*O. sativa*	OH	NP	NP	--	--	--	--	310	--	--	No	No
MB 0316	*C. dactylon*	MS	NP	NP	--	--	--	--	950	--	--	No	No
Other Known Fungi													
Phialophora graminicola		RI/R. Smiley	Simple	NP	--	--	--	--	310	410	400	NT	NT
G. cylindrosporus-1				NP	--	--	--	--	310	--	400	NT	NT
Magnaporthe grisea (*O. sativa*)					--	--	--	--	310	225, 410	400	NT	NT
Microdochium nivale (*A. stolonifera*)		OH			--	--	--	--	310	--	--	NT	NT

[n] Molecular primer sets evaluated in this study were designed by Rachdawong et al. (2002) for the avenacinase and avenacinase-like genes and those developed by Fouly and Wilkinson (2000) for the 18S rRNA gene.

[o] Mean ascospore sizes reported are the average of at least 90 ascospore measurements made from at least 3 perithecia.

[p] The expectations given for each variety include the morphological and pathogenic characters given by Walker (1972, 1981) and the amplicon sizes given by Rachdawong et al. (2002) for the avenacinase-specific primer sets and by Fouly and Wilkinson (2000) for the 18S rRNA-specific primer sets.

Table 2.1 (continued)

Table 2.1 (continued)

[q] The fragment produced by the amplification of genomic DNAs from Gga isolates was reported by Fouly and Wilkinson to be 300 bp, however, fragments of 225 bp were measured in this study.

[r] Abbreviations used: NP = not produced; NT = not tested; + = expected amplicon was produced; -- = no amplicon was expected or no amplicon was produced.

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[u] Amplicons sizes are listed when amplicons were produced when not expected.

[v] Erik Stromberg, Department of Plant Pathology, Physiology, and Weed Science, Virginia Polytechnic Institute and State University, Blacksburg, VA 24061.

[w] Richard Smiley, Columbia Basin Agricultural Research Center, Oregon State University, Pendleton, OR 97801.

[x] Peter Dernoeden, Natural Resources and Landscape Architecture, University of Maryland, College Park, MD 20742.

[y] Monica Elliott, Fort Lauderdale Research and Education Center, University of Florida, Ft. Lauderdale, FL 33314.

[z] Carol Stiles, University of Florida, Department of Plant Pathology, Fifield Hall, Gainesville, FL 32611.

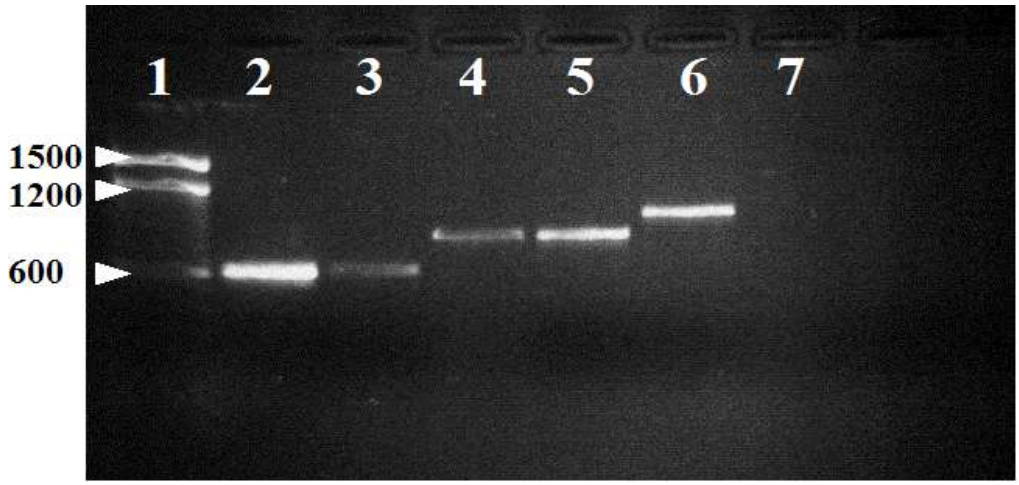

Figure 2.1. Amplicons typical of PCR reactions with the avenacinase primer sets. The following primer sets were used: Gga:AV3 (lanes 2-3; 617 bp amplicon), Ggt:AV3 (lanes 4-5; 870 bp amplicon), and Ggg:AV3 (lanes 6-7; 1,086 bp amplicon). Lane 1 contains an 100 bp DNA ladder. The following DNAs were amplified: MB 013 (lane 2), MB 0217 (lane 3), MB 026 (lane 4), MB 029 (lane 5), MB 024 (lane 6), and MB 015 (lane 7).

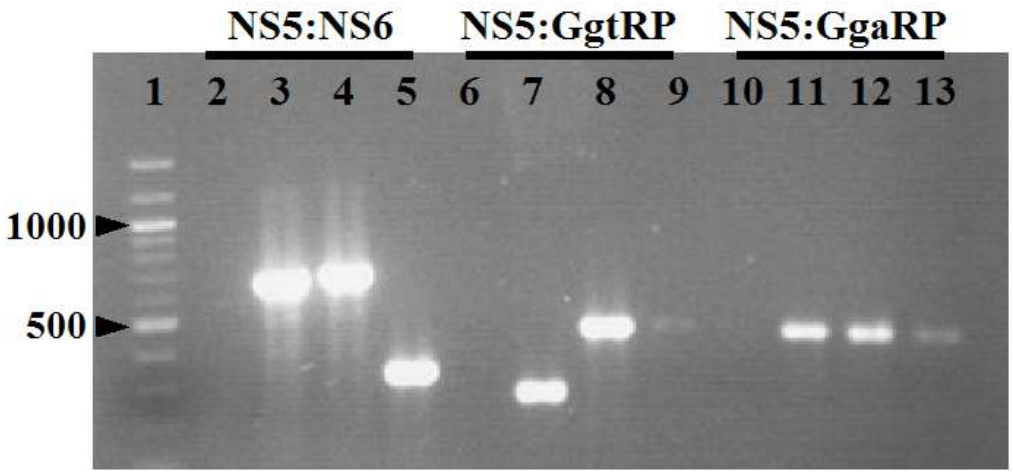

Figure 2.2. Characteristic amplicons produced with the 18S rDNA primer sets. The following primer sets were used: NS5:NS6 (lanes 2-5) , NS5:Ggt-RP (lanes 6-10); and NS5:Gga-RP (lanes 11-13). Lane 1 contains an 100-bp DNA ladder; lanes 2, 6, 10 contain no DNA; lanes 3, 7, 11 were amplified with DNA from MB 013; lanes 4, 8, 12 were amplified with DNA from MB 029; and lanes 5, 9, 13 were amplified with DNA from MB 015.

CHAPTER 3

THE EFFECT OF ROOT ZONE MIX COMPOSITION ON TAKE-ALL INCIDENCE OF CREEPING BENTGRASS

3.1 INTRODUCTION

Take-all of creeping bentgrass (*Agrostis stolonifera* L. syn. *A. palustris* Huds.) is caused by *Gaeumannomyces graminis* var. *avenae* (Turner) Dennis (Gga; 23, 24). Gga is one of several turfgrass pathogens that produces darkly pigmented ectotrophic runner hyphae, infects and colonizes the roots of graminaceous hosts, and produces vascular dysfunction leading to bleached patches of dead turfgrass. Although it has limited saprophytic ability in the soil, Gga can survive on the roots of weedy grass species in areas where creeping bentgrass is grown (4, 24). The disease was first reported in 1937 on golf greens and lawns by Schoevers in Europe (17) and by the early 1960s, reports of take-all patch on bentgrass were observed in the pacific northwest of the United States (10). Applications of lime (17, 24), herbicides (21, 23), soil fumigants (33), and nitrate-based inorganic fertilizers (11) have been shown to exacerbate the disease.

Younger stands of creeping bentgrass are more susceptible to disease outbreaks particularly if they are grown on sandy soils (17). Similar to take-all on wheat, severe outbreaks of take-all on bentgrass are followed in succeeding years by a decline in patch incidence (24). Sarniguet and Lucas (22) found the occurrence of take-all patch on bentgrass induced qualitative and quantitative changes in the soil bacterial populations.

Specifically, the centers of patches recovering from take-all had up to 8 times more fluorescent pseudomonads as compared with unaffected areas. Additionally, the *Pseudomonas* spp. recovered from the centers of patches were more antagonistic to Gga as compared with those from patch margins or unaffected areas. Nilsson and Smith (17) suggested that older stands of turfgrass are unaffected by this disease due to an established bacterial community. Applications of lime or soil fumigants may disrupt the established flora leading to outbreaks in previously take-all free turfgrass stands (17, 33).

The occurrence of take-all patch on creeping bentgrass in the U.S. coincided with the transition from the use of native soils to sand-amended mixes in putting green construction. Throughout the 1960s, researchers amended turfgrass root zone mixes with sand in attempts to reduce compaction and increase the poor water infiltration rates associated with high clay content native soils. Over the past 20 years, mixes for putting green construction have become sand-based with low percentages of organic matter. Organic amendments to sand-based mixes are generally chosen to optimize the physical and chemical properties of root zone mixes (26). Sphagnum peat is most widely used as it is readily available. However, compost amendments have increased in use because of their potential to enhance the disease suppression on creeping bentgrass. Several studies have shown that compost topdressings can control dollar spot (2, 15), brown patch (14) and gray snow mold (16). Fewer studies have investigated the effect of compost amendments incorporated into the root zone mix in reducing creeping bentgrass diseases such as Pythium root rot (6). The objective of this study was to determine the effect of root zone mix composition on take-all incidence of creeping bentgrass. An additional

objective was to develop a rapid growth chamber assay for take-all on creeping bentgrass which would facilitate the assessment of fungicide, fertilizer, and biological amendments on take-all incidence.

3.2 MATERIALS AND METHODS

3.2.1 Maintenance and cultivation of fungal isolates.

G. graminis isolates used in this study (Table 3.1) were maintained on 1/5 strength potato dextrose agar (1/5 PDA; Becton Dickinson, Cockeysville, MD) amended with 100 μg ml^{-1} penicillin and streptomycin sulfate (Sigma Chemical Corp., St. Louis, MO). Isolates were stored as previously described. Isolates were transferred monthly to freshly prepared media and maintained in culture for a maximum of six months (six generations) before re-isolation from stored cultures. Isolates were identified to the variety level using morphological, molecular and pathogenic characteristics and chosen for this study because they exhibited traits typical of Gga (Table 2.1).

3.2.2 Production of millet seed inoculum.

Colonized white proso millet (*Panicum milaceum* L.) seed was used as inoculum for all assays. Eighty milliliters of millet seed was added to a 250-ml Erlenmeyer flask with 60 ml of distilled water. The seed was soaked overnight and the excess water was decanted. Flasks were plugged with cotton, autoclaved for 50 min and cooled in a laminar flow hood for 4 to 6 h prior to inoculation with 12 agar plugs (8-mm diam.) taken from the margin of an actively growing culture of Gga. Flasks were incubated at room temperature for 14-21 days and were shaken every 4-5 days to ensure all of the millet seed was

colonized. Inoculum was dried in paper bags at room temperature (23-25ºC) for 3 to 4 days and shaken daily to break up clumps. Forty to fifty colonized seeds were plated on 1/5 PDA and 1/5 PDA amended with rifampicin (100 μg ml^{-1}; Sigma Chemical Corp., St. Louis, MO) prior to use to ensure no contamination was present and millet seeds were colonized. Only those inocula with no contamination and greater than 95% colonized seeds were used. Inocula were stored at room temperature used for up to 6 weeks after production.

3.2.3 Effect of isolate and inoculum dose on take-all incidence and severity.

Assays were established using conetainers (3.8-cm diam. x 21-cm depth; SC-10 Super Cell, Ray Leach Cone-tainers, Stuewe and Sons, Corvallis, OR) plugged with cotton and held upright in a tray (RL98Tray, Ray Leach Cone-tainers, Stuewe and Sons, Corvallis, OR). Rooting media was produced for each conetainer by thoroughly mixing colonized millet seed into 150 ml of sterilized mason sand (autoclaved for 90 min). The ability of Gga isolates MB 013 and MB 0114 to incite disease on creeping bentgrass and oats was assessed using the following doses of inoculum per conetainer: 0.0, 0.1, 0.5, 1.0, 2.0, and 5.0 g. The inoculated sand was covered with 5 ml of sterilized sand and was seeded with creeping bentgrass cv. Penneagle at a rate of 24.4 g m^{-2} or 4 oat seeds (*Avena sativa* cv. Armor). Seeds were covered with 5 ml of sand and fertilized with 4.88 g m^{-2} of starter fertilizer (20-27-5; N-P-K; Scotts Co., Marysville, OH). Conetainers were randomized within the tray, watered, and covered with plastic wrap until germination occurred. Oat assays were thinned to 2 seedlings per conetainer after germination. Conetainers were

incubated in a growth chamber set to provide 12 h photoperiod (12 h 18ºC; 12 h 12ºC) for 28 days and watered as needed.

Disease was assessed at 28 days. Measurements made on creeping bentgrass included turfgrass height (cm), symptomatic area (%), root length (cm), and root disease incidence. Reported root lengths were the mean root length from 10 randomly selected plants. Disease incidence was calculated by dividing the total number of visibly diseased roots (roots with discolored vascular tissues) by the total number of roots observed from 50 plants. Oat disease assessments were the same as those made for bentgrass, except disease severity, rather than disease incidence, was assessed. The qualitative disease rating scale of Weller and Cook (32) was used in which: 0 = no disease; 1 = one or two lesions on the roots of a given plant; 2 = 50-100% of the roots with one or more lesions each; 3 = all roots with lesions and some evidence of infection on the stem; 4 = lesions abundant and beginning to coelesce on the stem; and 5 = plants dead or nearly so. This experiment was blocked over time with seven replicate blocks. The experiment was analyzed as a randomized complete block.

The effect of isolate and inoculum dose on host plant height, root length, symptomatic area, and root disease incidence on creeping bentgrass was determined with analysis of variance (ANOVA) using Minitab statistical software (Minitab Inc., College Sta., PA). Symptomatic area and disease incidence data were arcsine-transformed prior to ANOVA. Mean separations were based on the differences in means for plant height and root length and transformed means for symptomatic area and disease incidence using the least significant difference (LSD; P=0.05).

The marginal effects nonparametic analysis of Brunner and Pari (5) was used to determine the effects of isolate and inoculum dose on oat severity rating using SAS statistical software (SAS Institute Inc., Cary, NC). Data were rank transformed prior to analysis and the least significant difference (LSD; P=0.05) for the rank transformation of the data was determined when factors or interactions were significant.

3.2.4 Effect of root zone mix composition on take-all incidence.

Composted biosolids (Com-Til, City of Columbus Department of Sewerage and Drainage, Lockbourne, OH) were screened through a 0.64-cm mesh sieve to remove large wood chips then incorporated into silica or mason sands to generate root zone mixes with 0, 10, or 20% compost (v/v). A mason sand:Sphagnum peat mix (4:1; v/v) was also used. Mixes and sands were subjected to chemical and elemental analyses (Table 3.2; CLC Labs, Columbus, OH) and were not autoclaved prior to use. Millet seed colonized by Gga isolate MB 013 was used for inoculum. Two inoculum doses (0.0 and 1.0 g per conetainer) were used in conjunction with the seven root zone mixes to produce 14 treatments. Assays were set-up as previously described using Penneagle creeping bentgrass seed and starter fertilizer. At 28 days, after turf height and symptomatic area were assessed, the turf foliage exceeding the rim of the conetainer was cut off, collected, and dried in an oven at 60^{o}C for 48 h to determine the foliar dry weight. Disease incidence was also assessed as previously described. This experiment was blocked over time with five replications. The experiment was analyzed as a randomized complete block.

The effect of treatment (root zone mix with or without inoculum) on turfgrass height, mean root length, symptomatic area and root disease incidence was determined with a one-way ANOVA. Percentage data were arcsine-transformed prior to ANOVA. The non-parametric Kruskal Wallis test (25) was used to determine the effect of treatment on foliar dry weight. Mean separations were based on the differences in means for plant height and root length, transformed means for symptomatic area and disease incidence, and the mean ranks for foliar dry weight using LSD (P=0.05). Median foliar dry weights corresponding to mean ranks for each isolate were shown.

3.3 RESULTS

3.3.1 Effects of isolate and inoculum dose on take-all incidence and severity.

The main effect of inoculum dose significantly (P<0.001) affected turfgrass height, root length and symptomatic area. As the amount of inoculum increased, mean turfgrass height and root length decreased and the area of turfgrass expressing symptoms increased (data not shown).

An interaction occurred between the two isolates and inoculum dose for root disease incidence on creeping bentgrass. Between isolates, no difference was detected in the mean root disease incidences at 0.0, 2.0, or 5.0 g doses, however, isolate MB 013 caused significantly greater (P<0.001) disease incidence at 0.1, 0.5 and 1.0-g doses as compared with isolate MB 0114 (Figure 3.1A). There were strong correlations between the observed symptomatic area and the measured root disease incidence on creeping bentgrass for each isolate (MB 013 R^2=91.0% and MB 0114 R^2=90.6%; Figure 3.2).

On oat, both isolate and dose had a significant effect on plant height and root length. Oat plants grown in sand amended with isolate MB 0114 inoculum had significantly (P=0.05) shorter foliage and roots as compared to plants grown with MB 013 inoculum. Generally, as inoculum dose increased, oat plant height and root length decreased (data not shown).

There was an interaction between isolate and dose for the observed symptomatic area and disease severity of oats. As dose increased so did the observed symptomatic plant area when plants were inoculated with isolate MB 0114, however, with isolate MB 013 the observed symptomatic area was not significantly different among the 0.5, 1.0, 2.0, and 5.0-g doses (Figure 3.3). Greater disease severity ratings were given to plants inoculated with MB 0114 as compared with MB 013 (Figure 3.1B, C).

3.3.2 Effect of root zone mix composition.

Significantly (P=0.05) shorter creeping bentgrass plants grew in the straight sand mixes as compared with the mixes that were amended with organic matter. Inoculating mixes generally produced shorter turfgrass. Similar turfgrass heights were observed for the uninoculated peat-amended mix and the 10% (v/v) compost-amended mixes. Root zone mixes amended with 20% compost produced the tallest bentgrass plants which averaged 12 and 14 cm in the inoculated and uninoculated treatments, respectively (data not shown).

Unamended sands and the peat-amended mix produced significantly (P<0.001) less foliar dry weight as compared with compost-amended mixes (H=51.74; df=13; P<0.001; Table 3.3). Compost-amended mixes, irrespective of inoculation and compost percentage,

produced comparable foliar dry weights. Although not significant, higher foliar dry weights were obtained from the 100% and 90% mason mixes receiving inoculum. No vascular discoloration was observed on any of the sampled seedlings from uninoculated treatments. (Table 3.3). The mason:compost (4:1; v/v) mix had significantly (P=0.05) less root disease incidence as compared with all other inoculated treatments, except the mason:compost (9:1; v/v) mix (Table 3.3).

3.4 DISCUSSION

This study clearly shows that root zone mix components (i.e., sand type and organic matter amendment) influence take-all incidence on creeping bentgrass (Table 3.3). The 4:1 (v/v) mason sand:compost mix significantly (P=0.05) reduced disease incidence and, although not significant, less disease was observed in the mason sand mixes as compared with the silica sand mixes. Additionally, the 4:1 (v/v) mason sand:compost mix had significantly (P=0.05) fewer diseased roots as compared with the 4:1 (v/v) mason sand:Sphagnum peat mix indicating that organic matter amendment type significantly effects take-all incidence. Comparisons of the chemical and elemental analyses show that the 4:1 mason sand:organic amendment mixes had similar chemical properties and few differences in the elemental concentrations other than higher P and K concentrations in the compost-amended mix. Similar elemental concentrations were also observed in the 4:1 (v/v) silica sand:compost mix in which no decrease in disease incidence was detected indicating the shift in take-all conduciveness was probably not due to elemental properties of the mixes.

One possible explanation for the shift in disease incidence may be differences in the microbial communities present within each root zone mix. Previous studies with sand-based turfgrass root zone mixes have shown that the culturable microbial community supported within a compost-amended turfgrass root zone mix had a greater culturable bacterial diversity (i.e., a greater number of total culturable bacterial taxa and different proportions of each taxa) as compared with a Sphagnum peat-amended mix (Thomas et al., unpublished). Microbial populations have been shown to control or reduce take-all incidence on wheat through several mechanisms including antibiosis (27, 28), competition (9, 20) and breakdown of infested crop residues (Asher, 1981). Any one or a combination of these mechanisms may have reduced the incidence of take-all in the 4:1 (v/v) mason sand:compost mix.

Similar to previous studies with compost-amendments on turfgrass systems, the amendments resulted in an increase in the plant available nutrients present in the root zone mixes (Table 3.2) which produced significantly (P=0.05) taller creeping bentgrass plants and greater foliar dry weights (Table 3.3; 12, 13, 18, 29). Although equivalent compost amendments to silica and mason sands resulted in similar chemical and elemental analyses, a noticeable difference was observed in the amount of plant available calcium and the cation exchange capacity. Mason sands are quarried from rock containing limestone and as the lime breaks down there is a release of calcium resulting in the high concentrations. The cation exchange capacity was determined using the sum of the cations (i.e., sum of K^+, Ca^{2+} and Mg^{2+}). There was a decrease in the CEC of the mason mixes as the compost percentage increased due to a decrease in the available Ca^{2+}, which was not compensated for by the increase in K^+ or Mg^{2+} (Table 3.2). However, mason sand

mixes, irrespective of compost amendment, had greater cation exchange capacities as compared with the silica sand-based mixes (Table 3.2) indicating that these mixes could hold more cations (nutrients) available for plant uptake and use. Given the low CEC values for the silica mixes, the nutrients imparted by the compost amendments may have leached out of the mixes prior to uptake and use by creeping bentgrass resulting in nutrient-stressed plants and greater take-all incidences. Nutrient deficiencies in wheat have been shown to result in severe yield losses due to take-all (11). The chemical and physical properties of root zone mixes should be compared prior to selection and use in putting greens as these results indicate silica sand-based mixes may be more expensive to maintain due to increased disease risk and lower nutrient retention.

There was an irregular trend observed in the percent organic matter determined in the silica sand-amended mixes (Table 3.2) as the percent organic matter in the 9:1 (v/v) silica sand:compost mix was 1.8% whereas it was 1.6% in the 4:1 (v/v) silica sand:compost mix. Although the opposite was expected, there are several possibilities which may explain the observed values. The compost was sieved (0.64 cm) prior to incorporation into sands. In addition, all mixes were subjected to a second sieving prior to chemical analysis through a 2-mm sieve. Both sieving procedures may have resulted in the lower organic matter percentage in the 4:1 mix as larger compost particles may have been added to this mix as compared to the 9:1 mix which were subsequently removed during the second sieving. Other possibilities include poor subsampling of the mixes for analysis, differences in water content, or organic matter degradation.

When determining the appropriate inoculum dose during assay development, it is not surprising that plant height and root length decreased as the area of symptomatic foliage

and root infection increased with increasing inoculum doses (Figure 3.2). No difference was observed between Gga isolates on creeping bentgrass height, root length, or symptomatic area. However, there was an interaction between isolate and dose on the observed root disease incidence as isolate MB 013 caused significantly (P=0.05) higher levels of root incidence at inoculum doses 0.1, 0.5, and 1.0 g as compared with isolate MB 0114. These results indicate that isolate MB 013 was more aggressive than isolate MB 0114 on creeping bentgrass and these differences in aggressiveness are similar to those previously reported by Datnoff et al. (7) for *G. graminis* var. *graminis* on St. Augustinegrass. Interestingly at higher inoculum doses (i.e., 2.0 and 5.0 g), mean root disease incidences were comparable between isolates (Figure 3.1A) illustrating that differences in isolate aggressiveness can be masked by high doses of inoculum.

It is possible that there could have been differences in the colonization of the inoculum, however, data from the oat assays in which the same batch of inocula were used show a reverse pattern in which isolate MB 0114 incited greater disease severity than isolate MB 013 (Figure 3.1B, C). The symptomatic oat area was not significantly (P=0.05) different and similar levels of disease severity were observed among plants receiving the 0.5 to 5.0 g inoculum colonized by isolate MB 013 whereas plants inoculated with isolate MB 0114 had higher levels of symptomatic foliage and disease severity with increasing inoculum doses (Figure 3.1B, C, 3.3). These results indicate there is a degree of pathogenic variation and perhaps host specificity among these fungal pathogens. The level of avenacinase activity expressed by each isolate may have influenced oat infection and colonization as avenacinase has been shown to be the key pathogenic determinant of *G. graminis* isolates on oats (3, 19). Avenacinase detoxifies the oat root saponin, avenacin,

enabling Gga isolates to overcome this host defense compound and infect oat roots. Gga isolate MB 013 may express lower levels of avenacinase activity which slowed the disease progression as compared with isolate MB 0114. In addition, other factors such as natural variation within pathogen populations of growth and colonization rates and extended periods of sub-culturing may have affected the ability of isolate MB 013 to infect and colonize oat roots.

Given that strong correlations were observed between the percent symptomatic bentgrass area and root disease incidence (R^2=91.0% for Gga isolates MB 0114 and MB 013, respectively; Figure 3.2) it may be possible to assess the relative root disease incidence without visually inspecting roots. This would greatly reduce the amount of time required to assess take-all incidence.

There was a difference in the root disease incidence observed between the unautoclaved mason mix treatment used in the compost-amended study (Table 3.3) and the autoclaved mason mix treatment receiving the 1.0-g inoculum dose (Figure 3.1A). The mean root incidence on creeping bentgrass was 36.5% in the sterilized mason sand whereas in the unautoclaved sand the mean root incidence was 21.9%. These differences are most likely the result of changes in the microbiological properties of the root zone mix induced by autoclaving and they support the conclusions by previous researchers (17,33) that sterilized soils have a poorly developed microflora which is less suppressive to take-all infection.

The assay described here could be used to quickly assess the efficacy of fungicides, fertilizers and biocontrol agents on take-all incidence of creeping bentgrass in 28 days. Additionally, inoculum doses or incubation times could be adjusted to facilitate in-depth

studies on the changes induced by associated microbial communities on the incidence of take-all patch or the impact of biocontrol agents or chemical applications on the native microflora and take-all development.

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Isolate	Host	Location	Source
MB 013	*Agrostis stolonifera* L.	New Jersey	B. Clarke[y]
MB 0114	*A. stolonifera*	Maryland	N. Tisserat[z]

[y] Bruce Clarke, Rutgers University, Department of Plant Pathology, Foran Hall, New Brunswick, NJ 08901-8520.

[z] Ned Tisserat, Kansas State University, Plant Pathology, Throckmorton Hall, Manhattan, KS 66506

Table 3.1. *Gaeumannomyces graminis* var. *avenae* isolates used in this study.

	Mason			Silica			Peat	Compost
	100:0[t]	**9:1**[t]	**4:1**[t]	**100:0**[t]	**9:1**[t]	**4:1**[t]	**4:1**[u]	**(100%)**
Chemical								
Organic matter (%)[v]	1.2	1.5	2.2	0.4	1.8	1.6	1.9	68.2
pH	7.2	7.2	7.1	7.2	7.3	7.2	7.3	8.0
Cation exchange capacity	10.6	9.6	8.9	1.4	2.7	2.6	12.3	NT
Elemental								
P[w]	21[x]	254	450	11	391	494	5	76
K[y]	36	121	225	12	176	167	32	1317
Ca	1958	1701	1456	208	306	281	2240	54
Mg	88	98	126	34	84	89	118	17
Fe[z]	2.2	4.0	6.7	5.5	6.0	7.5	6.0	35.3
Mn	3.0	2.5	4.0	0.7	3.7	4.7	3.0	1.3
Zn	0.7	2.4	5.6	0.7	5.9	8.9	0.6	74.5
Cu	0.4	0.8	0.8	0.1	0.9	1.3	0.7	2

Table 3.2. Chemical and elemental analyses of the root zone mixes used prior to use in take-all assays.

[t] Sand:compost; v/v.

[u] Mason sand:Sphagnum peat; v/v.

[v] Organic matter percentage was determined according to USGA specifications at 440ºC.

[w] Phosphorus was determined using the Bray and Kurtz P-1 method (8).

[x] Elemental analysis values expressed as ppm.

[y] K, Ca, and Mg concentrations were determined using the neutral ammonium acetate after extraction procedure (31).

Table 3.2 (continued)

Table 3.2 (continued)

[z] Fe, Mn, Zn, and Cu concentrations were determined using the inductively coupled plasma (ICP) method after acid digestion (30).

Root zone Mix	Inoculum (g)[w]	Disease Incidence[x]	Foliar Dry Weight (mg)[y]
Mason (100%)	0.0	0.0 a[z]	83.8 ab
Mason (100%)	1.0	21.9 cd	106.0 abc
Mason : Compost (9:1: v/v)	0.0	0.0 a	141.8 bcd
Mason : Compost (9:1: v/v)	1.0	20.2 bc	180.6 d
Mason : Compost (4:1; v/v)	0.0	0.0 a	189.2 d
Mason : Compost (4:1; v/v)	1.0	16.3 b	174.4 d
Silica (100%)	0.0	0.0 a	75.8 a
Silica (100%)	1.0	26.9 d	74.9 a
Silica : Compost (9:1; v/v)	0.0	0.0 a	196.2 d
Silica : Compost (9:1; v/v)	1.0	26.9 d	122.8 abcd
Silica : Compost (4:1; v/v)	0.0	0.0 a	182.7 d
Silica : Compost (4:1; v/v)	1.0	26.4 cd	163.4 cd
Mason : Sphagnum Peat (4:1; v/v)	0.0	0.0 a	100.0 abc
Mason : Sphagnum Peat (4:1; v/v)	1.0	23.4 cd	71.6 a

Table 3.3. The effect of root zone mix composition and inoculum dose creeping bentgrass root disease incidence and foliar dry weight.

[w] Inoculum was millet seed colonized by Gga isolate MB 013.

[x] Mean disease incidence of creeping bentgrass roots was calculated by dividing the total number of visibly diseased roots (roots with discolored vascular systems) by the total number of roots observed from 50 plants.

[y] Multiple comparisons of mean ranks are based on the Kruskal Wallis test. Corresponding median foliar dry weights are shown. Values followed by the same letter within a column are not significantly different at P=0.05.

Table 3.3 (continued)

Table 3.3 (continued)

[z] Multiple comparisons of means are based on arcsine-transformed values. However, mean percentages are shown. Values followed by the same letter within the column are not significantly different based on the LSD (*P*=0.05).

Figure 3.1. The effect of isolate and inoculum dose on root disease incidence of creeping bentgrass and disease severity of oats.

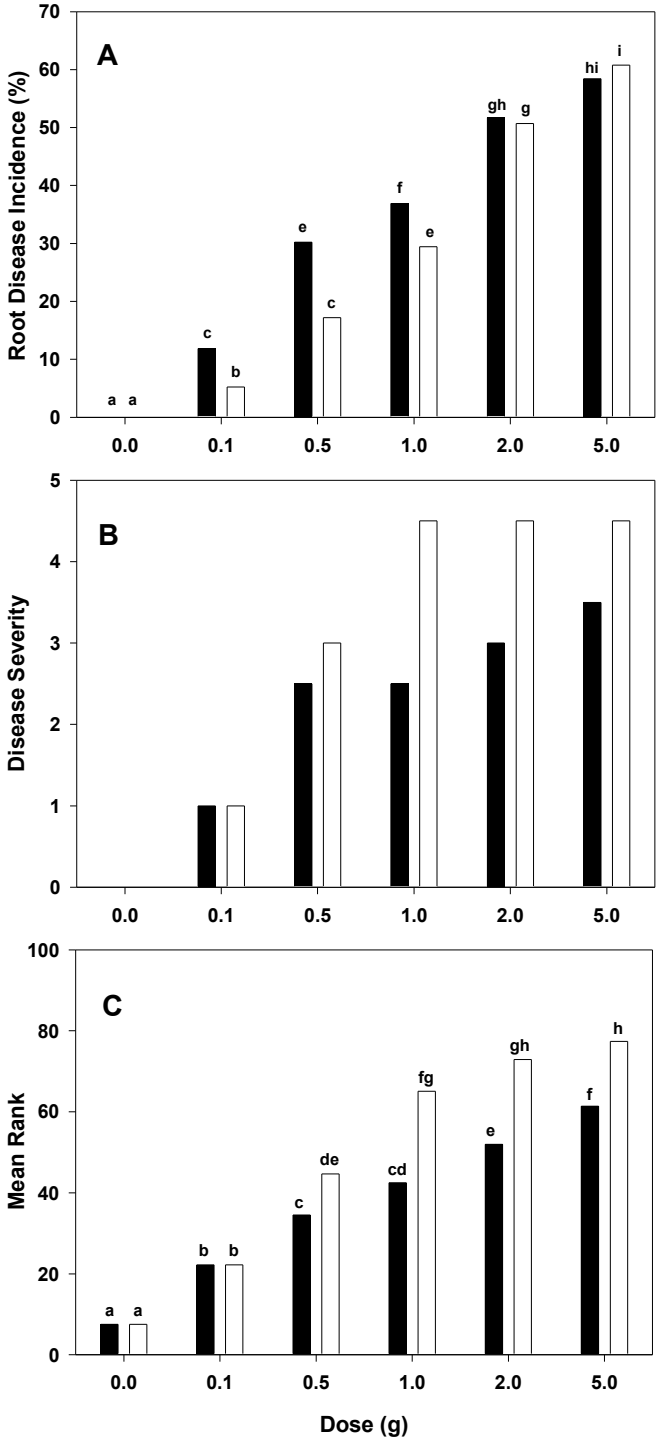

Figure 3.1 (continued)

Figure 3.1 (continued)

Black bars represent isolate MB 013 and white bars represent isolate MB 0114.

A. The root disease incidence on creeping bentgrass was calculated by dividing the total number of visibly diseased roots (roots with discolored vascular systems) by the total number of roots observed from 50 plants. Multiple comparisons of means are based on arcsine-transformed values. However, mean percentages are shown. Values followed by the same letter are not significantly different based on the LSD (P=0.05).

B. Disease severity was assessed using a rating scale (32) in which: 0 = no disease; 3 = all roots with lesions and some evidence of infection on the stem; and 5 = plants dead or nearly so. Severity rating values were rank transformed prior to analysis and the corresponding median ratings are displayed.

C. The marginal effects nonparametic analysis of Brunner and Pari (5) was used to determine the effects of isolate and inoculum dose on oat severity rating. Data were rank transformed prior to analysis and the least significant difference (LSD; P=0.05) for the rank transformation of the data was determined when factors or interactions were significant. Corresponding mean ranks are shown.

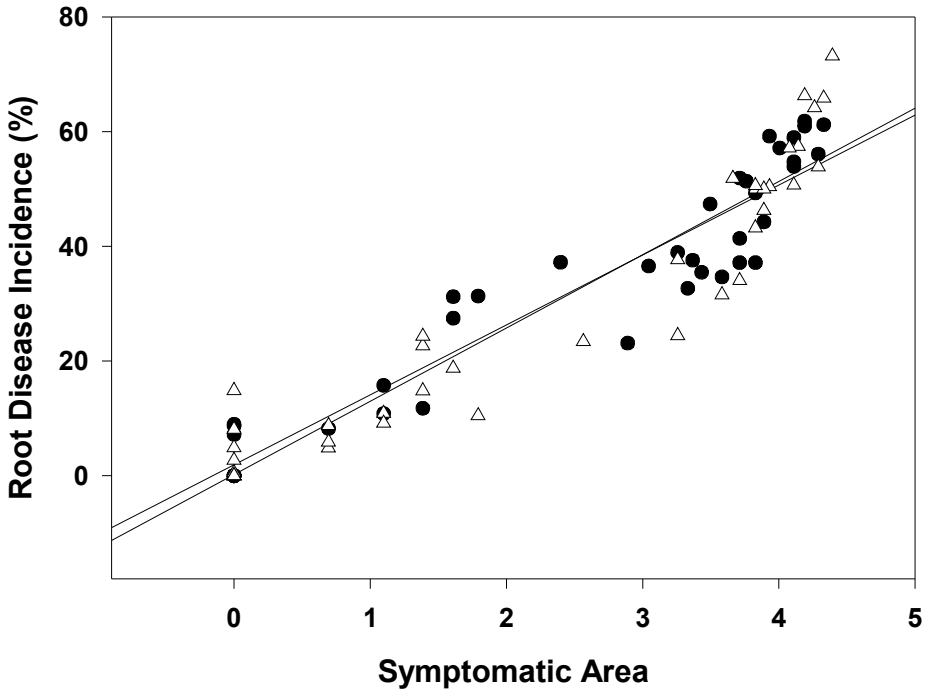

Figure 3.2. Regression analyses of the observed symptomatic area versus root disease incidence for Gga isolates MB 013 and MB 0114. Symptomatic area was transformed and expressed in the following manner: $\log_e$ (symptomatic area +1). Symbols: Δ = MB 0114 and ● = MB 013. R^2 values are 90.6% for MB 0114 and 91.0% for MB 013.

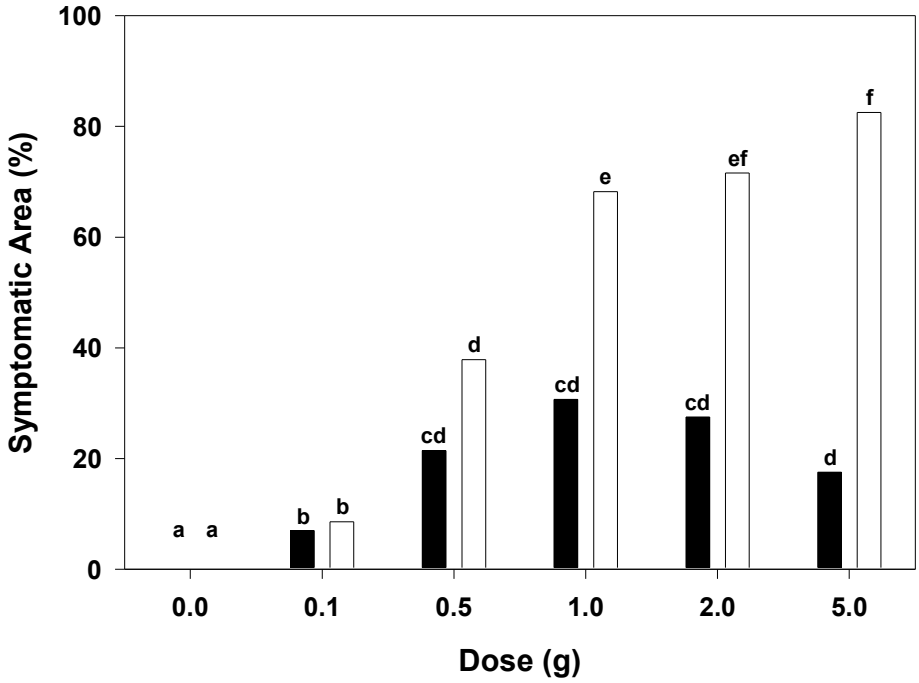

Figure 3.3. The effect of isolate and inoculum dose on the observed symptomatic oat area. Multiple comparisons of means are based on arcsine-transformed values. However, mean percentages are shown. Values followed by the same letter are not significantly different based on the LSD (P=0.05).

CHAPTER 4

PATHOGENICITY OF *GAEUMANNOMYCES GRAMINIS* ON CREEPING BENTGRASS.

4.1 INTRODUCTION

Take-all, caused by *Gaeumannomyces graminis* (Sacc.) v. Arx & Olivier, is a devastating root disease of cereal crops and turfgrasses worldwide. Three varieties of *G. graminis* have been described based on differences in hyphopodium type and ascospore length (16). *G. graminis* var. *graminis* (*Ggg*) infects rice (*Oryza sativa* L.) and warm-season grasses, such as St. Augustinegrass (*Stenotaphrum secundatum* (Walt.) Kuntze), and is easily differentiated from the other varieties based on the production of lobed hyphopodia. *G. graminis* var. *avenae* (Turner) Dennis (*Gga*) and *G. graminis* var. *tritici* Walker (*Ggt*) infect cool-season graminaceous hosts such as wheat (*Triticum aestivum* L.) and barley (*Hordeum vulgare* L.). *Gga* can also infect oats (*Avena sativa* L.) and is the variety reported as the causal agent of take-all patch of creeping bentgrass (*Agrostis stolonifera* L. syn. *A. palustris* Huds.) (10, 11). *Gga* and *Ggt* are morphologically indistinguishable in culture as both produce simple hyphopodia and perithecia are rarely produced.

Pathogenicity of *G. graminis* isolates on oats has been shown to be mediated by the avenacin-avenacinase interaction (1, 7, 8, 14, 15). Avenacin compounds are fungitoxic saponins produced and stored in the epidermal cells of oat roots (7). The fungitoxic

properties of these compounds arise from their ability to form complexes with fungal membrane sterols, leading to leaky cell membranes and fungal cell death (4). Four avenacin compounds have been described (2). The most prevalent and fungitoxic is avenacin A-1 which contains a *N*-methylanthranilate residue that fluoresces bright blue under UV light (2, 14). *Gga* produces avenacinase which detoxifies avenacin enabling this variety to overcome the oat root defenses and incite disease (3, 15). Although homologous avenacinase-like genes have recently been identified in *Ggt* and *Ggg* (9), *Ggt* isolates are incapable of infecting *Avena* spp. that produce avenacin (8, 7). Similarly, *Gga* isolates with disrupted avenacinase genes are unable to infect oats (1). Based on these results, Osbourn and co-workers concluded that avenacinase activity is the key determinant of *G. graminis* pathogenicity on oats.

The host-pathogen interaction between creeping bentgrass-*G. graminis* has been less investigated than that of oats-*G. graminis*. Clinical diagnosis of take-all patch of creeping bentgrass is typically made based on the observations of foliar symptoms, the presence of dark ectotrophic runner hyphae on the roots and vascular discoloration (13). *G. graminis* identification to the variety level based on hyphopodium and ascospore length is rarely confirmed and leads to many questions regarding the identity of the causal agent of take-all patch on creeping bentgrass. The objective of this study was to investigate the pathogenicity of *G. graminis* on creeping bentgrass. Specifically, to assess the production of avenacin in creeping bentgrass and the ability of *G. graminis* isolates to infect bentgrass.

4.2 MATERIALS AND METHODS

4.2.1 Root extracts.

Creeping bentgrass cultivars Crenshaw, L-93, Penneagle, Penncross, Penn G-2, and Providence were used. Ten milliliters of oat (cv. Armor), wheat (cv. Norm), and bentgrass seed were surface sterilized in 0.525% sodium hypocholorite solution containing 0.1% Tween 20 for 5 min, rinsed three times in sterile distilled water and placed on 0.3% water agar in petri dishes to germinate (10 petri dishes per cultivar). Dishes were incubated in a clear plastic bag at room temperature with natural light for 3-7 days. Twenty milligrams of root tip tissue (0-5 mm root sections) from oat, wheat, and creeping bentgrass were placed in 1.6 ml microcentrifuge tubes containing 200 µl of 50% aqueous methanol. Due to differences in root mass, batches of 10, 20, and 200 root tips from oat, wheat, and bentgrass, respectively, comprised the 20-mg samples. Root tip samples were stored at -20^{o}C until avenacin extraction.

Avenacin was extracted using a procedure similar to that described previously by Osbourn et al. (7). Harvested roots were ground with a pestle and hand drill for 30 s, the plant material was pelleted by centrifugation (45 s, 6400 rpm) and 100 µl of the supernatant was transferred to a new microcentrifuge tube. Roots were re-extracted three times, each with an additional 100 µl 50% aqueous methanol. A total of 400 µl of extract was collected per batch of roots. For each cultivar or species tested, a new pestle was used, extractions were performed in triplicate, pooled, and diluted 20-fold in aqueous methanol (v/v). Prior to dilution, crude root extracts were visualized under UV light (302 nm) and fluorescence was noted. Emission spectra were measured with an LS-5 Fluorescence Spectrophotometer (Perkin Elmer, Wellesley, MA) using an excitation

wavelength of 357 nm and an emission wavelength of 430 nm. This entire process was repeated three times for each creeping bentgrass cultivar, oats and wheat.

Prior to spectrophotometric analysis of creeping bentgrass and wheat root extracts, a dilution series of oat root extract was generated (final dilution of 1/2000) to determine the lower detection limit of the instrument. Fluorescence readings showed oat dilutions greater than 400-fold were not significantly different from the aqueous methanol solvent. Extracts of wheat and bentgrass were analyzed as 20-fold dilutions and as spiked samples which contained 20-fold diluted wheat or bentgrass and 100-fold diluted oat extract. The spiked samples were produced to ensure all samples would have fluorescence readings significantly greater than the solvent control which would allow for detection of otherwise undetectable levels of avenacin and ensure there was no quenching of the fluorescence in any of the samples.

4.2.2 TLC analysis and avenacin bioassay.

The TLC and avenacin bioassays were performed as described by Osbourn et al. (6). All bentgrass cultivars, wheat and oats were used in this assay. Roots were extracted with 50% aqueous methanol as described earlier. One hundred microliters of the crude root extracts were loaded in 10-µl aliquots onto two 250-µm thickness polyester silica gel TLC plates (2-25 µm mean particle size, Sigma Chemical Corp., St. Louis, MO). Plates were developed with chloroform:methanol:water (13:6:1; v/v/v). Fluorescent compounds were visualized under UV light (302 nm) and the location of each fluorescent spot was marked on the reverse side of the plate. An R_f value was calculated for each spot.

Gga isolate MB 013 and *Ggt* isolate MB 027 (Table 4.1) were used to assess the presence of inhibitory compounds in root extracts. Five milliliters of sterile potato dextrose broth (Becton Dickinson, Cockeysville, MD) was sprayed onto the developed plates to provide a suitable substrate for *G. graminis* growth. Each plate was inoculated with 15 agar plugs (8-mm diam.) taken from the margin of actively growing cultures and incubated in sterile plastic bags for two weeks at room temperature in the dark. Observations of fungal colonization of the plates were made at 7 and 14 days. Areas void of fungal growth at 14 days were scraped off the plate and eluted with 200 µl 50% aqueous methanol. Eluates were visualized under UV light (302 nm) to check for fluorescence and stored at -20ºC until HPLC analysis.

4.2.3 HPLC analysis of root extracts.

Root extracts from oats, wheat, and the creeping bentgrass cultivar 'Penneagle' were analyzed by HPLC using a procedure similar to that described by Papadopoulou et al. (8). Compounds eluted from areas on the TLC plates inhibiting fungal growth were also analyzed. Samples were analyzed using a Waters (Milford, MA) 2690 separations module and a 474 scanning fluorescence detector. The system was managed by a workstation running version 3.20 of Waters Millennium HPLC software. Injections, consisting of 20 µl of the 20-fold diluted root extracts or the eluate from the TLC plate, were separated on a Waters Xterra™ RP18, 5 µm, 4.6 x 150 mm column under isocratic conditions in 75% methanol at a flow rate of 1 ml min^{-1}. The fluorescence detector was set at an excitation

wavelength of 357 nm and an emission wavelength of 430 nm. The autosampler temperature was 4°C and column temperature was 30°C for all analyses. Total run time was 14 min per sample.

4.2.4 Maintenance and cultivation of fungal isolates.

G. graminis isolates used in this study (Table 4.1)were maintained on 1/5 strength potato dextrose agar (1/5 PDA; Becton Dickinson, Cockeysville, MD) amended with 100 μg ml^{-1} penicillin and streptomycin sulfate (Sigma Chemical Corp., St. Louis, MO). Isolates were stored as previously described by Thomas et al, unpublished. Isolates were transferred monthly to freshly prepared media and maintained in culture for a maximum of six months (six generations) then re-isolated from stored cultures.

Isolates were identified to the variety level by Thomas et al. (unpublished) using morphological, molecular and pathogenic characteristics and chosen for this study because they exhibited traits typical of *Gga* or *Ggt*.

4.2.5 Pathogenicity assays.

Millet seed inoculum and conetainer assays were established as described by Thomas et al. (unpublished). Prior to use, the viability of each batch of inoculum was determined by plating 40-50 colonized millet seeds on 1/5 PDA and 1/5 PDA amended with 100 μg ml^{-1} rifampicin (Sigma Chemical Corp., St. Louis, MO). Only batches of inocula with >95% colonized seeds and no fungal contamination were used.

The creeping bentgrass cultivar Penneagle, oats (cv. Armor) and wheat (cv. Norm) were used to establish conetainer assays. Conetainers were incubated in a growth

chamber set to provide 12 h photoperiod (12 h 18°C; 12 h 12°C) for 28 days. Assays were watered every other day.

Disease was assessed at 28 days. Measurements made on creeping bentgrass included turfgrass height (cm), percent symptomatic foliage, root length (cm), and root disease incidence. Disease incidence was calculated by dividing the total number of visibly diseased roots (roots with discolored vascular systems) by the total number of roots observed from 50 plants. Oat and wheat disease assessments were the same as those made for bentgrass, except disease severity, instead of disease incidence, was measured. The disease rating scale of Weller and Cook (17) was used in which: 0 = no disease; 1 = one or two lesions on the roots of a given plant; 2 = 50-100% of the roots with one or more lesions each; 3 = all roots with lesions and some evidence of infection on the stem; 4 = lesions abundant and beginning to coelesce on the stem; and 5 = plants dead or nearly so. This experiment was repeated five times, and each repetition was considered a replication. Each replication was treated as a block and the experiment was analyzed as a randomized complete block.

4.2.6 Statistical Analysis.

Statistical analyses were performed using Minitab Statistical Software (Minitab Inc., College Sta., PA). The non-parametric Kruskal Wallis test (12) was used to determine the effect of plant species or cultivar on root extract fluorescence and mean separations were based on the differences in mean ranks for root extracts using the least significant difference (LSD) at P=0.05. Median fluorescence measurements corresponding to mean ranks for each plant species or cultivar were reported.

The effect of isolate on host plant height, root length, symptomatic area, and root disease incidence of creeping bentgrass was determined with analysis of variance (ANOVA). Symptomatic area and disease incidence data were arcsine-transformed prior to ANOVA. Mean separations were performed based on LSD (P=0.05) of means for plant height and root length and transformed means for symptomatic area and disease incidence.

The non-parametric Kruskal Wallis test (12) was also used to determine the effect of isolate on wheat and oat disease severity. Mean separations were based on the differences in mean ranks for each isolate using LSD (P=0.05). Median disease ratings corresponding to mean ranks for each isolate were displayed. The Spearman rank correlation (12) between mean disease incidence on bentgrass and median disease severity rating in wheat across all isolates was calculated to determine the consistency of isolate pathogenicity across susceptible host species.

4.3 RESULTS

4.3.1 Avenacin production in creeping bentgrass.

Crude oat root extracts fluoresced bright blue under UV light indicating avenacin compounds were extracted. None of the crude root extracts from any of the creeping bentgrass cultivars or wheat fluoresced under UV (data not shown).

Fluorescence measured with the spectrophotometer in the 20-fold diluted oat extract sample was significantly greater (H=47.68, df=16, P<0.001) than the aqueous methanol control and all other plant samples (Table 4.2). The fluorescence measured from the 20-fold diluted root extracts of the creeping bentgrass cultivars and wheat was not

significantly different from the aqueous methanol control. No differences were detected between the spiked bentgrass and spiked wheat samples and the 100-fold diluted oat sample.

No fluorescence was observed for either wheat or creeping bentgrass when root extracts were separated on TLC and visualized under long wave UV. A bright blue spot was observed in the crude oat root extracts (Figure 4.1A). The R_f value of the spots under the TLC conditions was 0.80. *Gga* isolate MB 013 completely colonized the developed TLC plate (Figure 4.1C) whereas a zone of inhibition was observed in the oat lane on the plate colonized by *Ggt* isolate MB 027 (Figure 4.1B).

In the HPLC analyses, peaks with retention times of ~2.0 and ~2.2 min were common to wheat, bentgrass, oats, and the eluate from the TLC plate (Figure 4.2). No other peaks were observed in either the creeping bentgrass or the wheat root extract (data not shown). Two peaks with retention times of ~2.6 and ~3.2 min were prominent in both the TLC eluate and the diluted oat root extract.

4.3.2 Pathogenicity of *G. graminis* isolates

All *Gga* isolates were pathogenic on creeping bentgrass, wheat and oats (Table 4.3). *Ggt* isolates incited disease on wheat and creeping bentgrass. Creeping bentgrass plants inoculated with *Ggt* isolates, with the exception of MB 026 and MB 027, had significantly (P=0.05) longer roots than those inoculated with *Gga* isolates. With all hosts, generally as disease incidence or disease severity increased, the observed symptomatic area increased but mean plant height and root length decreased (data not shown).

Significant differences were observed in the levels of disease incidence on creeping bentgrass ($P<0.001$) and disease severity on oat (H=56.79, df=21, $P<0.001$) and wheat (H=64.58, df=21, $P<0.001$) among isolates of the same variety. Several of the *Ggt* isolates (e.g. MB 026, MB 027 and MB 0314) caused disease on creeping bentgrass at levels comparable to *Gga*. Most of the *Gga* isolates caused as severe or greater disease on wheat as compared with *Ggt* isolates. The ability of *G. graminis* isolates to cause disease incidence on creeping bentgrass was strongly correlated with the isolates' ability to cause disease severity on wheat based on a Spearman rank correlation of 0.93. None of the *Ggt* isolates tested were able to infect oats.

4.4 DISCUSSION

Avenacin was not detected in any of the creeping bentgrass cultivars evaluated in this study(Table 4.2, Figure 4.1), indicating avenacinase activity is not required for creeping bentgrass infection by *G. graminis*. Crude oat root extracts, but not creeping bentgrass or wheat, fluoresced bright blue under long wave UV (2, 14), indicating avenacin compounds had been extracted from root tips using the methanol extraction protocol described by Osbourn et al. (7). Similarly, fluorescent compounds were only detected in the oat root extracts when samples were analyzed by fluorimetry, TLC and HPLC (Table 4.2, Figure 4.1A, Figure 4.2). *Gga* isolate MB 013 completely colonized the developed TLC plate whereas growth of isolate MB 027 was inhibited by the fluorescent spot on the developed TLC plate (Figure 4.1B, C). This observation is consistent with the fungitoxic property of avenacin towards sensitive fungi such as *Ggt* (2, 7, 15). No inhibition of fungal growth by *Gga* or *Ggt* was observed in the wheat or creeping bentgrass extract

lanes on the TLC plates. HPLC analyses showed two prominent peaks (compounds) in the oat root extract and the eluate from the TLC plate which were not present in wheat or creeping bentgrass root extracts. These two peaks most likely correspond to avenacin compounds A-1 and B-1 which contain fluorescent *N*-methylanthranilate residues (2, 8). These fluorimetric, TLC and HPLC results are similar to those previously reported for wheat and oat root tip extracts (6, 7, 15) and clearly show creeping bentgrass does not produce avenacin.

Since avenacin is not produced in creeping bentgrass, avenacinase activity does not appear to be required for pathogenicity of *G. graminis* isolates on creeping bentgrass. The results of the infectivity assays support this conclusion, as all of the *Ggt* isolates were capable of inciting disease on creeping bentgrass (Table 4.3). Several *Ggt* isolates (e.g., MB 026, MB 027, and MB 0314) caused similar levels of root infection to that caused by *Gga* isolates.

Significant differences were observed in the levels of disease severity on wheat among isolates of the same variety, illustrating the natural variation present in pathogen populations. These differences were also observed in an isolate's ability to infect creeping bentgrass, however, those isolates that caused higher levels of disease incidence on bentgrass also caused higher levels of disease severity on wheat (r_s=0.93).

Interestingly, in previous studies (Thomas et al., unpublished), all of the *G. graminis* isolates recovered from symptomatic creeping bentgrass were identified as var. *avenae*, illustrating the prevalence of this variety as the causal agent of take-all patch on creeping bentgrass. Perhaps this is the result of differences in the abilities of each variety to colonize weedy grass species which may serve as asymptomatic reservoirs of the

pathogen. *Gga* has been shown to have a greater weedy grass host range than *Ggt* (11) which may maintain *Gga* populations as lands are renovated and developed into golf courses.

Although it has been shown to have little affect on other plant saponins (5), avenacinase may serve an as yet unknown function in the infection process of creeping bentgrass. The root extraction procedure used in this study was specific for avenacin, so there could be other defense compounds produced by creeping bentgrass, which *Gga* is capable degrading, but slow the infection process by *Ggt* that were not detected.

Although several *Ggt* isolates caused similar levels of disease as compared with *Gga*, most *Ggt* isolates caused low levels of root infection (Table 4.3). If *Ggt* causes take-all patch on creeping bentgrass under field conditions, the area affected may not be great enough to warrant sample taking, isolate recovery and identification. Field studies are in progress to assess the ability of *Ggt* isolates to infect creeping bentgrass under natural conditions.

This study shows that the creeping bentgrass-*G. graminis* interaction is not mediated by the host's production of avenacin or the pathogen's production of avenacinase and that *Ggt* may cause take-all patch of creeping bentgrass.

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Isolate	Host	Origination Information	
		Location	Source
Gaeumannomyces graminis var. *avenae*			
MB 001	*Agrostis stolonifera* L.	Ohio	This study
MB 002	*A. stolonifera*	Ohio	This study
MB 013	*A. stolonifera*	New Jersey	B. Clarke[w]
MB 014	*A. stolonifera*	Pennsylvania	This study
MB 019	*A. stolonifera*	Ohio	N. Tisserat[x]
MB 0214	*A. stolonifera*	Ohio	This study
MB 032	*A. stolonifera*	Ohio	This study
MB 0317	*Agrostis* spp.	Indiana	R. Smiley[y]
var. *tritici*			
MB 0126	*Triticum aestivum* L.	Ohio	This study
MB 025	*T. aestivum*	Virginia	E. Stromberg[z]
MB 026	*T. aestivum*	Montana	E. Stromberg
MB 027	*T. aestivum*	Virginia	E. Stromberg
MB 0311	*T. aestivum*	Washington	R. Smiley
MB 0312	*T. aestivum*	Washington	R. Smiley
MB 0313	*T. aestivum*	Washington	R. Smiley
MB 0314	*T. aestivum*	Washington	R. Smiley

Table 4.1. List of *Gaeumannomyces graminis* isolates used in this study.

[w] Bruce Clarke, Department of Plant Pathology, Rutgers University, New Brunswick, NJ 08901.

[x] Ned Tisserat, Department of Plant Pathology, Kansas State University, Manhattan, KS 66506.

Table 4.1 (continued)

Table 4.1 (continued)

[y] Richard Smiley, Columbia Basin Agricultural Research Center, Oregon State University, Pendleton, OR 97801.

[z] Erik Stromberg, Department of Plant Pathology, Physiology, and Weed Science, Virginia Polytechnic Institute and State University, Blacksburg, VA 24061.

Extracts[w]	Fluorescence[x]	Spiked Extracts[y]	Fluorescence
Methanol	2.0 a[z]		
Wheat	3.4 a	Spiked Wheat	21.7 b
Oat	97.9 c	Oat (1/100 dilution)	24.2 b
Creeping bentgrass cultivars			
Crenshaw	2.5 a	Spiked Crenshaw	23.8 b
L-93	2.7 a	Spiked L-93	24.2 b
Penn G-2	2.6 a	Spiked Penn G-2	23.6 b
Penncross	2.4 a	Spiked Penncross	23.2 b
Penneagle	2.4 a	Spiked Penneagle	23.2 b
Providence	2.6 a	Spiked Providence	24.1 b

Table 4.2. Fluorimetric analysis of root extracts from wheat, creeping bentgrass and oats.

[w] Wheat, oat, and bentgrass root extracts were diluted 20-fold (v/v) in 50% aqueous methanol.

[x] Excitation wavelength = 357 nm and emission wavelength = 430 nm.

[y] Spiked extracts contained 20-fold diluted wheat or bentgrass extract and 100-fold diluted oat extract.

[z] Multiple comparisons of mean ranks are based on the Kruskal Wallis test. Corresponding median fluorescent measurements of root extracts are shown. Values followed by the same letter are not significantly different at P=0.05.

Isolate	Host		
	Bentgrass[w] (incidence)	Wheat[x] (severity)	Oat[x] (severity)
Control	0 a[y]	0.0 a[z]	0.0 a[z]
G. graminis var. *avenae*			
MB 001	20 def	3.5 abcd	2.0 ab
MB 002	27 fgh	5.0 cd	2.5 ab
MB 013	33 h	5.0 cd	3.0 b
MB 014	18 cde	3.5 bcd	2.5 ab
MB 019	18 cde	2.5 abc	1.5 ab
MB 0214	45 i	5.0 d	5.0 b
MB 032	48 i	5.0 d	5.0 b
MB 0317	29 gh	5.0 d	4.0 b
G. graminis var. *tritici*			
MB 0126	7 ab	3.0 abcd	0.0 a
MB 025	1 a	2.5 abc	0.0 a
MB 026	24 efg	4.5 bcd	0.0 a
MB 027	14 bcd	4.0 bcd	0.0 a
MB 0311	2 a	1.5 ab	0.0 a
MB 0312	1 a	1.5 ab	0.0 a
MB 0313	1 a	1.0 ab	0.0 a
MB 0314	12 bc	3.5 bcd	0.0 a

Table 4.3. Take-all incidence and severity on graminaceous hosts.

[w] Mean disease incidence of creeping bentgrass roots was calculated by dividing the total number of visibly diseased roots (roots with discolored vascular systems) by the total number of roots observed from 50 plants.

[x] Median disease severity rating on wheat and oats. Severity was assessed on a rating scale of 0-5, in which 0=no disease and 5=a dead plant.

Table 4.3 (continued)

Table 4.3 (continued)

[y] Multiple comparisons of means are based on arcsine-transformed values. However, mean percentages are shown. Values followed by the same letter are not significantly different based on the LSD (P=0.05).

[z] Two oat and wheat plants were rated per conetainer and the average disease severity rating was used in analysis. Multiple comparisons of mean ranks are based on the Kruskal Wallis test. Corresponding median severity ratings are shown. Values followed by the same letter within a column are not significantly different at P=0.05.

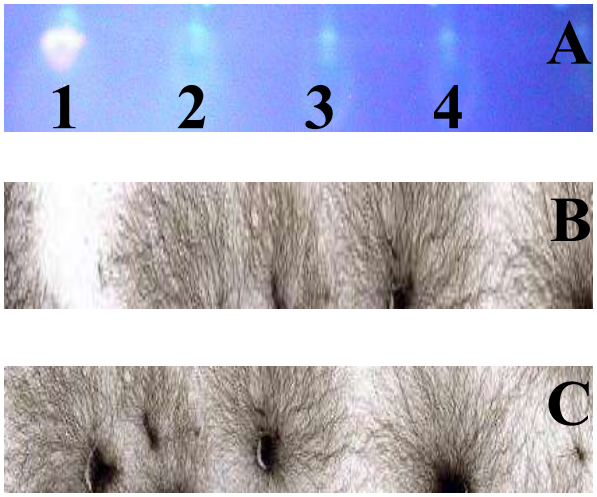

Figure 4.1. TLC analysis and fungal bioassay of oat, wheat, and creeping bentgrass root extracts. A: Developed TLC plate under UV illumination. Crude root extracts (100 µl) were separated on TLC plates using chloroform:methanol:water (13:6:1, v/v/v) solvent. Lanes: 1: oat root extracts; 2: wheat root extract; 3: 'Penneagle' creeping bentgrass root extracts; 4: 50% aqueous methanol. Fluorescent compounds correspond to the oat lane. No other fluorescent compounds were observed. B: Growth of *Ggt* isolate MB 027 on the developed plate. No growth occurred in the area where fluorescence was observed. C: Growth of *Gga* isolate MB 013 on the developed plate. No inhibition was observed.

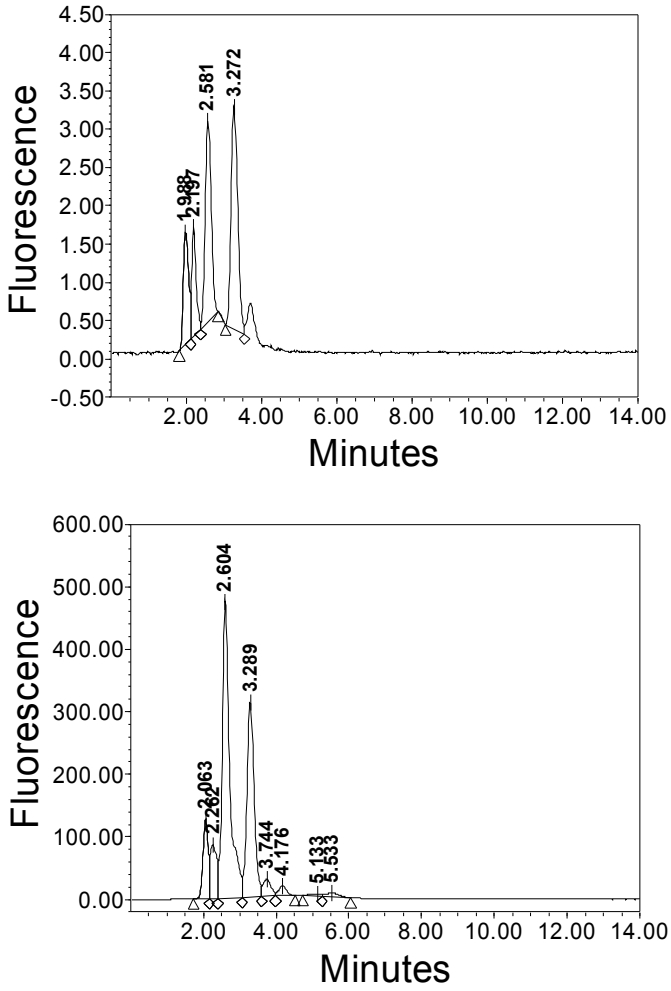

Figure 4.2. HPLC analysis of oat root extract and the fluorescent eluate from the TLC plate. Top: The oat root extract was diluted 20-fold (v/v) prior to analysis; Bottom: The fluorescent spot with R_f= 0.80 from the TLC plate was eluted in 200 µl 50% aqueous methanol. Injection volumes for both samples was 20 µl. The eluate from the TLC plate resulted in a more concentrated solution as indicated by the difference in the fluorescence scale (y-axis).

CHAPTER 5

FOLLOWING THE DISEASE PROGRESSION OF AN ECTOTROPHIC ROOT INFECTING FUNGUS.

5.1 INTRODUCTION

Soil-borne root-infecting fungi can be damaging to susceptible hosts. Control of diseases caused by root-infecting fungi can be difficult as symptom expression within the host may be delayed until periods of unfavorable environmental conditions, such as heat or drought stress. By the time symptom expression occurs, up to 50% or more of the host plant's roots may be infected. Some examples of diseases caused by root-infecting fungi and fungal-like organisms include take-all of wheat, Pythium root rot, Verticillium wilt of raspberry, and Fusarium wilt of pea.

Gaeumannomyces graminis is an ascomycete fungus that infects and colonizes the roots of graminaceous plants. Three morphological variants of the fungus have been described, but all three varieties produce specialized appressoria, called hyphopodia, and darkly pigmented runner hyphae. The hyphopodia function as organs of attachment to hold the fungus onto host roots. The darkly pigmented ectotrophic runner hyphae grow along the outside of the root and disseminate the pathogen by growing from root to root. Take-all of wheat, caused by *Gaeumannomyces graminis* var. *tritici*, is a devastating disease on wheat grown in the Pacific Northwest region of the United States. The disease cycle of the fungus is fairly simple as the pathogen survives, disseminates, and infects

through mycelial growth (Figure 5.1). G. *graminis* var. *tritici* survives in infected host tissues (crop residues) which remain in the field from season to season. However, *G. graminis* var. *tritici* has limited saprophytic abilities and will remain viable only as long as the wheat residue remains intact. It may also survive between wheat crops on the roots of alternative weedy grass hosts. When a new wheat crop is planted, the fungus grows out from the infected crop residue and infects newly seeded plants. The fungus will grow ectotrophically producing hyphopodia and infection hyphae which the penetrate the root and the colonization process begins. Fungal mycelium grows through the cortex of host roots, around plant cells, and eventually into the stele. Once *G. graminis* var. *tritici* begins colonizing the stele, vascular dysfunction occurs and the uptake of water and nutrients from the soil is reduced. Vascular discoloration, a key diagnostic symptom, is produced by fungal colonization of the stele (Figure 5.2). Spread of the fungus from plant to plant occurs as the dark ectotrophic runner hyphae grows from root to root. Once a host is colonized, perithecia are typically produced (Figure 5.3) on the lower leaf sheaths and stems of the plant. Ascospores are not considered to play an important role in this disease cycle.

Similar disease cycles occur for other *G. graminis*-incited diseases, such as *G. graminis* var. *avenae* on creeping bentgrass and oats and *G. graminis* var. *graminis* on rice. The objective of this exercise was to familiarize students with the root infection process through the establishment of host pathogenicity test tube assays. Test tubes were used to permit the observation of both host and pathogen growth, their interaction and the progression of the disease cycle. This assay is also a good way to produce perithecia of *G. graminis*. (Laboratory Instructor's Note #1)

5.1.1 Objective

To become familiar with the infection and colonization process of a root-infecting fungus

5.2 MATERIALS AND METHODS

5.2.1 For instructor or laboratory preparers. (Laboratory Instructor's Note #2)

5.2.1.1 List of materials needed for instructor or laboratory preparers.

- 10% Bleach solution (0.525% sodium hypochlorite) containing 0.1% Tween 20 (400 ml)
- Distilled water
- Wheat seeds (200 seeds per 50 students)
- Whatman no. 1 filter paper (4-10 cm discs) or 2 paper towels
- Self closing plastic bag
- Agar
- Disposable 25-ml pipets and pipet bulb
- Creeping bentgrass cv. Penneagle seed (10 ml)
- Cultures of *Gaeumannomyces graminis* var. *avenae* (Lab Instructor's Note # 3)
- No. 3 cork corer (8-mm diam.)
- 16 X 100 mm borosilicate test tubes (pre-sterilized); 4 per student

5.2.1.2 Preparation of materials for students by instructor.

1. Cultures of *G. graminis* var. *avenae* should be transferred to fresh 1/5 strength potato dextrose agar 7-10 days prior to use. This ensures the plates are completely colonized and actively growing. Using good aseptic technique, cut agar disks into the media using a no. 3 cork borer. Two plugs will be needed per student.
2. Pre-germination of wheat seeds. Surface sterilize wheat seeds in 200 ml of the 10% bleach solution for 5 min, then thoroughly rinse with 300 ml of distilled water three times.
3. Place wheat seeds on moistened filter paper or paper towels and spread out. Place paper and seeds in a plastic bag, seal closed with air in the bag, and incubate for 3-4 days at room temperature under natural light (Lab Instructor's Note #4, 5).
4. Prepare 1 liter of 0.3% water agar (3 g agar per liter) per 45 tubes to be used. Autoclave for 20 min then dispense 20 ml into each test tube. Allow tubes to cool for a minimum of 2 hours prior to use. Tubes can be covered with parafilm or sterile test tube caps and stored at room temperature for 3-4 days before use.
5. An hour before lab, surface sterilize the creeping bentgrass seed following step 2. Place rinsed seed in a petri dish on a piece of filter paper to dry. Shake the dish gently to break up any clumps of seeds.

5.2.2 For students.

5.2.2.1 List of materials needed by students.

- 4 test tubes containing 0.3% water agar
- A culture of *G. graminis* var. *avenae* with agar discs pre-cut

- Sterilized toothpicks or dissecting probe
- Alcohol lamp (if using a dissecting probe)
- Wooden applicator sticks
- Pre-germinated wheat seeds
- Surface sterilized creeping bentgrass seed
- Tweezers
- Parafilm
- Test tube rack

5.2.2.2 Preparation of assay tubes.

1. Label test tubes with the following information: name, date, fungal isolate, host and lab section. Label two tubes as controls and two as inoculated.
2. Set up 2 control tubes: Into one test tube, sprinkle a pinch of the sterilized creeping bentgrass seed. Seal the tube with parafilm and place into the test tube rack.
3. Surface sterilize tweezer tips over an alcohol flame for a few seconds. Allow the tweezers to cool for a few seconds, then gently pick up a pre-germinated wheat seed and place it roots-down onto the top of the water agar layer in the test tube. Seal the tube with parafilm and place in the test tube rack.
4. Set up 2 inoculated tubes: If using a probe, sterilize the tip over an alcohol burner and cool for a few seconds before using. With the tip of the probe or a sterile toothpick, gently lift an agar disk from the colonized culture plate and drop it onto the water agar.
5. Gently press the agar disk with a wooden stick to submerge it about an 1" (2.5 cm) into the water agar.

6. Repeat Steps 4 and 5 with the second tube.
7. Seed the tubes following Steps 2 and 3 for the 2 inoculated tubes. Place all tubes in a test tube rack and place in a growth chamber set to provide a 16 h photoperiod and 15ºC.
8. Once wheat seedlings have produced leaves long enough to stick out of the tubes, remove parafilm and plug the opening with a cotton plug permitting the wheat leaves to grow out of the tube.
9. Remove parafilm 14 days after assay set-up from the bentgrass tubes. (Figure 5.4) (Laboratory Instructor's Note #6)
10. Observe plant development and root colonization weekly under a dissecting microscope through the glass tubes and note the progression of the disease on the chart. Be sure to note the growth of mycelium from the agar plug, infection and colonization of the host and symptom expression.
11. Observations of perithecia. Obtain a pair of tweezers, alcohol lamp, a microscope slide, a cover slip and water dropper bottle.
12. Surface sterilize the tweezer tips over an alcohol flame and cool for a few seconds. Remove the parafilm or cotton plug from the inoculated tube and using the tweezers, gently retrieve bentgrass or wheat plants with perithecia. Place the plant tissue onto the microscope slide and add a drop of water. Add the cover slip and observe the perithecium under the compound microscope. If asci and ascospores are not ejected, a little pressure can be applied to the cover slip to break open the perithecium.

5.3 RESULTS

Be sure to note pathogen and host growth, the date root infection is first observed on each host, and any signs or symptoms as they are produced in Table 5.1. (Laboratory Instructor's Note #7)

Date	*Host*	*Observations*

Table 5.1. Observations of host growth and disease progression.

5.4 FOLLOW-UP QUESTIONS

1. Give two examples of how aseptic technique was used in this experiment. Why do you think this was important?
2. Were there differences in the rate of root infection and perithecial development between creeping bentgrass and wheat?
3. What was the purpose of including non-inoculated tubes? Were there any differences observed between the non-inoculated and the inoculated tubes? If so, what were they?
4. Describe where the perithecia were produced on each host. Given this location, what role do you think perithecia and ascospores play in the disease cycle?

5.5 LAB INSTUCTOR'S NOTES

1. Perithecia production requires an incubation period of at least 6 weeks. Assays may be set-up weeks in advance to ensure students will observe all steps of the *G. graminis* disease cycle. See Note #6 below.
2. Depending on lab size and supplies available, students may work individually, in pairs or small groups. Adjust the number of test tubes and wheat seeds needed accordingly.
3. Isolates recently collected from infected plant material work best. *G. graminis* var. *avenae* was chosen for this exercise because it will infect both wheat and creeping bentgrass. All isolates tested with this method are maintained on 1/5 strength PDA. It is unknown what impact, if any, full strength media would have on cultures and this procedure.
4. Depending on environmental conditions and seed age, this time may vary. Test the timing of 10-12 seeds under normal lab conditions to determine when to perform this step. Seedlings should not become rooted into the filter paper or the paper towels because it then becomes difficult to inoculate the tubes.
5. Petri dishes with small discs of filter paper or paper towels can be used in place of the plastic bag. This may be a preferred method as 8-12 seeds can be placed in one dish, and the dishes can be distributed to groups or tables of students.
6. Test tubes do not need to be covered with parafilm until 28 days after set-up and only if perithecia are desired. Perithecial formation begins as small spherical mycelial balls, that form perithecial necks during maturation. Depending on isolates, perithecial initials may be observed as early as 28 days with mature perithecia by 35-42 days.
7. It is normal to see the water agar disappear over time as the water is utilized by the

plants. The decrease in media will be more noticeable in the wheat tubes. Additional water agar (cooled-more like a lumpy gel) can be added to wheat tubes if necessary, but this may delay maturation of perithecia.

5.6 LITERATURE CITED

Smiley, R.W., Dernoeden, P.H., and Clarke, B.B. 1992. Compendium of turfgrass diseases, 2nd Ed. APS Press, St. Paul, MN. 98pp.

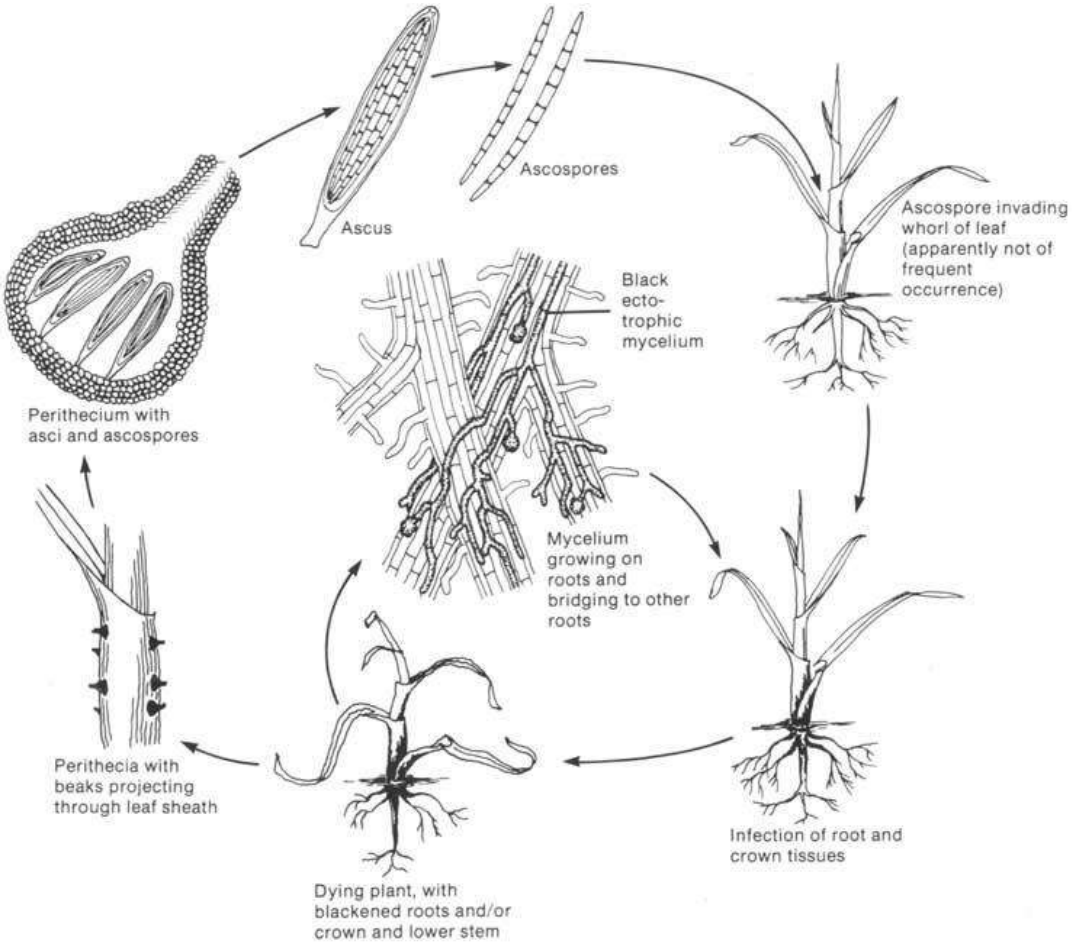

Figure 5.1. Disease cycle for take-all patch on graminaceous hosts. Taken from the Compendium of Turfgrass Diseases (Smiley et al., 1992).

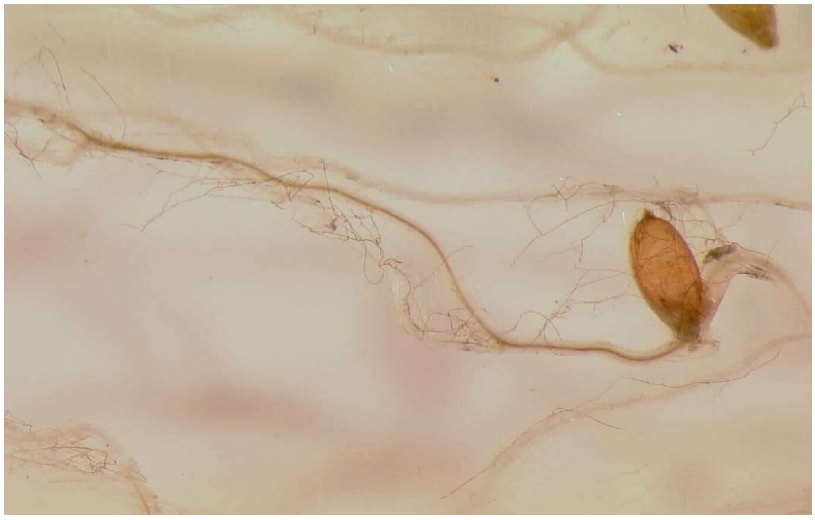

Figure 5.2. Vascular discoloration and runner hyphae typical of *G. graminis* root infection on creeping bentgrass.

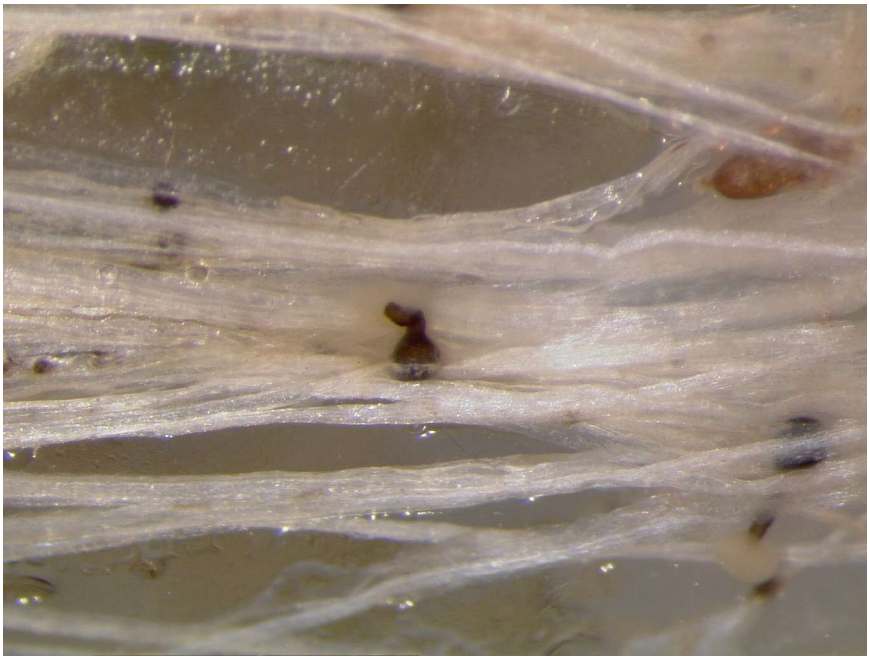

Figure 5.3. Mature perithecium of *G. graminis* embedded in creeping bentgrass tissues.

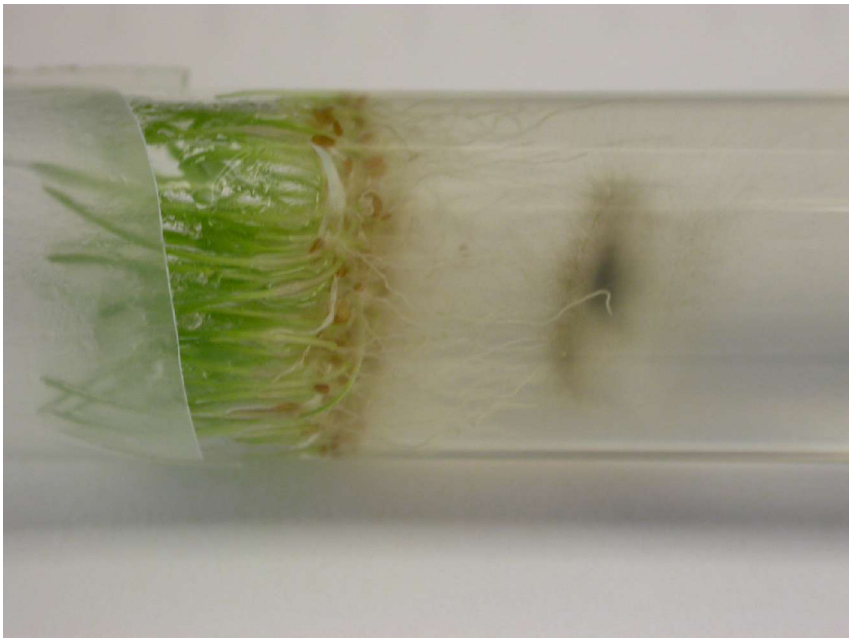

Figure 5.4. Set-up of creeping bentgrass pathogenicity assay. Note root growth through the inoculum plug.

CHAPTER 6

THE DEVELOPMENT OF A REAL-TIME QUANTITATIVE PCR ASSAY FOR THE DETECTION OF *GAEUMANNOMYCES GRAMINIS*

6.1 INTRODUCTION

Take-all, caused by *Gaeumannomyces graminis* (Sacc.) Arx & Olivier, can be a devastating disease of cereal and grass crops worldwide. The fungus is a soil-borne ascomycete that colonizes the roots of susceptible hosts leading to the production of stunted plants and whiteheads in cereal crops and to straw-colored thinning patches within stands of turfgrasses. Three morphological variants have been described within the species and are differentiated based on hyphopodium type and ascospore length (19). *G. graminis* var. *avenae* (E.M. Turner) Dennis (*Gga*) which is characterized by simple hyphopodia and longer mean ascospore lengths (i.e., 100-130 μm; 19), causes disease primarily on oats (*Avena sativa* L.; 17) and creeping bentgrass (*Agrostis palustris* Huds.; 16, 17), however, it may also infect wheat (*Triticum aestivum* L.) and barley (*Hordeum vulgare* L.). *G. graminis* var. *tritici* Walker (*Ggt*) incites disease on wheat and barley and is characterized by shorter mean ascospore lengths (i.e., 70-105 μm; 19) and simple hyphopodia. *G. graminis* var. *graminis* (*Ggg*), which is generally differentiated based on the production of lobed hyphopodia, has ascospore lengths comparable to Ggt (i.e., 80-105 μm; 19) and causes disease on warm season grasses and rice (*Oryza sativa* L.).

Positive identification of *G. graminis* varieties is complicated by the fact that the varieties are indistinguishable in culture and overlaps in the definitive morphological characters has been observed by many researchers (3, 17, 19, 23, 25; Chapter 2) and have led to isolate identifications of intermediate or indeterminate.

There is no known resistance available against *G. graminis* in any of the hosts affected. Isolating and quantifying the fungus directly from soil samples has been shown to be difficult and unreliable in assessing the presence of *G. graminis* in a given soil site. In addition, all three varieties can survive on the roots of weedy grass species in areas surrounding fields, paddies, or golf courses. Often infections on weedy grass hosts are asymptomatic (1, 7, 11) and usually its not until a susceptible economically important host is planted that the presence of *G. graminis* is confirmed. However, if *G. graminis* is known to be present in a given soil prior to seeding with a susceptible host, cultural practices such as tilling or removal of weedy grass species can be performed to reduce the amount of viable inoculum (24).

Identification and detection of bacterial and fungal species has been facilitated by the advent of molecular techniques over the past 15 years. Assays developed and used for the identification or detection of *G. graminis* have included ELISA (4), RAPD (5, 22) and RFLP analyses (6, 12, 20, 21). PCR primer sets specific to mitochondrial DNA (9, 10, 15) and internal transcribed spacer (ITS) DNA between rDNA genes (2, 8) have also been designed in an effort to accurately identify *G. graminis* varieties. However, in all these processes, some degree of cross reactivity either between the varieties or with other ectotrophic root pathogens have prevented the adoption of these assays for clinical diagnosis purposes (4, 9, 10, 20, 21). Additionally, the Ggg population has proven to be

too variable to give consistent results (5, 6, 21, 22). Rachdawong et al. (14) recently reported on the production of *G. graminis* variety-specific primer sets in which the three varieties of *G. graminis* could be distinguished from closely related *Gaeumannomyces* species and from each other. This study was initiated to develop a variety specific real-time PCR assay for Gga based on the sequence dissimilarities among the avenacinase gene of Gga and the avenacinase-like genes of Ggt and Ggg. The development of a quantitative assay will hopefully permit the investigation into population dynamics of the *G. graminis* varieties under various soil and control conditions leading to improved disease control methods.

6.2 MATERIALS AND METHODS

6.2.1 Maintenance and cultivation of fungal isolates.

Fungal isolates used in this study are listed in Table 2.1. All fungal isolates were maintained on 1/5 strength potato dextrose agar (PDA) as previously described in Chapter 2.

6.2.2 Preparation of fungal genomic DNA.

DNA extracted and used in previous molecular studies (Chapter 2) was stored at -20ºC until use in this study.

6.2.3 Cloning and sequencing avenacinase and avenacinase-like fragments.

The primer set AV1:AV3 (see Figure 6.1 for sequences and annealing sites) was used to amplify 1.4 kb regions from the 5'-end of the avenacinase and avenacinase-like genes

(11). PCR was carried out in a 20-µl reaction mixture which contained 1x reaction buffer, 200 µM dNTPs, 4.5 mM $MgCl_2$, 50 ηg of each primer, 1.0 U Platinum *Taq* DNA polymerase (Invitrogen Corp., Carlsbad, CA) and 20-30 ηg DNA. PCR was performed in a thermal cycler (PTC-200, Peltier Thermal Cycler, M.J. Research, Inc, Reno, NV) with the following cycle: an initial denaturation of 3 min at 95°C, then 30 cycles of denaturation at 94°C for 45 sec, annealing at 59°C for 1 min, and extension at 72°C for 2.5 min. A final extension period for 7 min at 72°C occurred prior to a final incubation at 4°C. PCR products were separated by electrophoresis on 0.8% agarose gels, visualized with ethidium bromide and photographed with the Gel DOC-IT DNA imaging system (Ultra-Violet Products, Inc., Upland, CA).

Amplicons from Gga isolates MB 014, MB 0113, MB 001, MB 004, MB 0114, MB 013, MB 0215; Ggg isolates MB 015, MB 0219; and Ggt isolates MB 0126 and MB 0218 were excised from the agarose gels, purified with the GFX PCR DNA & Gel Band Purification Kit (Amersham Biosciences Corp., Piscataway, NJ), and cloned into the pGEM-T Easy vector (pGEM-T Easy Vector System I, Promega Corporation, Madison, WI). Competent DH10β *E. coli* cells were transformed via electroporation with recombinant plasmids. Bacterial colonies were blue-white selected on LB agar (pH=8.0) amended with ampicillin (100 µg ml^{-1}), X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) and IPTG (isopropyl-β-D-thiogalactopyranoside) grown on 37°C for 18 to 24 h. Ten randomly selected blue colonies per isolate were transferred with a pipet tip to 250-ml Erlenmeyer flasks containing 50 ml LB broth (pH=8.0) amended with ampicillin (100 µg ml^{-1}) and incubated in an orbital shaker at 37°C for 18 to 24 h. Plasmids were extracted and purified with the QIAprep Spin Miniprep Kits (QIAGEN, Inc., Valencia,

CA). Plasmids were digested with *Not* 1 (New England Biolabs, Beverly, MA) and digestion products were separated on 0.8% agarose gels to confirm they have the insert then sent to the Plant-Microbe Genomics Facility at The Ohio State University for sequencing using a dye-terminator sequencing cycle (BigDye Terminator Cycle Sequencing, Applied Biosystems, Inc., Foster City, CA). The sequencing was performed with an ABI 3700 DNA Analyzer (Applied Biosystems). Four internal primers (see Figure 6.1 for annealing sites and sequences of Ave 467 R, Ave 450 F, Ave 884 F, and Ave 908 R) were designed and used in the sequencing reactions to ensure both strands were read twice. One amplicon was sequenced per isolate. Sequences were aligned and amino acid sequences deduced with the Sequencher program (Gene Codes Corp., Ann Arbor, MI).

6.2.4 Design of real-time PCR primers and probe.

Highly homologous 250-350 bp subsequences within the consensus avenacinase sequence were subjected to analysis by the Primers Express Program (PE Applied Biosystems, Foster City, CA) to generate Gga-specific primer and probe sets for use in the TaqMan format (probe hydrolysis). The primer set identified included forward primer Gga 723F (5'-GCCTGCACCAAGCACTTCA-3') and reverse primer Gga 809R (5'-ACGAGATGGCCTCAACCGT-3') for use with the probe (5'-FAM-AGGAGGAGCAGCGCAACCCCA-TAMRA-3'). Primer sequences are presented in Figure 6.3. Primers and probes were synthesized by Invitrogen (Invitrogen Corp., Carlsbad, CA).

6.2.5 Amplification with TaqMan primer sets.

Specificity of the Gga 723F:Gga 809R primer set was assessed with PCR reactions with DNA from Gga, Ggt and Ggg isolates. PCR was carried out in a 15-µl reaction mixture which contained 1x reaction buffer, 200 µM dNTPs, 3 mM $MgCl_2$, 0.3 µM of each primer, 0.375 U Platinum *Taq* DNA polymerase (Invitrogen Corp., Carlsbad, CA) and 10 ηg DNA. PCR was performed in a thermal cycler (PTC-200, Peltier Thermal Cycler, M.J. Research, Inc, Reno, NV) with the following cycle: an initial denaturation of 3 min at 95°C, then 30 cycles of denaturation at 94°C for 45 sec, annealing and extension at 65°C for 1 min. A final extension period for 3 min at 72°C occurred prior to a final incubation at 4°C. PCR products were separated by electrophoresis on 3.0% agarose gels, visualized with ethidium bromide and photographed with the Gel DOC-IT DNA imaging system (Ultra-Violet Products, Inc., Upland, CA).

6.2.6 Real-time PCR amplifications with SYBR green.

SYBR green was used to monitor DNA amplification. Reactions were carried out in a 15-µl reaction mixtures which contained 1X SYBR green reaction buffer containing dNTPs and $MgCl_2$ (LightCycler DNA Master SYBR Green 1 kit, Roche Applied Science, Indianapolis, IN), 0.3 µM of each primer, 0.375 U Platinum *Taq* DNA polymerase (Invitrogen Corp., Carlsbad, CA) and 50 pg DNA. PCR was performed using a LightCycler (LightCycler 2.0 Instrument, Roche Applied Science, Indianapolis, IN) with the following cycle: an initial denaturation at 95°C for 15 sec then amplification with a two-step cycle of 94°C for 0 sec, annealing and extension at 60°C for 15 secs (50 cycles). Samples were always run in duplicate and each run contained two TE controls.

6.2.7 Real-time PCR amplifications using probe hydrolysis.

PCR was carried out in a 15-µl reaction mixture which contained 1X reaction buffer (10X contains BSA and 10 mM $MgCl_2$; Idaho Technology, Salt Lake City, UT), 200 µM dNTPs, 1.5 mM $MgCl_2$, 0.3 µM of each primer, 0.1 µM probe, 0.375 U Platinum *Taq* DNA polymerase (Invitrogen Corp., Carlsbad, CA) and 50 pg DNA. PCR was performed as previously described for the SYBR green reactions using a Roche LightCycler instrument.

6.3 RESULTS

6.3.1 Sequences of avenacinase and avenacinase-like fragments.

Most of the Gga isolates (e.g., MB 001, MB 004, MB 013, and MB 0114) sequenced in this study had greater than 99% similarity with the consensus 1.4 kb avenacinase sequence (Figure 6.1). Isolates MB 014 and MB 0113 had the most variable sequence for Gga isolates which were 97% and 98% identical to the consensus avenacinase sequence, respectively. Both Ggt avenacinase-like sequences were 97% homologous to the avenacinase sequence of Gga. The sequences from Ggg isolates MB 015 and MB 0219 had over 200 base changes resulting in only 85% homology with the consensus sequence of Gga. Almost 75% of the base changes observed in the sequences from Ggt isolates MB 0126 and MB 0218 were also present in the avenacinase-like sequences of isolates previously identified as Ggg by Rachdawong et al. (14).

Comparisons made against previously reported sequence data by Rachdawong et al. (14) for the avenacinase and avenacinase-like genes show the Gga-specific reverse primer sequence to be conserved in Gga isolates (Figure 6.1). Gga isolates MB 014 and MB

0113 contained a single nucleotide change within this primer sequence which did not affect amplification (Table 2.1). The specificity of the Gga reverse primer resulted from polymorphisms at bases 776 (G to A), 791 (A to G) and 792 (C to G) in Ggg and Ggt. The sequence data generated in this study show all of these polymorphisms to be maintained in Ggt populations and two of them in the Ggg isolates. The Ggt-specific reverse primer sequence was conserved in Ggt isolate MB 0126 but not isolate MB 0218. The nucleotide base change at the 3'-end from T to C which aided in Ggt-variety specificity was found to be common in Gga based on avenacinase sequences. Five nucleotide changes were observed in each Ggg avenacinase-like sequence in the region encoding the Ggg-specific reverse primer (14). The avenacinase-like gene in Ggt isolate MB 0218 contained the single nucleotide polymorphism which comprised the specificity for Ggg.

Amino acid sequences were extremely similar among the Gga isolates (Figure 6.2). Three isolates varied by two or less amino acids (e.g., MB 004, MB 013, and MB 0114). Gga isolates MB 0215 and MB 001 both had a base pair insertion that resulted in reading frame shifts and much different amino acid sequences. Similar to the nucleotide sequence data, the Gga isolates MB 014 and MB 0113 had a similar number of amino acid changes as the Ggt isolates (i.e., 15 to 17). The amino acid sequences for Ggg isolates MB 015 and MB 0219 were much more variable as compared to those of Gga and Ggt.

6.3.2 Development of Gga-specific real-time PCR assay.

All Gga isolates produced 86 bp amplicons (Figure 6.4) when genomic DNA was amplified with the primer set Gga723F:Gga809R. Most Ggg and Ggt isolates failed to

produce any amplicons when amplified with the Gga-specific primer set, however, several isolates did produce faint bands comparable in length to those produced by Gga (Figure 6.4).

All reactions performed using SYBR green to monitor DNA amplification exhibited an exponential increase in dsDNA concentration (Figure 6.6). Reactions containing Gga DNA had the earliest crossing times (C_T values) which were generally 15 cycles before Ggg or Ggt samples and 25 cycles sooner than TE (no DNA controls) reactions. Melting curve analysis revealed the amplicons produced by TE, and DNA from Ggt and Ggg isolates had similar T_m values (melting temperatures) of 81.0ºC. The T_m for amplicons produced by Gga DNA samples was 88.6ºC (Table 6.1). Melting curve analysis of Gga DNA dilution reactions indicated that the desired 86 bp amplicon was the primary PCR product at DNA concentrations greater than 50 fg (data not shown).

All reactions containing Gga DNA exhibited amplification via an increase in fluorescence when probe hydrolysis was used to monitor DNA amplification (Figure 6.5). In addition, reactions containing DNA from Ggt and Ggg also experienced an exponential increase in fluorescence typical of DNA amplification and probe hydrolysis. Control capillaries which contained TE instead of DNA exhibited fluorescence irregularly throughout the development and optimization of the assay. In attempts to reduce the non-specific amplification or the primer-probe concatemerization leading to the changes in fluorescence and false positive signal, the concentrations of all reagents were varied. No amplification would occur without the addition of dNTPs, Taq polymerase, a $MgCl_2$ concentration greater than 1.5 mM, or buffer. $MgCl_2$ concentrations were varied 1 to 5 mM and as the concentration increased, the C_T values for both Gga and Ggt became

smaller. Varying primer concentrations did not reduce the amplification signal observed for DNA from Ggt.

The utilization of both SYBR green and probe hydrolysis permitted similar amplification conditions to be tested in an environment that could be subjected to melting curve analysis. PCR reactions performed in duplicate TE, Gga, and Ggt DNA samples that were then subjected to melting curve analysis showed each DNA type (i.e., TE, Gga and Ggt) generated an unique amplicon (Figure 6.6).

6.4 DISCUSSION

6.4.1 Avenacinase and avenacinase-like sequences

Similar to the conclusions by previous researchers, Gga and Ggt seem to be more closely related to each other as compared with Ggg as the avenacinase sequences from Gga and the avenacinase-like sequences from Ggt were 97% identical (Figure 6.1). Several Gga isolates contained either an insertion or deletion that resulted in a frame shift and much different amino acid sequence (e.g., MB 001 and MB 0215). Only one amplicon per isolate was sequenced. As such, the nucleotide changes and resultant shifts in amino acid sequence many be an artifact of the amplification step, that is a product of the Taq polymerase and not the true sequence.

Corresponding amino acid sequences were also quite similar (Figure 6.2). Previous work by Osbourn et al. (13) showed that the amino acid sequences of avenacinase and tomatinase enzymes were similar and both possess two aspartic acid residues (276-279 V-S-D-W and 292-295 G-L-D-M) which are believed to be the active catalytic sites for saponin deglucosylation. As expected, all seven Gga isolates with the exception of MB

0215, contained both active residues. Interestingly both Ggt isolates sequenced in this study also contained the aspartic acid residues. Further discussion should be prefaced with the knowledge that only 1.4 kb out of 3.5 kb that encode avenacinase genes were sequenced and there may be a decrease in homology between Gga and Ggt. However, given the similarities in the first 1.4 kb and the presence of the active deglucosylation sites, perhaps the few amino acid differences affect the tertiary structure of Ggt avenacinase-like enzymes resulting in an inability of avenacin to to bind to avenacinase. Since many researchers believe that Gga developed the ability to infect oats through acquisition of the avenacinase activity, the avenacinase-like gene may be the precursor to the avenacinase gene which arose through mutations in response to the selective avenacin pressure from oats.

The sequence information presented in this study also confirms conclusions made previously that Ggg isolate MB 024 is Ggt. Sequencing data from isolate MB 0126 was similar to that generated for Ggt isolates, however, the sequence from isolate MB 0218 was more similar to that reported by Rachdawong et al. (14) for Ggg isolates. As previously discussed, Ggg isolate MB 024 was identified by Turner as *G. graminis* and Rachdawong et al. (14) used it as Ggg based on sequence dissimilarities to Ggt isolates. These results presented here, in addition to those previously presented (14), show there are two subpopulations of Ggt which differ slightly in their avenacinase-like sequence. The differences in nucleotide sequences resulted in slightly different amino acid sequences and the exact effect of these differences is unknown. Rachdawong et al. (14) reported *G. graminis* isolates recovered from symptomatic wheat roots and producing amplicons with the Ggg-specific primer set were less aggressive on wheat as compared

with those isolates generating amplicons with the Ggt-specific primer set.

The avenacinase-like sequences from Ggg isolates generated in this study clearly show that the reason for the lack of amplicons from Ggg isolates when amplified with the Ggg-variety specific primer set designed by Rachdawong et al. (14) as five base changes were present in the sequences from Ggg isolates within the Ggg-specific primer site (Figure 6.1). Similar to previous work with Ggg isolates, the sequence information generated was quite variable as compared with Gga and Ggt (i.e., there were over 200 base pair differences) but also within the variety. Due to the dissimilarities of the sequences from Ggg, the avenacinase-like gene is not a good target for the development of Ggg-specific primer sets.

6.4.2 Development of a real-time PCR assay for Gga.

All Gga isolates produced 86 bp amplicons indicating the primer sequences were conserved among the Gga population (Figure 6.4). Given the high sequence homology amongst Gga isolates (Figure 6.1), these results are not surprising. The faint bands produced by several amplifications with DNA from Ggt and Ggg isolates indicate some non-specific amplification is occurring. However, the annealing and amplification time will be reduced in the LightCycler assay and the non-specific products should not be generated or detected within the 50 amplification cycles.

Based on the results with SYBR green monitoring DNA amplification, primer dimers were concluded to be the amplicons produced in reactions with DNA from Ggt and Ggg isolates as all amplicons produced by TE, Ggt, and Ggg had extremely similar T_m values (Table 6.1). The earlier C_T values for reactions containing DNA from Ggg or Ggt as

compared with reactions containing TE (i.e., no DNA controls) was probably due to a stabilization of primer dimers by the presence of additional DNA. Given that primer dimers were the only non-specific PCR products detected, the assay was transitioned to using probe hydrolysis for monitoring DNA amplification.

Based on the results using probe hydrolysis, the Gga-specific real-time PCR assay could not be optimized to solely amplify DNA from Gga (Figure 6.6). Reactions with Ggt and Ggg DNA consistently produced fluorescence similar to Gga samples, only at later cycle numbers. The utilization of both SYBR green and probe hydrolysis to monitor DNA amplification permitted the T_m of any amplicons produced in the reactions with TE, Gga, and Ggt to be determined. Each origin of DNA resulted in unique amplicons (Figure 6.7), that is dsDNA produced by TE samples melted at 84ºC, Ggt amplicons at 88ºC and Gga amplicons at 89ºC. Contrary to the previous results with SYBR analysis, these results indicate that non-specific amplification is occurring in reactions with Ggt DNA. The only differences between the SYBR green reactions and the probe hydrolysis reactions were the reaction buffer and the presence of a fluorescently-labeled probe. The buffer used with the probe hydrolysis assays was purchased from Idaho Technology (Salt Lake City, UT) whereas Roche was the supplier of the buffer in the SYBR green kit. Differences in the salt concentrations within the buffers may have lead to the non-specific amplification observed in the hydrolysis assays and favored primer dimer amplification in the SYBR green assays. The probe could have enhanced or stabilized the formation of primer dimers or primer-probe concatamers in reactions containing TE in lieu of DNA.

Besides adjusting reagent concentrations within the master mixes to hinder the non-specific amplification observed by Ggt and Ggg, the annealing and amplification

parameters of the two-step cycle were adjusted. The melting condition of 95°C for 0 secs was maintained. The following annealing and amplification conditions were evaluated: 60°C for 8 sec; 65°C for 8 sec; 68°C for 8 sec; 60°C for 3 sec; 65°C for 3 sec; and 68°C for 3 sec. The only effect altering amplification and annealing conditions had was to shift the crossing point (C_T) of the fluorescence to later cycles. Non-specific amplification was still observed. At temperatures greater than 70°C, no probe hydrolysis occurred which is consistent with the loss of expression of the DNA polymerase exonuclease activity at these temperatures.

This study showed that real-time PCR assays have promise in identifying and differentiating microorganisms, however genetic similarities among pathogen populations can greatly increase the difficulty of developing specific assays. The number and placement of polymorphisms within the forward and reverse primer sites designed in this study were probably not great enough to ensure variety specificity (Figure 6.6, 6.7). One of the frustrations with the avenacinase gene sequences was the conserved regions high in GC content which could not be used for developing primers and probes due to a preference of 50% GC content and the high melting temperatures associated with these areas. Future studies should be directed at the transition of the Gga:AV3 primer set as described by Rachdawong et al. (14) to real-time primers for the detection of Gga. Given the success of this primer in differentiating *G. graminis* isolates (Table 2.1) this gene sequence has great potential in being selective for Gga.

Based on the avenacinase-like sequence information generated, this gene is not a good target for developing Ggt- or Ggg-specific primers and probes. Ggt populations have been

shown to have to sub-populations that differ in the avenacinase-like sequence and Ggg populations have been shown to have a highly variable genetic sequence.

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DNA (50 pg)	Amplicon Melting Temperature (ºC)
TE	81.27
G. graminis var. *avenae*	
MB 001	88.55
MB 004	88.56
MB 013	88.58
MB 014	88.97
MB 0110	89.04
MB 0114	88.50
MB 0116	88.51
MB 0215	88.40
G. graminis var. *tritici*	
MB 0126	80.50
MB 025	80.53
MB 027	80.86
MB 029	80.76
MB 0210	80.70
MB 0311	80.48
MB 0314	80.41
MB 0218	80.40
MB 024	80.48
G. graminis var. *graminis*	
MB 015	80.40
MB 031	80.61
MB 035	80.59
MB 036	80.62
MB 037	80.63
MB 038	80.97

Table 6.1. Amplicon melting temperatures from real-time PCR reactions performed with the primer set Gga 723F:Gga 809R. SYBR green was used to monitor amplification.

```
     Primer AV1>

1    AGATGTTGCGCTCAAGTGCTTTCGCTCTCCTCGCCTGGGCCTCCCTCTCGGAAGCCCAGTTCGGCATCAAGCATACTCAG Gga [U35463]
     AGATGTTGCGCTCAAGTGCTTTCGCTCTCCTCGCCTGGGCCTCCCTCTCGGAAGCCCACTTCGGCATCAAGCATACTCAG Gga [AF365953]
     AGATGTTGCGCTCAAGTGCTTTCGCTCTCCTCGCCTGGGCCTCCCTCTCGGAAGCCCAGTTCGGCATCAAGCATACTCAG MB 001
     AGATGTTGCGCTCAAGTGCTTTCGCTCTCCTCGCCTGGGCCTCCCTCTCGGAAGCCCAGTTCGGCATCAAGCATACTCAG MB 004
     AGATGTTGCGCTCAAGTGCTTTCGCTCTCCTCGCCTGGGCCTCCCTCTCGGAAGCCCAGTTCGGCATCAAGCATACTCAG MB 013
     AGATGTTGCGCTCAAGTGCTTTCGCTCTCTTCGCCTGGGCCTCCCTCTCGGAAGCCCAGTTCGGCATCAAGCATACTCAG MB 014
     AGATGTTGCGCTCAAGTGCTTTCGCTCTCCTCGCCTGGGCCTCCCTCTCGGAAGCCCAGTTCGGCATCAAGCATACTCAG MB 0113
     AGATGTTGCGCTCAAGTGCTTTCGCTCTCCTCGCCTGGGCCTCCCTCTCGGAAGCCCAGTTCGGTATCAAGCATACTCAG MB 0114
     AGATGTTGCGCTCAAGTGCTTTCGCTCTCCTCGCCTGGGCCTCCCTCTCGGAAGCCCAGTTCGGCATCAAGCATACTCAG MB 0215

     AGATGTTGCGCTCAAGTGCTTTCGCTCTCCTCGCTTGGGCCTCCCTCTCGGAAGCCCAGTTCGGCATCAAGCATACTCAG Ggg [AF365954]
     AGATGTTGCGCTCAAGTGCTTTCGCTCTCCTCGCTTGGGCCTCCCTCTCGGAAGCCCAGTTCGGCATCAAGCATACTCAG Ggt [AF365955]
     AGATGTTGCGCTCAAGTGCTTTTGCTCTCCTTGCCTGGGCTTCCCTCTCGGAAGCCCAGTTTGGCATCAAGCATACCCAG MB 015
     AGATGTTGCGCTCAAGTGCTTTTGCTCTCCTTGCTTGGGCTCCCCTCTCTGAAGCCCAGTTTGGCATCAAGCATACTCAG MB 0219

     AGATGTTGCGCTCAAGTGCTTTCGCTCTCCTCGCTTGGGCCTCCCTCTCGGAAGCCCAGTTCGGCATCAAGCATACTCAG Ggt [AF365958]
     AGATGTTGCGCTCAAGTGCTTTCGCTCTCCTCGCTTGGGCCTCCCTCTCGGAAGCCCAGTTCGGCATCAAGCATACTCAG Ggt [AF365956]
     AGATGTTGCGCTCAAGTGCTTTCGCTCTCCTCGCTTGGGCCTCCCTCTCGGAAGCCCAGTTCGGCATCAAGCATACTCAG Ggt [AF365957]
     AGATGTTGCGCTCAAGTGCTTTCGCTCTCCTCGCTTGGGCCTCCCTCTCGGAAGCCCAGTTCGGCATCAAGCATACTTAG MB 0126
     AGATGTTGCGCTCAAGTGCTTTCGCTCTCCTCGCTTGGGCCTTCCTCTCGGAAGCCCAGTTCGGCATCAAGCATACTCAG MB 0218
```

Figure 6.1. Partial nucleotide sequences of the avenacinase and avenacinase-like genes from *Gaeumannomyces graminis*. Sequence data was generated with the primer set AV1:AV3. The Genbank accession numbers (listed in brackets) indicate the sequence data reported by Osbourn et al. (10) and Rachdawong et al. (11) which were compared to the sequences generated in this study. Nucleotide differences are in bold print and introns are shown in lowercase. Primer annealing sites are underlined and arrows indicate the direction of alignment. Alignments were performed by the Sequencher program (Gene Codes Corp., Ann Arbor, MI) and dashes were use to maximize the alignment.

Figure 6.1

(continued)

Figure 6.1 (continued)

```
81   TATGGCACGAGCGAGCCTGTCTACCCGTCGC-gtacgttatcaacaagccgaaa-gcctt-ccgcagaccatcccacttttt Gga [U35463]
     TATGGCACGAGCGAGCCTGTCTACCCGTCGC-gtacgttatcaacaagccgaaa-gcctt-ccgcagaccatcccacttttt Gga [AF365953]
     TATGGCACGAGCGAGCCTGTCTACCCGTCGC-gtacgttatcaacaagccgaaa-gcctt-ccgcagaccatcccacttttt MB 001
     TATGGCACGAGCGAGCCTGTCTACCCGTCGC-gtacgttatcaacaagccgaaa-gcctt-ccgcagaccatcccacttttt MB 004
     TATGGCACGAGCGAGCCTGTCTACCCGTCGC-gtacgttatcaacaagccgaaa-gcctt-ccgcagaccatcccacttttt MB 013
     TATGGCACGAGCGAGCCTGTCTACCCGTCGC-gtacgttatcaacaagccgaaaagcctt-ccgcagaccatcccacttctttt MB 014
     TATGGCACGAGCGAGCCTGTCTACCCGTCGC-gtacgttatcaacaagccgaaaagcctt-ccgcagaccatcccacttttt MB 0113
     TATGGCACGAGCGAGCCTGTCTACCCGTCGC-gtacgttatcaacaagccgaaa-gcctt-ccgcagaccatcccacttttt MB 0114
     TATGGCTCGAGCGAGCCTGTCTACCCGTCGC-gtacgttatcaacaagccgaaa-gcctt-ccgcagaccatcccactttt- MB 0215

     TATGGCACGAGCGAGCCTGTCTACCCGTCGC-gtacgttgtcaacaagccaaaa-gcctt-ccgcagaccatcccacttttt Ggg [AF365954]
     TATGGCACGAGCGAGCCTATCTACCCGTCGC-gtgcgttgtcaacaagccaaaa-gcctt-ccgcagaccatcccacttttt Ggt [AF365955]
     TATGACACGAGCGAGCCTGTCTATCCGTCGC-gtgcgtttttcggcagcccccaaaagcttcctcaaagaccatcctactcccct MB 015
     TATGACACGAGCGAGCCTGTCTATCCGTCGC-gtatgtttccaacaggccaaaaagcctcctcaccgaccatcgtactcccgt MB 0219

     TATGGCACGAGCGAGCCTGTCTACCCGTCGC-gtacgttgtcaacaagccaaaa-gcctt-ccgcagaccatcccacttttt Ggt [AF365958]
     TATGGCACGAGCGAGCCTGTCTACCCGTCGC-gtacgtagtcaacaagccaaaa-gcctt-ccgcagaccatcccacttttt Ggt [AF365956]
     TATGGCACGAGCGAGCCTGTCTACCCGTCGC-gtacgttgtcaacaagccaaaa-gcctt-ccgcagaccatcccacttttt Ggt [AF365957]
     TATGGCACGAGCGAGCCTGTCTACCCGTCGC-gtacgtcgtcaacaagccaaaa-gcctt-ccgcagaccatcccacttttt MB 0126
     TATGGCACAAGCGAGCCTGTCTACCCGTCGCagtacgttgtcaacaagccaaaa-gcctt-ccgaagaccatcccacttttt MB 0218
```


Figure 6.1 (continued)

Figure 6.1 (continued)

```
161  ----ctgtctcgtacttgtgctaatcttctcgcac-ctctagCCGAAATCTCTGGCTCTGGAGGATGGGAAGCTGGCCTGG Gga [U35463]
     ----ctgtctcgtacttgtgctaatcttctcgcac-ctctagCCGAAATCTCTGGCTCTGGAGGATGGGAAGCTGGCCTGG Gga [AF365953]
     ----ctgtctcgtacttgtgctaatcttctcgcac-ctctagCCGAAATCTCTGGCTCTGGAGGATGGGAAGCTGGCCTGG MB 001
     ----ctgtctcgtacttgtgctaatcttctcgcac-ctctagCCGAAATCTCTGGCTCTGGAGGATGGGAAGCTGGCCTGG MB 004
     ----ctgtctcgtacttgtgctaatcttctcgcac-ctctagCCGAAATCTCTGGCTCTGGAGGATGGGAAGCTGGCCTGG MB 013
     t---ctgtctcgtacttgtgctaatcttctcgcac-ctctagCCGAAATCTCTGGCTCTGGAGGATGGGAAGCTGGCCTGG MB 014
     t---ctgtctcgtacttgtgctaatcttctcgcac-ctctagCCGAAATCTCTGGCTCTGGAGGATGGGAAGCTGGCCTGG MB 0113
     ----ctgtctcgtacttgtgctaatcttctcgcac-ctctagCCGAAATCTCTGGCTCTGGAGGATGGGAAGCTGGCCTGG MB 0114
     ---cctgtgtcgtacttgtgctaatcttctcgcac-CTCTAgCCGAAATCTCTGGCTCTGGAGGATGGGAAGCTGGCCTGG MB 0215

     tt--ctgtctcgtacttgtgctaatctgctcgcac-ctctagCCGAAATCTTGGCTCTGGAGGATGGGAAGCTGGCCTGG Ggg [AF365954]
     t---ctgtctcgtccttgtgctaatctgctcgcac-ctctagCCGAAATCTCGGCTCTGGAGGATGGGAAGCTGGCCTGG Ggt [AF365955]
     t-cgtaccctaggacaagtactgatccgccgcacttctagCCAAAATCTCCGGTGCTGGAGGATGGGAAGCCGCTCTCG MB 015
     t-cgtgccccgggggcttgtactgatccgccgtacttctagCCAAAATCTCCGGCGCTGGAGGATGGGAAGCCGCCCTCG MB 0219

     ttt-ctgtctcgtacttgtgctaatctgctcgcac-ctctagCCGAAATCTCGGCTCTGGAGGATGGGAAGCTGGCCTGG Ggt [AF365958]
     ttt-ctgtctcgtacttgtgctaatctgctcgcac-ctctagCCGAAATCTCGGCTCTGGAGGATGGGAAGCGGGCCTGG Ggt [AF365956]
     ttttctgtctcgtacttgtgctaatctgctcgcac-ctctagCCGAAATCTCGGCTCTGGAGGATGGGAAGCTGGCCTGA Ggt [AF365957]
     ttttctgtctcgtacttgtgctaatctgctcgcac-ctctagCCGAAATCTCGGCTCTGGAGGATGGGAAGCTGGCCTGG MB 0126
     tt--ctgtctcgtacttgtgctaatctGctcgcac-ctctagCCGAAATCTTGGCTCTGGAGGATGGGAAGCTGGCCTGG MB 0218
```


Figure 6.1 (continued)

Figure 6.1 (continued)

```
                                                                  Ggg-specific primer>
241  CCAAAGCCAAGGACTTCGTCGCGCAACTGACGCCCGAGGAGAAGGCGAACATGGTCACAGG-CACCCCCGGTCCCTGCGTG Gga [U35463]
     CCAAAGCCAAGGACTTCGTCGCGCAACTGACGCCCGAGGAGAAGGCGAACATGGTCACAGG-CACCCCCGGTCCCTGCGTG Gga [AF365953]
     CCAAAGCCAAGGACTTCGTCGCGCAACTGACGCCCGAGGAGAAGGCGAACATGGTCACAGG-CACCCCCGGTCCCTGCGTG MB 001
     CCAAAGCC**C**AGGACTTCGTCGCGCAACTGACGCCCGAGGAGAAGGCGAACATGGTCACAGG-CACCCCCGGTCCCTGCGTG MB 004
     CCAAAGCCAAGGACTTCGTCGCGCAACTGACGCCCGAGGAGAAGGCGAACATGGTCACAGG-CACCCCCGGTCCCTGCGTG MB 013
     CCAAAGCCAAGGACTTCGTCGCGCAACTGACGCCCGAGGAGAAGGCGAACATGGTCACAGG-CACCCCCGGTCCCTGCGTG MB 014
     CCAAAGCCAAGGACTTCGTCGCGCAACTGACGCCCGAGGAGAAGGCGAACATGGTCACAGG-CACCCCCGGTCCCTGCGTG MB 0113
     CCAAAGCCAAGGACTTCGTCGCGCAACTGACGCCCGAGGAGAAGGCGAACATGGTCACAGG-CACCCCCGGTCCCTGCGTG MB 0114
     CCAAAGCCAAGGACTTCGTCGCGCAACTGACGCCCGAGGAGAAGGCGAACATGGTCACAGG-CACCCCCGGTCCCTGCGTG MB 0215

     CCAAAGCCAAGGACTTCGTCGCGCA**G**CTGACGCCCGAGGAGAAGGCGAACATGGTCAC**G**GG-CACCCCCGGTCCCTGCGT**A** Ggg [AF365954]
     CCAAAGCCAAGGACTTCGTCGCGCA**G**CTGACGCCCGAGGAGAAGGCGAACATGGTCAC**G**GG-CACCCCCGGTCCCTGCGT**A** Ggt [AF365955]
     CCAA**G**GCCAA**A**GACTT**T**GTCGCG**G**A**G**CTGACGC**T**CGAGGAGAAGGCGAACATGGTCAC**T**GG**G**CACCC**GT**GGTCC**T**TG**T**GT**C** MB 015
     CCAA**G**GCCAA**A**GACTTCGTCGCG**G**A**G**CTGACGC**T**CGAGGAGAAGGCGAACATGGTCACAGG-**T**ACCC**GT**GGTCC**T**TGCGT**C** MB 0219

     CCAAAGCCAAGGACTTCGTCGCGCA**G**CTGACGCCCGAGGAGAAGGCGAACATGGTCAC**G**GG-CACCCCCGGTCCCTGCGTG Ggt [AF365958]
     CCAAAGCCAAGGACTTCGTCGCGCA**G**CTGACGCCCGAGGAGAAGGCGAACATGGTCAC**G**GG-CACCCCCGGTCCCTGCGTG Ggt [AF365956]
     CCAAAGCCAAGGACTTCGTCGCGCA**G**CTGACGCCCGAGGAGAAGGCGAACATGGTCAC**G**GG-CACCCCCGGTCCCTGCGTG Ggt [AF365957]
     CCAAAGCCAAGGACTTCGTCGCGCA**G**CTGACGCCCGAGGAGAAGGCGAACATGGTCAC**G**GG-CACCCCCGGTCCCTGCGTG MB 0126
     CCAAAGCCAAGGACTTCGTCGCGCA**G**CTGACGCCCGAGGAGAAGGCGAACATGGTCAC**G**GG-CACCCCCGGTCCCTGCGT**A** MB 0218
```


Figure 6.1 (continued)

Figure 6.1 (continued)

```
321  GGCAACATCGCCCCCGTGCCGCGCCTCAACTTCACCGGCCTGTGCCTACAGGACGGGCCGGCCACCCTCCGCCAGGCCAC Gga [U35463]
     GGCAACATCGCCCCCGTGCCGCGCCTCAACTTCACCGGCCTGTGCCTACAGGACGGGCCGGCCACCCTCCGCCAGGCCAC Gga [AF365953]
     GGCAACATCGCCCCCGTGCCGCGCCTCAACTTCACCGGCCTGTGCCTACAGGACGGCCCGGCCACCCTCCGCCAGGCCAC MB 001
     GGCAACATCGCCCCCGTGCCGCGCCTCAACTTCACCGGCCTGTGCCTACAGGACGGCCCGGCCACCCTCCGCCAGGCCAC MB 004
     GGCAACATCGCCCCCGTGCCGCGCCTCAACTTCACCGGCCTGTGCCTACAGGACGGCCCGGCCACCCTCCGCCAGGCCAC MB 013
     GGCAACATCGCCCCCGTGCCGCGCCTCAACTTCACCGGCCTGTGCCTACAGGACGGCCCGGCCGCCCTCCGCCAGGCCAT MB 014
     GGCAACATCGCCCCCGTGCCGCGCCTCAACTTCACCGGCCTGTGCCTACAGGACGGCCCGGCCGCCCTCCGCCAGGCCAT MB 0113
     GGCAACATCGCCCCCGTGCCGCGCCTCAACTTCACCGGCCTGTGCCTACAGGACGGCCCGGCCACCCTCCGCCAGGCCAC MB 0114
     GGCAACATCGCCCCCGTGCCGCGCCTCAACTTCACCGGCCTGTGCCTACAGGACGGCCCGGCCACCCTCCGCCAGGCCAC MB 0215

     AGCAACATCGCCCCCGTGCCGCGCCTCAACTTCACCGGCCTGTGCCTACAGGACGGCCCGGCCACCCTCCGCCAGGCCAC Ggg [AF365954]
     GGCAACATCGCCCCCGTGCCGCGCCTCAACTTCACCGGCCTGTGCCTACGGGACGGCCCGGCCACCCTCCGCCAGGCCAC Ggt [AF365955]
     GGCAACATCGCCCCTGTGCTACGCCTCAACTTCACCGGCCTGTGCCTACAGGACGGCCCGGCCGCCATCCGCCAGGCCGT MB 015
     GGCAACATCGCCCCTGTGCCGCGCCTCAACTTCACCGGCCTGTGCCTGCAGGATGGCCCGGCCGCCCTCCGCCAGGCCGT MB 0219

     GGCAACATCGCCCCCGTGCCGCGCCTCAACTTCACCGGGCTGTGCCTACAGGACGGCCCGGCCACCCTCCGCCAGGCCAC Ggt [AF365958]
     GGCAACATCGCCCCCGTGCCGCGCCTCAACTTCACCGGGCTGTGCCTACAGGACGGCCCGGCCACCCTCCGCCAGGCCAC Ggt [AF365956]
     GGCAACATCGCCCCCGTGCCGCGCCTCAACTTCACCGGGCTGTGCCTACAGGACGGCCCGGCCACCCTCCGCCAGGCCAC Ggt [AF365957]
     GGCAACATCGCCCCCGTGCCGCGCCTCAACTTCACCGGGCTGTGCCTACAGGACGGCCCGGCCACCCCCCGCCAGGCCAC MB 0126
     GGCAACATCGCCCCCGTGCCGCGCCTCAACTTCACCGGCCTGTGCCTACAGGACGGCCCGGCCACCCTCCGCCAGGCCAC MB 0218
```


Figure 6.1 (continued)

Figure 6.1 (continued)

```
401  TTACGTCACCGTCTTCCCGGGCGGTGTCAGCGCGGCTTCGTCGTGGGACAAGGACCTCATCTACAAGCACGGCGTGCTGA Gga [U35463]
     TTACGTCACCGTCTTCCCGGGCGGTGTCAGCGCGGCTTCGTCGTGGGACAAGGACCTCATCTACAAGCACGGCGTGCTGA Gga [AF365953]
     TTACGTCACCGTCTTCCCGGGCGGTGTCAGCGCGGCTCCGTCGTGGGACAAGGACCTCATCTACAAGCACGGCGTGCTGA MB 001
     TTACGTCACCGTCTTCCCGGGCGGTGTCAGCGCGGCTTCGTCGTGGGACAAGGACCTCATCTACAAGCACGGCGTGCTGA MB 004
     TTACGTCACCGTCTTCCCGGGCGGTGTCAGCGCGGCTTCGTCGTGGGACAAGGACCTCATCTACAAGCACGGCGTGCTGA MB 013
     TTACGTCACCGTCTTCCCGGGCGGTGTCAGCGCGGCTTCGTCGTGGGACAAGCACCTCATCCACAAGCACGGCGTGCTGA MB 014
     TTACGTCACCGTCTTCCCGGGCGGTGTCAGCGCGGCTTCGTCGTGGGACAAGCACCTCATCCACAAGCACGGCGTGCTGA MB 0113
     TTACGTCGCCGTCTTCCCGGGCGGTGTCAGCGCGGCTTCGTCGTGGGACAAGGACCTCATCTACAAGCACGGCGTGCTGA MB 0114
     TTACGTCACCGTCTTCCCGGGCGGTGTCAGCGCGGCTTCGTCGTGGGACAAGGACCTCATCTACAAGCACGGCGTGCTGA MB 0215

     TTACGTCACCGTCTTCCCGGGCGGTGTCAGCGCGGCTTCGTCGTGGGACAAGGACCTCATCTACAAGCACGGCGTGCTGA Ggg [AF365954]
     TTACGTCACCGTCTTCCCCGGCGGTGTCAGCGCGGCTTCGTCGTGGGACAAGGACTTCATTTACAAGCGCGGCGTGCTGA Ggt [AF365955]
     TTACGCCAGCGTTTTCCCGGGCGGTGTCAGCGCTGCTTCGTCGTGGGATAGGGACCTCAACTACCAGCACGGCGCGCTGA MB 015
     TTACGCCAGCGTTTTCCCGGGCTGTGTCAGCGCTGCTTCGTCGTGGGATAAGGACCTCATCTACCAGCACGGCGCGCTGA MB 0219

     TTACGTCACCGTCTTCCCGGGCGGTGTCAGCGCGGCTTCGTCGTGGGACAAGGACCTCATCTACAAGCACGGCGTGCTGA Ggt [AF365958]
     TTACGTCACCGTCTTCCCGGGCGGTGTCAGCGCGGCTTCGTCGTGGGACAAGGACCTCATCTACAAGCACGGCGTGCTGA Ggt [AF365956]
     TTACGTCACCGTCTTCCCGGGCGGTGTCAGCGCGGCTTCGTCGTGGGACAAGGACCTCATCTACAAGCACGGCGTGCTGA Ggt [AF365957]
     TTACGTCACCGTCTTCCCGGGCGGTGTCAGCGCGGCTTCGTCGTGGGACAAGGACCTCATCTACAAGCACGGCGTGCTGA MB 0126
     TTACGTCACCGTCTTCCCGGGCGGTGTCAGCGCGGCTTCGTCGTGGGACAAGGACCTCATCTACAAGCACGGCGTGCTGA MB 0218
```


Figure 6.1 (continued)

Figure 6.1 (continued)

```
              Ave 450 F>                 Ggt-specific primer>
481  TGGCCGAGGAGTTCCGTGACAAGGGGTCCCACGTCATCCTCGGCCCTGTAATTGGTCCCCGTGG-AAGGTCCCCGTACGCC Gga [U35463]
     TGGCCGAGGAGTTCCGTGACAAGGGGTCCCACGTCATCCTCGGCCCTGTAATTGGTCCCCGTGG-AAGGTCCCCGTACGCC Gga [AF365953]
     TGGCCCGAGAGTTCCGTGACAAGGGCTCCCACGTCATCCTCGGCCCTGTAATTGGTCCCCTTGG-AAGGTCCCCGTACGCC MB 001
     TGGCCCGAGAGTTCCGTGACAAGGGCTCCCACGTCATCCTCGGCCCTGTAATTGGTCCCCTTGG-AAGGTCCCCGTACGCC MB 004
     TGGCCCGAGAGTTCCGTGACAAGGGCTCCCACGTCATCCTCGGCCCTGTAATTGGTCCCCTTGG-AAGGTCCCCGTACGCC MB 013
     TGGCCCAAGAGTTCCGTGACAAGGGCTCTCACGTCATCCTCGGCCCTGTAATTGGCCCCCTTGG-AAGGTCCCCGTACGGC MB 014
     TGGCCCAAGAGTTCCGTGACAAGGGCTCTCACGTCATCCTCGGCCCTGTAATTGGCCCCCTTGG-AAGGTCCCCGTACGGC MB 0113
     TGGCCCGAGAGTTCCGTGACAAGGGCTCCCACGTCATCCTCGGCCCTGTAATTGGTCCCCTTGG-AAGGTCCCCGTACGCC MB 0114
     TGGCCCGAGAGTTCCGTGACAAGGGCTCTCACGTCATCCTCGGCCCTGTAATTGGCGCCCTTGG-AAGGTCCCCGTACGCC MB 0215

     TGGCCCGAGAGTTCCGTGACAAGGGCTCTCACATCATCCTCGGCCCTGTAATTGGTCCCCTTGG-AAGGTCCCCGTACGCC Ggg [AF365954]
     TGGCCCGAGAGTTCCGTGACAAGGGCTCTCACATCATCCTCGGCCCTGTAATTGGTCCCCTTGG-AAGGTCCCCGTACGCC Ggt [AF365955]
     TGGCCCAAGAATTCCGTGACAAGGGCTCCCACGTTATCCTGGGGCCTGTCATTGGCGCCCTAGG-AAGGTCCCCGTACGCC MB 015
     TGGCCCAAGAGTTCCGTGACAAGGGCTCTCACGTCATCCTGGGCCCTGTCATTGGTGCC-TTGGGAAGGTCCC-GTACGCC MB 0219


     TGGCCCGAGAGTTCCGTGACAAGGGCTCTCACATCATCCTCGGCCCCGTAATTGGCCCCCTTGG-AAGGTCCCCGTACGCC Ggt [AF365958]
     TGGCCCGAGAGTTCCGTGACAAGGGCTCTCACATCATCCTCGGCCCCGTAATTGGCCCCCTTGG-AAGGTCCCCGTACGCC Ggt [AF365956]
     TGGCCCGAGAGTTCCGTGACAAGGGCTCTCACATCATCCTCGGCCCCGTAATTGGCCCCCTTGG-AAGGTCCCCGTACGCC Ggt [AF365957]
     TCGGCCGAGAGTTCCGTGACAAGGGCTCTCACATCATCCTCGGCCCCGTAATTGGCCCCCTTGG-AAGGTCCCCGTACGCC MB 0126
      TGGCCCGAGAGTTCCGTGACAAGGGCTCTCACATCATCCTCGGCCCTGTAATTGGTCCCCTTGG-AAGGTCCCCGTACGCC MB 0218
                       <Ave 467 R
```


Figure 6.1 (continued)

Figure 6.1 (continued)

```
561  GGGCGCAACTGGGAGGGATTCTCCCC-GACTTCGTACCTCGCGGGCGTCATGGCAGAGCAGACGGTCAAGGGGATGCAGgt Gga [U35463]
     GGGCGCAACTGGGAGGGATTCTCCCC-GACTTCGTACCTCGCGGGCGTCATGGCAGAGCAGACGGTCAAGGGGATGCAGgt Gga [AF365953]
     GGGCGCAACTGGGAGGGATTCTCCCCCGACT-CGTACCTCGCGGGCGTCATGGCAGAGCAGACGGTCAAGGGGATGCAGgt MB 001
     GGGCGCAACTGGGAGGGATTCTCCCCCGACT-CGTACCTCGCGGGCGTCATGGCAGAGCAGACGGTCAAGGGGATGCAGgt MB 004
     GGGCGCAACTGGGAGGGATTCTCCCCCGACT-CGTACCTCGCGGGCGTCATGGCAGAGCAGACGGTCAAGGGGATGCAGgt MB 013
     GGGCGCAACTGGGAGGGATTCTCTCCCGACT-CGTACCTCGCGGGCGTCATGGTAGAGCAGACGGTCAAGGGGATGCAGgt MB 014
     GGGCGCAACTGGGAGGGATTCTCCCCCGACT-CGTACCTCGCGGGCGTCATGGTAGAGCAGACGGTCAAGGGGATGCAGgt MB 0113
     GGGCGCAACTGGGAGGGATTCTCCCCCGACT-CGTACCTCGCGGGCGTCATGGCAGAGCAGACGGTCAAGGGGATGCAGgt MB 0114
     GGGCGCAACTGGGAGGGATTCTCCCCCGACT-CGTACCTCGCGGGCGTCATGGTAGAGCAGACGGTCAAGGGGATGCAGgt MB 0215

     GGGCGCAACTGGGAGGGATTCTCCCCCGACT-CGTACCTCGCGGGCGTCCTGGCAGAGCAGACGGTCAAGGGGATGCAGgt Ggg [AF365954]
     GGGCGCAACTGGGAGGGATTCTCCCCCGACT-TGTACCTCGCGGGCGTCCTGGCAGAGCAGACGGTCAAGGGGATGCAGgt Ggt [AF365955]
     GGGCGCAACTGGGAGGGATTCTCCCCCGACT-CGTACCTGTCGGGCGTCCTGGTAGAGGAGACGGTCAAGGGCATGCAGgt MB 015
     GGGCGTAACTGGGAGGGATTCTCCCCCGACT-CGTACCTGTCGGGCGTCCTGGTAGAGGAGACAGTCAAGGGGATGCGGgt MB 0219

     GGGCGCAACTGGGAGGGATTCTCCCCCGACT-CGTACCTCGCGGGCGTCCTGGCAGAGCAGACGGTCAAGGGGATGCAGgt Ggt [AF365958]
     GGGCGCAACTGGGAGGGATTCTCCCCCGACT-CGTACCTCGCGGGCGTCCTGGCAGAGCAGACGGTCAAGGGGATGCAGgt Ggt [AF365956]
     GGGCGCAACTGGGAGGGATTCTCCCCCGACT-CGTACCTCGCGGGCGTCCTGGCAGAGCAGACGGTCAAGGGGATGCAGgt Ggt [AF365957]
     GGGCGCAACTGGGAGGGATTCTCCCCCGACT-CGTACCTCGCGGGCGTCCTGGCAGAGCAGACGGTCAAGGGGATGCAGgt MB 0126
     GGGCGCAACTGGGAGGGATTCTCCCCCGACT-CGTACCTCGCGGGCGTCCTGGCAGAGCAGACGGTCAAGGGGATGCAGgt MB 0218
```


Figure 6.1 (continued)

Figure 6.1 (continued)

```
641  aagga**c**ccc-tctcca**c**caacatgtcggcgccgagcctatt**a**ccccgtaatactgacactt--gacagTCGGTCGGCGTGCA Gga [U35463]
     aagga**c**ccc-tctcca**c**caacatgtcggcgccgagcctatt**a**ccccgtaatactgacactt--gacagTCGGTCGGCGTGCA Gga [AF365953]
     aaggagccc-tctccagcaacatgtcggcgccgagcctatt-ccccgtaatactgacacttt-gacagTCGGTCGGCGTGCA MB 001
     aaggagccc-tctccagcaacatgtcggcgccgagcctatt-ccccgtaatactgacacttt-gacagTCGGTCGGCGTGCA MB 004
     aaggagccc-tctccagcaacatgtcggcgccgagcctatt-ccccgtaatactgacacttt-gacagTCGGTCGGCGTGCA MB 013
     aaggagccc**c**tctccagcaacatgtcggcgccgagcctatt-ccc**t**gtaatactgacactttt**g**acagTCGGTCGGCGTGCA MB 014
     aaggagccc**c**tctccagcaacatgtcggcgccgagcctatt-ccc**t**gtaatactgacactttt**g**acagTCGGTCGGCGTGCA MB 0113
     aaggagccc-tctccagcaacatgtcg**a**cgccgagcctatt-ccccgtaatactgacacttt-gacagTCGGTCGGCGTGCA MB 0114
     aaggagccc-tctccagcaacatgtcggcgccgagcctatt-ccc**t**gtaatactgacacttt-gacagTCGGTCGGCGTGCA MB 0215

     aagg**g**gccc-tctccagcaacatgt**t**ggcgccgagcctatt-ccc**t**gtaatactgacacttt-gacagTCGGTCGGCGTGCA Ggg [AF365954]
     aagg**g**gccc-tctccagcaacatgt**t**ggcgccgagcctatt-ccc**t**gtaatactgacacttt-gacagTCGGTCGGCGTGCA Ggt [AF365955]
     aaggagccc-tctccagcaaca**ccc**cggc**a**ccg**t**gcctatt**t**ccc-gt**g**a**c**actgac**g**ctt**c**-g**g**cagTCGGTCGG**T**GTGCA MB 015
     aagaagccc-tctccagcaacatg**c**cg**a**c**at**cg**ca**cctatt**t**-cc**a**gt**g**a**c**actgac**c**c**c**t**c**-gacagTCGGTCGG**T**GTGCA MB 0219

     aaggagccc-tctccagcaacatgtcggcgccgagcctatt-ccc**t**gtaatactgacacttt-gacagTCGGTCGGCGTGCA Ggt [AF365958]
     aaggagccc-tctccagcaacatgtcggcgccgagcctatt-ccc**t**gtaatactgacacttt-gacagTCGGTCGGC**A**TGCA Ggt [AF365956]
     aaggagccc-tctccagcaacatgtcggcgccgagcctatt-ccc**t**gtaatactgacacttt-gacagTCGGTCGGCGTGCA Ggt [AF365957]
     aaggagccc-tctccagcaacatgtcggcgccgagcctatt-ccc**t**gtaatactgacacttt-gac**g**gTCGGTCGGCGTGCA MB 0126
     aagg**g**gccc-tctccagcaacatgt**t**ggcgccgagcctatt-ccc**t**gtaatactgacacttt-gacagTCGGTCGGCGTGC**G** MB 0218
```


Figure 6.1 (continued)

Figure 6.1 (continued)

```
                                                                Gga-specific primer>
721  AGCCTGCACCAAGCACTTCATCGGCAATGAGCAGGAGGAGCAGCGCAACCCCACGGCGGTGGATGGCAAGACGGTTGAGG Gga [U35463]
     AGCCTGCACCAAGCACTTCATCGGCAATGAGCAGGAGGAGCAGCGCAACCCCACGGCGGTGGATGGCAAGACGGTTGAGG Gga [AF365953]
     AGCCTGCACCAAGCACTTCATCGGCAATGAGCAGGAGGAGCAGCGCAACCCCACGGCGGTGGATGGCAAGACGGTTGAGG MB 001
     AGCCTGCACCAAGCACTTCATCGGCAATGAGCAGGAGGAGCAGCGCAACCCCACGGCGGTGGATGGCAAGACGGTTGAGG MB 004
     AGCCTGCACCAAGCACTTCATCGGCAATGAGCAGGAGGAGCAGCGCAACCCCACGGCGGTGGATGGCAAGACGGTTGAGG MB 013
     AGCCTGCACCAAGCACTTCATCGGCAATGAGCAGGAGGAGCAGCGCAACCCCACGGCGGTGG**G**TGGCAAGACGGTTGAGG MB 014
     AGCCTGCACCAAGCACTTCATCGGCAATGAGCAGGAGGAGCAGCGCAACCCCACGGCGGTGG**G**TGGCAAGACGGTTGAGG MB 0113
     AGCCTGCACCAAGCACTTCATCGGCAATGAGCAGGAGGAGCAGCGCAACCCCACGGCGGTGGATGGCAAGACGGTTGAGG MB 0114
     AGCCTGCACCAAGCACTTCATCGGCAATGAGCAGGAGGAGCAGCGCAACCCCACGGCGGTGGATGGCAAGACGGTTGAGG MB 0215

     A**A**CCTGCACCAAGCACTTCATCGGCAATGAGCAGGAGGAGCAGCGCAACCCCACG**A**CGGTGGATGGCAAG**GG**GGTTGAGG Ggg [AF365954]
     A**A**CCTGCACCAAGCACTTCATCGGCAATGAGCAGGAGGAGCAGCGCAACCCCACG**A**CGGTGGATGGCAAG**GG**GGTTGAGG Ggt [AF365955]
     AGCCTGCACCAAGCACTTCATCGGCAATGAGCAGGAGGAGCA**A**CGCAACCCCACG**A**CGGTGGA**C**GGC**G**AGA**G**GGT**C**GAGG MB 015
     AGCCTG**T**ACCAAGCACTTCATCGG**T**AATGAGCAGGAGGAGCAGCGCAACCCCACG**A**CGGTGGATGGCAAGA**G**GGT**C**GAGG MB 0219

     A**A**CCTGCACCAAGCACT**A**CATCGGCAATGAGCAGGAGGAGCAGCGCAACCCCACG**A**CGGTGGATGGCAAG**GG**GGTTGAGG Ggt [AF365958]
     A**A**CCTGCACCAAGCACT**A**CATCGGCAATGAGCAGGAGGAGCAGCGCAACCCCACG**A**CGGTGGATGGCAAG**GG**GGTTGAGG Ggt [AF365956]
     A**A**CCTGCACCAAGCACT**A**CATCGGCAATGAGC**G**GGAGGAGCAGCGCAACCCCACG**A**CGGTGGATGGCA**G**G**GG**GGTTGAGG Ggt [AF365957]
     A**A**CCTGCACCAAGCACT**A**CATCGGCAATGAGCAGGAGGAGCAGCGCAACCCCACG**A**CGGTGGATGGCAAG**GG**GGTTGAGG MB 0126
     A**A**CCTGCACCAAGCACTTCATCGGCAATGAGCAGGAGGAGCAGCGCAACCCCACG**A**CGGTGGATGGCAAG**GG**GGTTGAGG MB 0218
```


Figure 6.1 (continued)

Figure 6.1 (continued)

```
801  CCATCTCGTCCAACATTGATGACCGCACAATGCACGAGGCCTACCTGTGGCCCTTTTACAACGCCGTCAGGGCCGGCACC Gga [U35463]
     CCATCTCGTCCAACATTGATGACCGCACAATGCACGAGGCCTACCTGTGGCCCTTTTACAACGCCGTCAGGGCCGGCACC Gga [AF365953]
     CCATCTCGTCCAACATTGATGACCGCACAATGCACGAGGCCTACCTGTGGCCCTTTTACAACGCCGTCAGGGCCGGCACC MB 001
     CCATCTCGTCCAACATTGATGACCGCACAATGCACGAGGCCTACCTGTGGCCCTTTTACAACGCCGTCAGGGCCGGCACC MB 004
     CCATCTCGTCCAACATTGATGACCGCACAATGCACGAGGCCTACCTGTGGCCCTTTTACAACGCCGTCAGGGCCGGCACC MB 013
     CCATCTCGTCCAACATTGATGACCGCACAATGCACGAGACCTACCTGTGGCCCTTTTACAATGCCGTCAGGGCCGGCACC MB 014
     CCATCTCGTCCAACATTGATGACCGCACAATGCACGAGACCTACCTGTGGCCCTTTTACAACGCCGTCAGGGCCGGCACC MB 0113
     CCATCTCGTCCAACATTGATGACCGCACAATGCACGAGGCCTACCTGTGGCCCTTTTACAACGCCGTCAGGGCCGGCACC MB 0114
     CCATCTCGTCCAACATTGATGACCGCACAATGCACGAGACCTACCTGTGGCCCTTTTACAACGCCGTCAGG-CCGGCACC MB 0215

     CCATCTCGTCCAACATTGACGACCGCACAATGCACGAGACCTACCTGTGGCCCTTTTACAACGCCGTCAGGGCCGGCACC Ggg [AF365954]
     CCATCTCGTCCAACATTGACGACCGCACAATGCACGAGACCTACCTGTGGCCCTTTTACAACGCCGTCAGGGCCGGCACC Ggt [AF365955]
     CCATTTCGTCAAACATCGACGACCGCACGATGCACGAGACCTACCTGTGGCCCTTCTACAACGCCGTTAGGGCCGGCACC MB 015
     CCATTTCGTCCAACATCGATGACCGCACAATGCACGAGACTTACCTGTGGCCCTTCTACAACGCCGTCAGGGCTGGCACC MB 0219

     CCATCTCGTCCAACATTGACGACCGCACAATGCACGAGACCTACCTGTGGCCCTTTTACAACGCCGTCAGGGCCGGCACC Ggt [AF365958]
     CCATCTCGTCCAACATTGACGACCGCACAATGCACGAGACCTACCTGTGGCCCTTTTACAACGCCGTCAGGGCCGGCACC Ggt [AF365956]
     CCATCTCGTCCAACATTGACGACCGCACAATGCACGAGACCTACCTGTGGCCCTTTTACAACGCCGTCAGGGCCGGCACC Ggt [AF365957]
     CCATCTCGTCCAACATTGACGACCGCACAATGCACGAGACCTACCTGTGGCCCTTTTACAACGCCGTCAGGGCCGGCACC MB 0126
     CCATCTCGTCCAACATTGACGACCGCACAATGCACGAGACCTACCTGTGGCCCTTTTACAACGCCGTCAGGGCCGGCACC MB 0218
```


Figure 6.1 (continued)

Figure 6.1 (continued)

```
        Ave 884 F>
881  ACCTCCATAATGTGCTCTTACCAGAGGATCAACGGCAGCTACGGTTGTCAGAACAGCAAGACCCTCAACGGGCTTCT-CA Gga [U35463]
     GCCTCCATAATGTGCTCTTACCAGAGGATCAACGGCAGCTACGGCTGCCAGAACAGCAAGACCCTCAACGGGCTTCT-CA Gga [AF365953]
     ACCTCCATAATGTGCTCTTACCAGAGGATCAACGGCAGCTACGGCTGCCAGAACAGCAAGACCCTCAACGGGCTTCT-CA MB 001
     ACCTCCATAATGTGCTCTTACCAGAGGATCAACAGCAGCTACGGCTGCCAGAACAGCAAGACCCTCAACGGGCTTCT-CA MB 004
     ACCTCCATAATGTGCTCTTACCAGAGGATCAACGGCAGCTACGGCTGCCAGAACAGCAAGACCCTCAACGGGCTTCT-CA MB 013
     GCCTCCATAATGTGCTCTTACCAGAGGATCAACGGCAGCTACGGCTGCCAGAACAGCAAGACCCTCAACGGGCTTCT-CA MB 014
     GCCTCCATAATGTGCTCTTACCAGAGGATCAACGGCAGCTACGGCTGCCAGAACAGCAAGACCCTCAACGGGCTTCT-CA MB 0113
     ACCTCCATAATGTGCTCTTACCAGAGGATCAACGGCAGCTACGGCTGCCAGAACAGCAAGACCCTCAACGGGCTTCT-CA MB 0114
     GCCTCCATAATGTGCTCTTACCAGAGGATCAACGGCAGCTACGGCTGCCAGAACAGCAAGACCCTCAACGGACTTCT-CA MB 0215

     ACGTCCATAATGTGCTCTTACCAGAGGATCAACGGCAGCTACGGCTGCCAGAACAGCAAGACCCTCAACGGGCTTCT-CA Ggg [AF365954]
     ACCTCCATAATGTGCTCTTACCAGAGGATCAACGGCAGCTACGGCTGCCAGAACAGCAAGACCTTCAACGGGCTTCTTCA Ggt [AF365955]
     ACCTCCATAATGTGCTCTTACCAGAGGATCAATGGCAGCTACGGCTGCCAGAACAGCAAGACCCTTAACGGGCTTCT-CA MB 015
     ACCTCCATAATGTGCTCTTACCAGAGGATCAACGGCAGCTACGGCTGCCAGAACAGCAAAACCCTCAACGGGCTTCT-CA MB 0219

     ACGTCCATAATGTGCTCTTACCAGAGGATCAACGGCAGCTACGGCTGCCAGAACAGCAAGACCCTCAACGGGCTTCT-CA Ggt [AF365958]
     ACGTCCATAATGTGCTCTTACCAGAGGATCAACGGCAGCTACGGCTGCCAGAACAGCAAGACCCTCAACGGGCTTTT-CA Ggt [AF365956]
     ACGTCCATAATGTGCTCTTACCAGAGGATCAACGGCAGCTACGGCTGCCAGAACAGCAAGACCCTCAACGGGCTTCT-CA Ggt [AF365957]
     ACGTCCATAATGTGCTCTTACCAGAGGATCAACGGCAGCTACGGCTGCCAGAACAGCAAGACCCTCAACGGGCTTCT-CA MB 0126
     ACCTCCATAATGTGCTCTTACCAGAGGATCAACGGCAGCTACGGCTGCCAGAACAGCAAGACCCTCAACGGGCTTCT-CA MB 0218
                      <Ave 908 R
```


Figure 6.1 (continued)

Figure 6.1 (continued)

```
961  AGACCGAGCTCGGCTTCCAGGGCTTCGTCGTGTCGGACTGGtgcgtggctacctccctctaccagatgaaacatgcagtg Gga [U35463]
     AGAC**T**GAGCTCGGCTTCCAGGGCTTCGTCGTGTCGGACTGGtgcgtggctacctccctctaccagatgaaacatgcagtg Gga [AF365953]
     AGACCGAGCTCGGCTTCCAGGGCTTCGTCGTGTCGGACTGGtgcgtggctacctccctctaccagatgaaacatgcagtg MB 001
     AGACCGAGCTCGGCTTCCAGGGCTTCGTCGTGTCGGACTGGtgcgtggctacctccctctaccagatgaaacatgtagtg MB 004
     AGACCGAGCTCGGCTTCCAGGGCTTCGTCGTGTCGGACTGGtgcgtggctacctccctctaccagatgaaacatgcagtg MB 013
     AGACCGAGCTCGGCTTCCAGGGCTTCGTCGTGTCGGACTGGtgcgtggctacctccctctaccagatgaaacatgcagtg MB 014
     AGACCGAGCTCGGCTTCCAGGGCTTCGTCGTGTCGGACTGGtgcgtggctacctccctctaccagatgaaaccatcagtg MB 0113
     AGACCGAGCTCGGCTTCCAGGGCTTCGTCGTGTCGGACTGGtgcgtggctacctccctctaccagatgaaacatgcagtg MB 0114
     AGACCGAGCTCGGCTTCCAGGGCTTCGTCGTGTCGGACTGGtgcgtggctacctccctctaccagatgaaacatgcagtg MB 0215

     AGACCGAGCTCGGCTTCCAGGGCTTCGTCGTGTCGGACTGGtgcgtggctacctccctctaccagatgaaacatgcagtg Ggg [AF365954]
     AGACCGAGCTCGGCTTCCAGGGCTTCGTCGTGTCGGACTGGtgcgtggctacctcc**t**tctaccagatgaaacatgcagtg Ggt [AF365955]
     A**A**ACCGAGCTCGGCTTCCAGGGCT**AT**GTC**A**TGTCGGACTGGt**a**cgt**ac**ctacc**g**c**ttgga**a**at**a**t**gc**ga**t--g**tgc--tg MB 015
     AGACCGAGCT**T**GGCTTCCAGGGCT**AT**GTC**A**TGTC**T**GACTGGt**a**cgtg**tt**tacc**g**c**ttggg**a**at**a**t**gc**ga**t**a--tgc--tg MB 0219

     AGACCGAGCTCGGCTTCCAGGGCTTCGTCGTGTCGGACTGGtgcgtggctacctccctctaccagatgaaacatgcagtg Ggt [AF365958]
     AGACCGAGCTCGGCTTCCAGGGCTTCGTCGTGTCGGACTGGtgcgtggctacctccctctaccagatgaaacatgcagtg Ggt [AF365956]
     AGACCGAGCTCGGCTTCCAGGGCTTCGTCGTGTCGGACTGGtgcgtggct**t**cctccctctaccagatgaaacatgcagtg Ggt [AF365957]
     AGACCGAGCTCGGCTTCCAGGGCTTCGTCGTGTCGGACTGGtgcgtggctacctccctctaccagatgaaacatgcagtg MB 0126
     AGACCGAGCTCGG**G**TTCCAGGGCTTCGTCGTGTCGGACTGGtgcgtggctacctccctctaccagatgaaacatgcagtg MB 0218
```


Figure 6.1 (continued)

Figure 6.1 (continued)

```
1041 ccttgttttt-gctaatggccatgacagGGCCGCTACCCATTCCGGAGTTGCCTCCATTGAGGCTGGTCTGGACATGAAC Gga [U35463]
     ccttgttttt-gctaatgg**t**cat**a**acagGGCCGCTACCCATTCCGGAGTTGCCTCCATTGAGGCTGGTCTGGACATGAAC Gga [AF365953]
     ccttgttttt-gctaatggccatgacagGGCCGCTACCCATTCCGGAGTTGCCTCCATTGAGGCTGGTCTGGACATGAAC MB 001
     ccttgttttt-gctaatggccatgacagGGCCGCTACCCATTCCGGAGTTGCCTCCATTGAGGCTGGTCTGGACATGAAC MB 004
     ccttgttttt-gctaatggccatgacagGGCCGCTACCCATTCCGGAGTTGCCTCCATTGAGGCTGGTCTGGACATGAAC MB 013
     ccttgttttt-gctaatggc**t**at**a**acagGGCCGCTACCCATTCCGGAGTTGCCT**T**CATTGAGGC**C**GGTCTGGACATGAAC MB 014
     ccttgttttt-gctaatggc**t**at**a**acagGGCCGCTACCCATTCCGGAGTTGCCTCCATTGAGGC**C**GGTCTGGACATGAAC MB 0113
     ccttgttttt-gctaatggccatgacagGGCCGCTACCCATTCCGGAGTTGCCTCCATTGAGGCTGGTCTGGACATGAAC MB 0114
     ccttgttttt-gctaatggccat**a**acagGGCCGCTACCCATTCCGGAGT**C**GCCTCCATTGAGGC**C**GGTCTGGACATGAAC MB 0215

     ccttgttttt-gctaatggccat**a**acagGGCCGCTACCCATTCCGGAGT**C**GCCTCCATTGAGGCTGGTCTGGACATGAAC Ggg [AF365954]
     ccttgttttt**t**gctaatggccat**a**acagGGCCGCTACCCATTCCGGAGT**C**GCCTCCATTGAGGCTGGTCTGGACATGAAC Ggt [AF365955]
     **-------**ttt**ta**ctaat**a**gcca**atc**cagG**ATG**GC**C**ACCCAT**G**CCGGAGT**CC**CC**G**CCAT**C**GAGGCTGGTCTGGACATGAAC MB 015
     **--------**tt**ac**tt**g**at**a**gcca**aat**cagG**ATG**GC**C**ACCCA**CG**CCGGAGT**CC**CC**G**CCAT**C**GAGGCTGGTCT**-**GACATGAAC MB 0219

     ccttgttttt-gctaatggccat**a**acagGGCCGCTACCCATTCCGGAGT**C**GCCTCCATTGAGGCTGGTCTGGACATGAAC Ggt [AF365958]
     ccttgttttt-gctaatggccat**a**acagGGCCGCTACCCATTCCGGAGT**C**GCCTCCATTGAGGCTGGTCTGGACATGAAC Ggt [AF365956]
     ccttgttttt-gctaatggccat**a**acagGGCCGCTACCCATTCCGGAGT**C**GCCTCCATTGAGGCTGGTCTGGACATGAAC Ggt [AF365957]
     ccttgttttt-gctaatggccat**a**acagGGCCGCTACCCATTCCGGAGT**C**GCCTCCATTGAGGCTGGTCTGGACATGAAC MB 0126
     ccttgttttt-gctaatggccataacagGGCCGCTACCCATTCCGGAGT**C**GCCTCCATTGAGGCTGGTCTGGACATGAAC MB 0218
```


Figure 6.1 (continued)

Figure 6.1 (continued)

```
1121 ATGCCCGGACCGCTTAATTTTTTTGCCCCAACCTTCGAGTCTTACTTTGGCAAGAACATCACCACTGCGGTCAACAACGG Gga [U35463]
     ATGCCCGGATCGCTCGATTTTTTTGCCCCAACCTTCGAGTCTTACTTTGGCAAGAACATCACCACTGCGGTCAACAACGG Gga [AF365953]
     ATGCCCGGACCGCTTAATTTTTTTGCCCCAACCTTCGAGTCTTACTTTGGCAAGAACATCACCACTGCGGTCAACAACGG MB 001
     ATGCCCGGACCGCTTAATTTTTTTGCCCCAACCTTCGAGTCTTACTTTGGCAAGAACATCACCACTGCGGTCAACAACGG MB 004
     ATGCCCGGACCGCTTAATTTTTTTGCCCCAACCTTCGAGTCTTACTTTGGCAAGAACATCACCACTGCGGTCAACAACGG MB 013
     ATGCCCGGATCGCTCGATTTTTTTGCCCC------CGAGTCTTACTTTGGCAAGAACATCACCACTGCGGTCAACAACGG MB 014
     ATGCCCGGATCGCTCGATTTTTTTGCCCC------CGAGTCTTACTTTGGCAAGAACATCACCACTGCGGTCAACAACGG MB 0113
     ATGCCCGGACCGCTTAATTTTTTTGCCCCAACCTTCGAGTCTTACTTTGGCAAGAACATCACCACTGCGGTCAACAACGG MB 0114
     ATGCCCGGATCGCTCGATTTTTTTGCCCCAACCTTCGAGTCTTACTTTGGCAAGAACATCACCACTGCGGTCAACAACGG MB 0215

     ATGCCCGGACCGCTCAATTTTTTTGCCCCAACCCTCGAGTCTTACTTTGGCAAGAACATCACCACTGCGGTCAACAACGG Ggg [AF365954]
     ATGCCCGGACCGCTCAATTTTTTTGCCCCAACCCTCGGGTCTTACTTTGGCAAGAACATCACCACTGCGGTCAACAACGG Ggt [AF365955]
     ATGCCCGGTCCGATCGAATTTCTCGGCACGACTGTCAAGTCGTATTTTGGTGGGAACGTCACCATTGCGGTCAACAACGG MB 015
     ATGCCCGGTCCGATCGAATTCCTCGGCACAACCGTCAAGTCTTACTTTGGCGGGAACATCACCATTGCGGTTAACAACGG MB 0219

     ATGCCCGGACCGCTCAATTTTTTTGCCCCAACCCTCGAGTCTTACTTTGGCAAGAACATCACCACTGCGGTCAATAACGG Ggt [AF365958]
     ATGCCCGGACCGCTCAATTTTTTTGCCCCAACCCTCGAGTCTTACTTTGGCAAGAACATCACCACTGCGGTCAATAACGG Ggt [AF365956]
     ATGCCCGGACCGCTCAATTTTTTTGCCCCAACCCTCGAGTCTTACTTTGGCAAGAACATCACCACTGCGGTCAATAACGG Ggt [AF365957]
     ATGCCCGGACCGCTCAATTTTTTTGCCCCAACCCTCGAGTCTTACTTTGGCAAGAACATCACCACTGCGGTCAATAACGG MB 0126
     ATGCCCGGACCGCTCAATTTTTTTGCCCCAACCCTCGGGTCTTACTTTGGCAAGAACATCACCACTGCGGTCAACAACGG MB 0218
```


Figure 6.1 (continued)

Figure 6.1 (continued)

```
1201 CACACTCTCCTCCCGGAGGGTCGACGAGATGATTGAGCGCATCATGACTCCCTACTTCGCCCTGGGTCAGGACAAGAACT Gga [U35463]
     CACACTCTCCTCCCGGAGGGTCGACGAGATGATTGAGCGCATCATGACTCCCTACTTCGCCCTGGGTCAGGACAAGAACT Gga [AF365953]
     CACACTCTCCTCCCGGAGGGTCGACGAGATGATTGAGCGCATCATGACTCCCTACTTCGCCCTGGGTCAGGACAAGAACT MB 001
     CACACTCTCCTCCCGGAGGGTCGACGAGATGATTGAGCGCATCATGACTCCCTACTTCGCCCTGGGTCAGGACAAGAACT MB 004
     CACACTCTCCTCCCGGAGGGTCGACGAGATGATTGAGCGCATCATGACTCCCTACTTCGCCCTGGGTCAGGACAAGAACT MB 013
     CACACTCTCCTCCCGGAGGGTCGACGAGATGATTGAGCGCATCATGACTCCCTACTTCGCCCTGGGTCAGGACAAGAACT MB 014
     CACACTCTCCTCCCGGAGGGTCGACGAGATGATTGAGCGCATCATGACTCCCTACTTCGCCCTGGGTCAGGACAAGAACT MB 0113
     CACACTCTCCTCCCGGAGGGTCGACGAGATGATTGAGCGCATCATGACTCCCTACTTCGCCCTGGGTCAGGACAAGAACT MB 0114
     CACACTCTCCTCCCGGAGGGTCGATGAGATGATTGAGCGCATCATGACTCCCTACTTCGCCCTGGGTCAGGACAAGAACT MB 0215

     CACACTCTCCTCCCGGAGGGTCGACGACATGATTGAGCGCATCATGACTCCCTACTTCGCCCTGGGTCAGGACAAGGACT Ggg [AF365954]
     CACACTCTCCTCCCGGAGGATCGACGACATGATTGAGCGCATCATGACTCCCTACTTCGCCCTGGGTCAGGACAAGGACT Ggt [AF365955]
     TTCTCTCTCCATCCAGAGGGTCGACGACATGATTGAGCGTATCATGGCGCCCTACTTCGCCCTAGGTCAGGACAAGAACT MB 015
     CTCTCTCTCCATCCAGAGGGTCGACGACATGATTGAGCGTATCATGGCGCCCTACTTCGCCCTGGGCCAGGACAAGAACT MB 0219

     CACACTCTCCTCCCGGAGGGTCGACGACATGATTGAGCGCATCATGACTCCCTACTTCGCCCTGGGTCAGGACAAGGACT Ggt [AF365958]
     CACACTCTCCTCCCGGAGGGTCGACGACATGATTGAGCGCATCATGACTCCCTACTTCGCCCTGGGTCAGGACAAGGACT Ggt [AF365956]
     CACACTCTCCCCCCCGGAGGGCGACGACATGATTGAGCGCATCATGACTCCCTACTTCGCCCTGGGTCAGGACAAGGACT Ggt [AF365957]
     CACACTCTCCTCCCGGAGGGTCGACGACATGATTGAGCGCATCATGACTCCCTACTTCGCCCTGGGTCAGGACAAGGACT MB 0126
     CACACTCTCCTCCCGGAGGGTCGACGACATGATTGAGCGCATCATGACTCCCTACTTCGCCCTGGGTCAGGACAAGGACT MB 0218
```


Figure 6.1 (continued)

Figure 6.1 (continued)

```
1281 ACCCCCCTGTCGACGGCTCCACGGTGTCCGTCGGCTTCTCGCAGCCCGGCTTCTGGAGCCACGAATTCCCCCTCGGCCCC Gga [U35463]
     ACCCCCCTGTCGACGGCTCCACGGTGTCCGTCGGCTTCTCGCGGCCCGGCTTCTGGAACCACGAATTCCCCCTCGGCCCC Gga [AF365953]
     ACCCCCCTGTCGACGGCTCCACGATGTCCGTCGGCTTCTCGCAGCCCGGCTTCTGGAGCCACGAATTCCCC-TCGGCCCC MB 001
     ACCCCCCTGTCGACGGCTCCACGGTGTCCGTCGGCTTCTCGCAGCCCGGCTTCTGGAGCCACGAATTCCCCCTCGGCCCC MB 004
     ACCCCCCTGTCGACGGCTCCACGGTGTCCGTCGGCTTCTCGCAGCCCGGCTTCTGGAGCCACGAATTCCCCCTCGGCCCC MB 013
     ACCCCCCTGTCGACGGCTCCACGGTGTCCGTCGGCTTCTCGCCGCCCGGCTTCTGGAACCACGAATTCCCCCTCGGCCCC MB 014
     ACCCCCCTGTCGACGGCTCCACGGTGTCCGTCGGCTTCTCGCCGCCCGGCTTCTGGAACCACGAATTCCCCCTCGGCCCC MB 0113
     ACCCCCCTGTCGACGGCTCCACGGTGTCCGTCGGCTTCTCGCAGCCCGGCTTCTGGAGCCACGAATTCCCCCTCGGCCCC MB 0114
     ACCCCCCTGTCGACGGCTCCACGGTGTCCGTCGGCTTCTCGCCGCCCGGCTTCTGGAACCACGAATTCCCCCTCGGCCCC MB 0215

     ACCCCCCTGTCGACGGCTCCACGGTGCCCATCGGCTACTTGCAGCCCGACGCCTGGAACCACGAATTCCCCCTCGGCCCC Ggg [AF365954]
     ACCCCCCTGTCGACGGCTCCACGGTGCCCATCGGCTTCTTGCAGCCCGACGTCTGGAGCCACGAATTCCCCCTCGGCCCC Ggt [AF365955]
     ACCCGCCTGTCGATGGCTCTACAGTGCCGCTCAGCTTCAACACCCGCGACTCCTGGGTTCACGACTTCCCCCTGGGCCCC MB 015
     ACCCCCCTGTCGATGGCTCCACCGTGCCGCTCAGCTTCAACACTCGCGACTCCTGGGTTCACGAATTCCCCCTCGGCCCC MB 0219

     ACCCCCCTGTCGACGGCTCCACGGTGCCCATCGGCTACTTGCAGCCCGACGCCTGGAACCACGAATTCCCCCTCGGCCCC Ggt [AF365958]
     ACCCCCCTGTCGACGGCTCCACGGTGCCCATCGGCTACTTGCAGCCCGACGCCTGGAACCACGAATTCCCCCTCGGCCCC Ggt [AF365956]
     ACCCCCCTGTCGACGGCTCCACGGTGCCCATCAGCTACTTGCAGCCCGACGCCTGGAACCACGAATTCCCCCTCGGCCCC Ggt [AF365957]
     ACCCCCCTGTCGACGGCTCCACGGTGCCCATCGGCTTCTTGCAGCCCGACGTCTGGAGCCACGAATTCCCCCTCGGCCCC MB 0126
     ACCCCCCTGTCGACGGCTCCACTGTGCCCATCGGCTTCTTGTAGCCCGGCGTCTGGAGCCACGAATTCCCCCTCGGCCCC MB 0218
```


Figure 6.1 (continued)

Figure 6.1 (continued)

```
                  <AV3(3' common primer)
1361 ACGGTCGACGTGCGCAGGAACCACCATGAGCA 1385 Gga [U35463]
     ATGGTTGACGTGCGCAGGAACCACCATGAGCA 1385 Gga [AF365953]
     ACGGTCGACGTGCGCAGGAACCACCATGAGCA 1384 MB 001
     ACGGTCGACGTGCGCAGGAACCACCATGAGCA 1385 MB 004
     ACGGTCGACGTGCGCAGGAACCACCATGAGCA 1384 MB 013
     ATGGTCGACGTGCGCAGGAACCACCATGAGCA 1383 MB 014
     ATGGTCGACGTGCGCAGGAACCACCATGAGCA 1384 MB 0113
     ACGGTCGACGTGCGCAGGAACCACCATGAGCA 1385 MB 0114
     ACGGTCGACCTGCGCAGGAACCACCATGAGCA 1384 MB 0215

     ACGGTCGACGTGCGCAGGAACCACCATGAGCA 1387 Ggg [AF365954]
     ACGGTCGACGTGCGCAGGAACCACCATGAGCA 1388 Ggt [AF365955]
     CTGGTTGACGTGCGCAGGAACCACCATGAGCA 1381 MB 015
     CTGGTTGACGCGCGCAGGAACCACCATGAGCA 1378 MB 0219

     ACGGTCGACGTGCGCAGGAACCACCATGAGCA 1388 Ggt [AF365958]
     ACGGTCGACGTGCGCAGGAACCACCATGAGCA 1389 Ggt [AF365956]
     ACGGTCGACGTGCGCAGGAACCACCATGAGCA 1390 Ggt [AF365957]
     ACGGTCGACGTGCGCAGGAACCACCATGAGCA 1390 MB 0126
     ACGGTCGACGTGCGCAGGAACCACCATGAGCA 1389 MB 0218
```

Figure 6.2. Deduced amino acid sequence corresponding to the avenacinase gene of Gga and avenacinase-like genes of Ggg and Ggt. Amino acid changes are indicated in bold print. Dashes were used to maximize alignments.

Figure 6.2 (continued)

Figure 6.2 (continued)

```
121 LIYKHGVLMAREFRDKGSHVILGPVIGPLGRSPYAGRNWEGFSPDSYLAGVMAEQTVKGM    Gga MB 001
    LIYKHGVLMAREFRDKGSHVILGPVIGPLGRSPYAGRNWEGFSPDSYLAGVMAEQTVKGM    Gga MB 004
    LIYKHGVLMAREFRDKGSHVILGPVIGPLGRSPYAGRNWEGFSPDSYLAGVMAEQTVKGM    Gga MB 013
    LIHKHGVLMAQEFRDKGSHVILGPVIGPLGRSPYGGRNWEGFSPDSYLAGVMVEQTVKGM    Gga MB 014
    LIHKHGVLMAQEFRDKGSHVILGPVIGPLGRSPYGGRNWEGFSPDSYLAGVMVEQTVKGM    Gga MB 0113
    LIYKHGVLMAREFRDKGSHVILGPVIGPLGRSPYAGRNWEGFSPDSYLAGVMAEQTVKGM    Gga MB 0114
    LIYKHGVLMAREFRDKGSHVILGPVIGALGRSPYAGRNWEGFSPDSYLAGVMVEQTVKGM    Gga MB 0215

    LIYKHGVLMAREFRDKGSHIILGPVIGPLGRSPYAGRNWEGFSPDSYLAGVLAEQTVKGM    Ggt MB 0126
    LIYKHGVLMAREFRDKGSHIILGPVIGPLGRSPYAGRNWEGFSPDSYLAGVLAEQTVKGM    Ggt MB 0218

    LNYQHGALMAQEFRDKGSHVILGPVIGALGRSPYAGRNWEGFSPDSYLSGVLVEETVKGM    Ggg MB 015
    LIYQHGALMAQEFRDKGSHVILGPVIGALGRSRTPGVTGRDSPPTRTCRASW-RRQSRGC    Ggg MB 0219


181 QSVGVQACTKHFIGNEQEEQRNPTAVDGKTVEAISSNIDDRTMHEAYLWPFYNAVRAGTT    Gga MB 001
    QSVGVQACTKHFIGNEQEEQRNPTAVDGKTVEAISSNIDDRTMHEAYLWPFYNAVRAGTT    Gga MB 004
    QSVGVQACTKHFIGNEQEEQRNPTAVDGKTVEAISSNIDDRTMHEAYLWPFYNAVRAGTT    Gga MB 013
    QSVGVQACTKHFIGNEQEEQRNPTAVGGKTVEAISSNIDDRTMHETYLWPFYNAVRAGTA    Gga MB 014
    QSVGVQACTKHFIGNEQEEQRNPTAVGGKTVEAISSNIDDRTMHETYLWPFYNAVRAGTA    Gga MB 0113
    QSVGVQACTKHFIGNEQEEQRNPTAVDGKTVEAISSNIDDRTMHEAYLWPFYNAVRAGTT    Gga MB 0114
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    QSVGVQTCTKHYIGNEQEEQRNPTTVDGKGVEAISSNIDDRTMHETYLWPFYNAVRAGTT    Ggt MB 0126
    QSVGVRTCTKHFIGNEQEEQRNPTTVDGKGVEAISSNIDDRTMHETYLWPFYNAVRAGTT    Ggt MB 0218

    QSVGVQACTKHFIGNEQEEQRNPTTVDGERVEAISSNIDDRTMHETYLWPFYNAVRAGTT    Ggg MB 015
    -SVGVQACTKHFIGNEQEEQRNPTTVDGKRVEAISSNIDDRTMHETYLWPFYNAVRAGTT    Ggg MB 0219
```


Figure 6.2 (continued)

Figure 6.2 (continued)

```
241 SIMCSYQRINGSYGCQNSKTLNGLLKTELGFQGFVVSDWAATHSGVASIEAGLDMNMPGP    Gga MB 001
    SIMCSYQRINSSYGCQNSKTLNGLLKTELGFQGFVVSDWAATHSGVASIEAGLDMNMPGP    Gga MB 004
    SIMCSYQRINGSYGCQNSKTLNGLLKTELGFQGFVVSDWAATHSGVASIEAGLDMNMPGP    Gga MB 013
    SIMCSYQRINGSYGCQNSKTLNGLLKTELGFQGFVVSDWAATHSGVAFIEAGLDMNMPGS    Gga MB 014
    SIMCSYQRINGSYGCQNSKTLNGLLKTELGFQGFVVSDWAATHSGVASIEAGLDMNMPGS    Gga MB 0113
    SIMCSYQRINGSYGCQNSKTLNGLLKTELGFQGFVVSDWAATHSGVASIEAGLDMNMPGP    Gga MB 0114
    P-CALTRGSTAATAARTARPSTDFSRPSSASRASSCRT-AATHSGVASIEAGLDMNMPGS    Gga MB 0215

    SIMCSYQRINGSYGCQNSKTLNGLLKTELGFQGFVVSDWAATHSGVASIEAGLDMNMPGP    Ggt MB 0126
    SIMCSYQRINGSYGCQNSKTLNGLLKTELGFQGFVVSDWAATHSGVASIEAGLDMNMPGP    Ggt MB 0218

    SIMCSYQRINGSYGCQNSKTLNGLLKTELGFQGYVMSDWMATHAGVPAIEAGLDMNMPGP    Ggg MB 015
    SIMCSYQRINGSYGCQNSKTLNGLLKTELGFQGYVMSDWMATHAGVPAIEAGLT*-CPVR    Ggg MB 0219


301 LNFFAPTFESYFGKNITTAVNNGTLSSRRVDEMIERIMTPYFALGQDKNYPPVDGSTMSV    Gga MB 001
    LNFFAPTFESYFGKNITTAVNNGTLSSRRVDEMIERIMTPYFALGQDKNYPPVDGSTVSV    Gga MB 004
    LNFFAPTFESYFGKNITTAVNNGTLSSRRVDEMIERIMTPYFALGQDKNYPPVDGSTVSV    Gga MB 013
    LDFFAP--ESYFGKNITTAVNNGTLSSRRVDEMIERIMTPYFALGQDKNYPPVDGSTVSV    Gga MB 014
    LDFFAP--ESYFGKNITTAVNNGTLSSRRVDEMIERIMTPYFALGQDKNYPPVDGSTVSV    Gga MB 0113
    LNFFAPTFESYFGKNITTAVNNGTLSSRRVDEMIERIMTPYFALGQDKNYPPVDGSTVSV    Gga MB 0114
    LDFFAPTFESYFGKNITTAVNNGTLSSRRVDEMIERIMTPYFALGQDKNYPPVDGSTVSV    Gga MB 0215

    LNFFAPTLESYFGKNITTAVNNGTLSSRRVDDMIERIMTPYFALGQDKDYPPVDGSTVPI    Ggt MB 0126
    LNFFAPTLGSYFGKNITTAVNNGTLSSRRVDDMIERIMTPYFALGQDKDYPPVDGSTVPI    Ggt MB 0218

    IEFLGTTVKSYFGGNVTIAVNNGSLSIQRVDDMIERIMAPYFALGQDKNYPPVDGSTVPL    Ggg MB 015
    SNSSAQPSSLTLAGTSPLRLTTALSPSRGSTT-LSVSWRPTSPWARTRTTPLSMAPPCRS    Ggg MB 0219
```


Figure 6.2 (continued)

Figure 6.2 (continued)

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361 GFSQPGFWSHEFPSAPRSTCAGTTMS 387   Gga MB 001
    GFSQPGFWSHEFPLGPTVDVRRNHHE       Gga MB 004
    GFSQPGFWSHEFPLGPTVDVRRNHHE       Gga MB 013
    GFSPPGFWNHEFPLGPMVDVRRNHHE       Gga MB 014
    GFSPPGFWNHEFPLGPMVDVRRNHHE       Gga MB 0113
    GFSQPGFWSHEFPLGPTVDVRRNHHE       Gga MB 0114
    GFSPPGFWNHEFPLGPTVDLRRNHHE       Gga MB 0215

    GFLQPDVWSHEFPLGPTVDVRRNHHE       Ggt MB 0126
    GFL-PGVWSHEFPLGPTVDVRRNHHE       Ggt MB 0218

    SFNTRDSWVHDFPLGPLVDVRRNHHE       Ggg MB 015
    ASTLATPGFTNSPSAPWLTRAGTTMS       Ggg MB 0219
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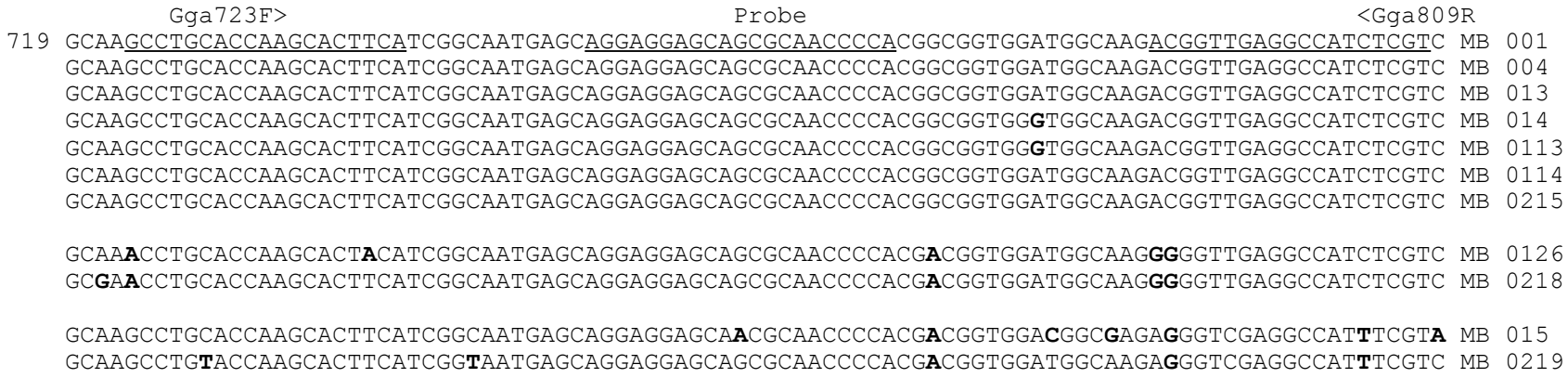

Figure 6.3. Primer sequences for the Gga-specific real-time PCR assay. Total amplicon size was 86 bp.

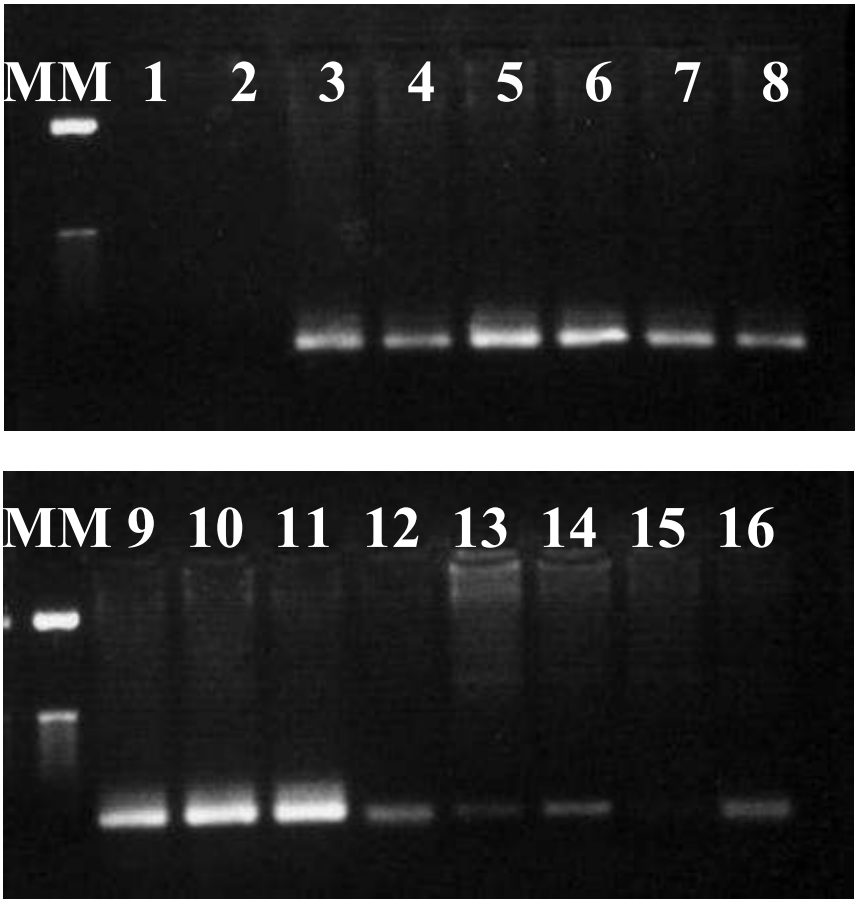

Figure 6.4. Amplicons produced in PCR reactions with primer set Gga723F:Gga809R with DNA from Gga, Ggt, and Ggg. Lanes: MM= molecular marker (25 bp ladder); 1=TE; 2=water; 3=MB 001; 4=MB 004; 5=MB 013; 6=MB 014; 7=MB 0110; 8=MB 0113; 9=MB 0114; 10=MB 0116; 11=MB 0215; 12=MB 0126; 13=MB 015; 14=MB 016; 15=MB 0219; and 16=MB 0218.

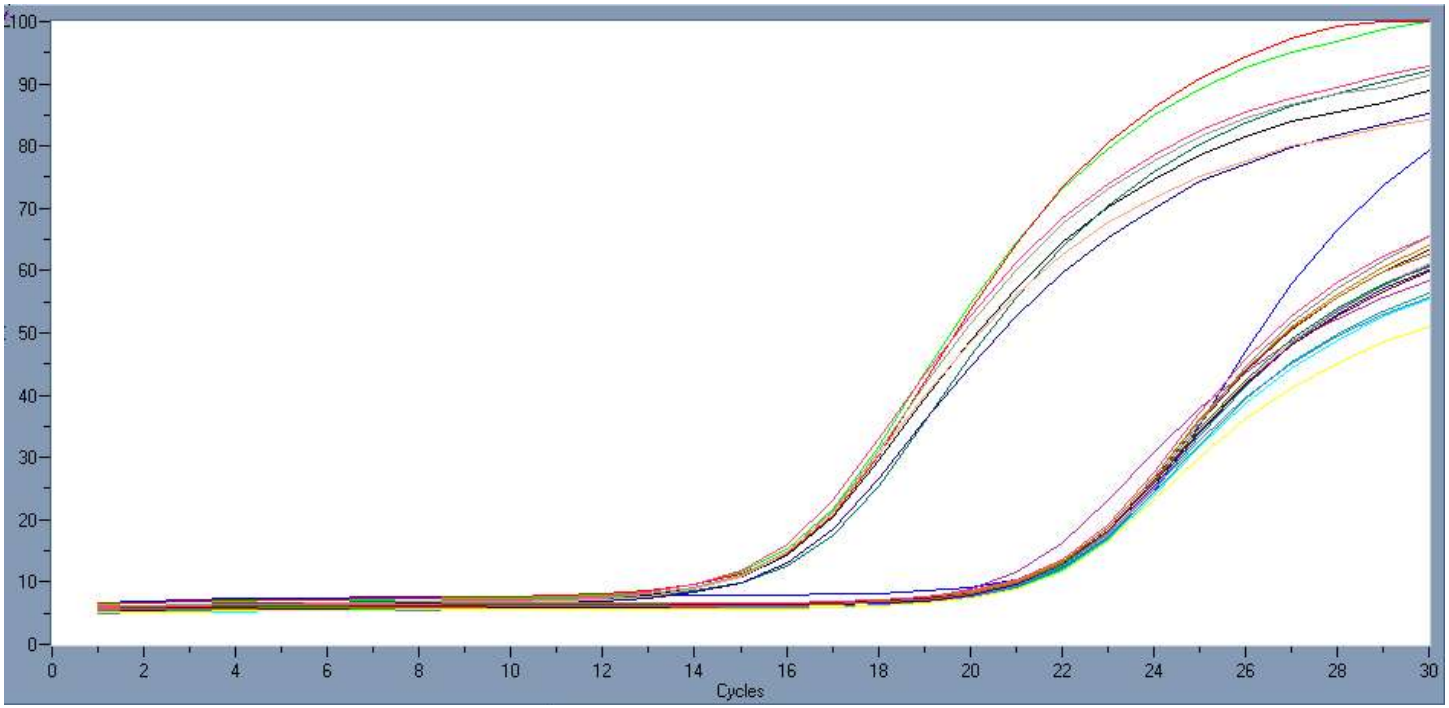

Figure 6.5. Curves generated with SYBR green to monitor dsDNA accumulation during amplification of genomic DNA from Gga, Ggt, and Ggg with the Gga-specific primer set, Gga723F:Gga809R.

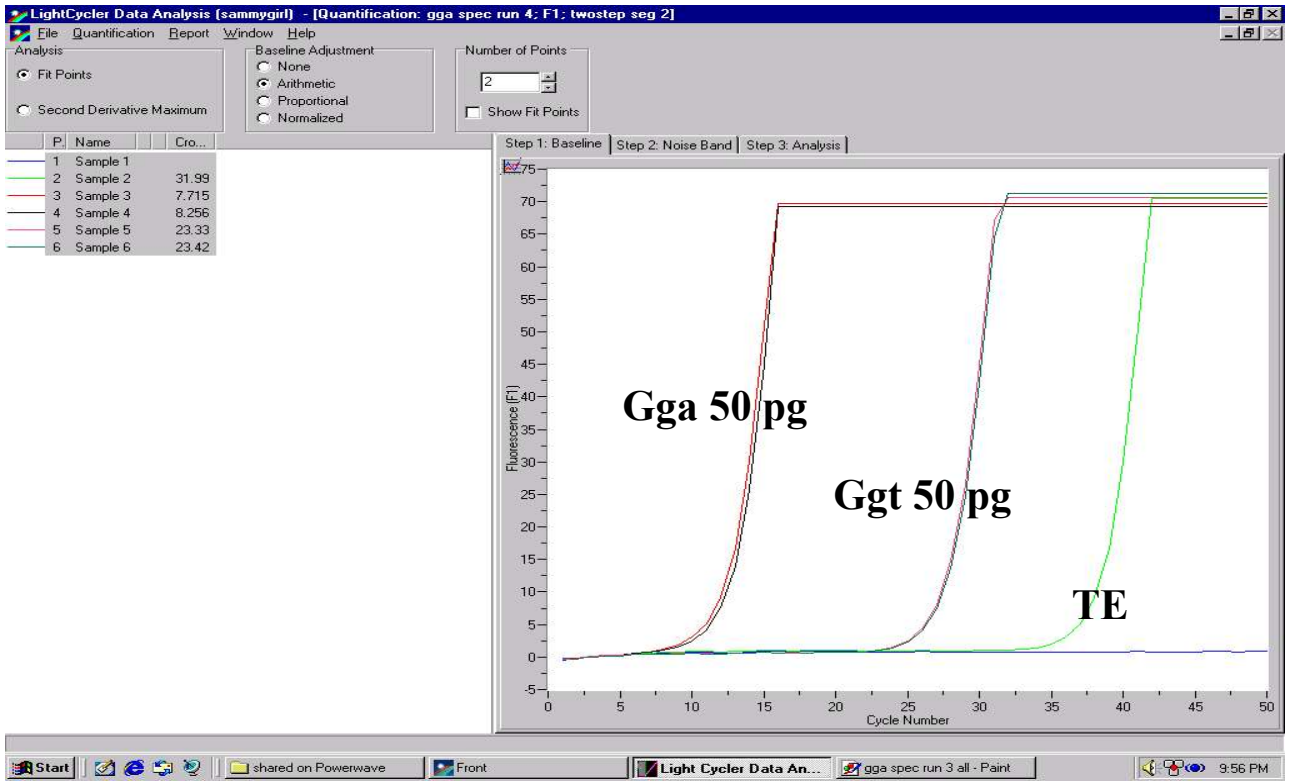

Figure 6.6. Curves generated with SYBR green under probe hydrolysis conditions to monitor dsDNA accumulation amplification of TE and genomic DNA from Gga isolate MB 013 and Ggt isolate MB 0126 with the Gga-specific primer set, Gga723F:Gga809R.

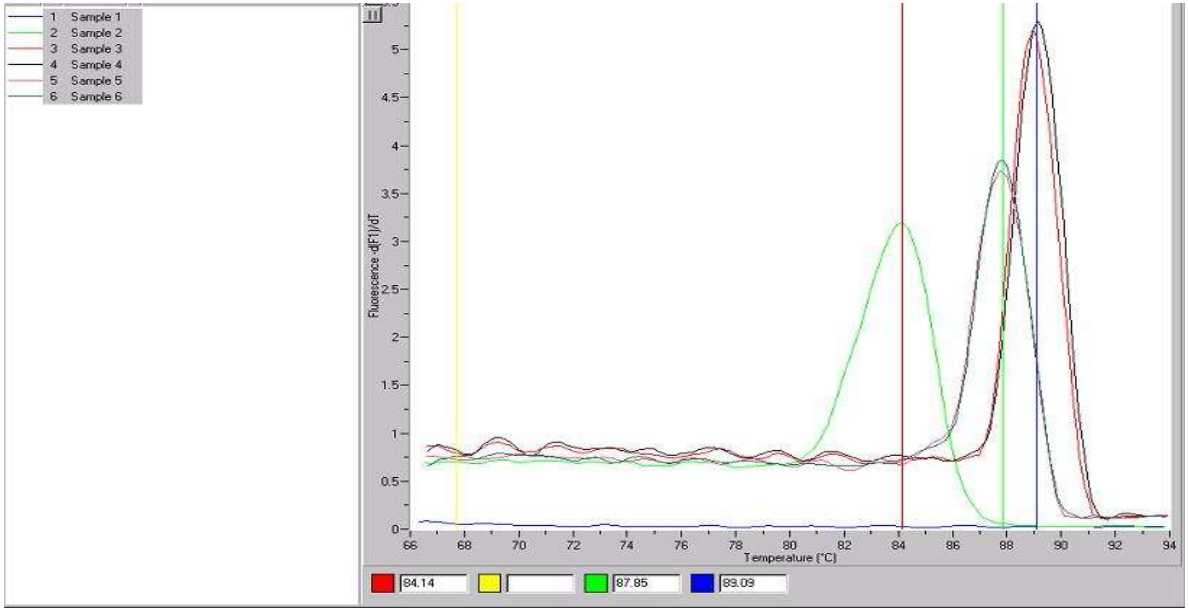

Figure 6.7. Melting curve analysis of amplicons generated with the Gga-specific primer set and Gga, Ggt and TE samples. SYBR green was used to monitor amplification. The vertical lines represent the melting point of the amplicon. Samples 1 and 2 are TE (T_m=84.14); Samples 3 and 4 are MB 013 (T_m=89.09) and Samples 5 and 6 are MB 0126 (T_m= 87.85).

CHAPTER 7

SUMMARY OF RESULTS

7.1 Morphological, molecular, and pathogenic identification of *Gaeumannomyces graminis*.

1. Morphological characters of all three G. graminis varieties overlap. Generally, Gga isolates produced longer ascospores as compared with Ggt and Ggg and Ggg isolates produced lobed hyphopodia.

2. Pathogenicity on oats was the easiest way to distinguish Gga isolates from Ggt and Ggg isolates.

3. The production of amplicons with the molecular primer set Gga:AV3 was perfectly correlated to pathogenicity on oats indicating this primer set can be used to distinguish Gga isolates from Ggt and Ggg.

4. All G. graminis isolates recovered from symptomatic creeping bentgrass were identified as Gga based on morphology, pathogenicity, and molecular characteristics.

5. Ggt isolates could be distinguished from greater than 90% of Ggg isolates using the molecular primer sets NS5:NS6 and NS5:GgtRP.

6. Ggg isolates could not be readily distinguished using any of the morphological assays or molecular primer sets,

7. Given that the overlap in morphological characteristics and the easiest distinction to

make between isolates was pathogenicity on oats and wheat, the isolates within G. graminis may be better separated into forma specialis groups.

7.2 The effect of root zone mix composition on take-all incidences of creeping bentgrass.

1. Sand type and organic matter amendment were shown to significantly (P=0.05) affect take-all incidence on creeping bentgrass.

2. Compost amendments to sands resulted in an increase in the plant available nutrients, significantly (P=0.05) taller plants and higher foliar dry weights.

3. Gga isolates varied in their ability to incite disease on creeping bentgrass and oats indicating a degree of host specificity. The differences in isolate aggressiveness could be overcome by increasing inoculum dose.

7.3 Pathogenicity of *Gaeumannomyces graminis* on creeping bentgrass.

1. Avenacin was not detected in root extracts of creeping bentgrass cultivars using fluorimetry, TLC and HPLC.

2. Ggt isolates are capable of inciting take-all patch on creeping bentgrass in growth chamber studies.

3. Avenacinase activity is not required for root infection of creeping bentgrass.

7.4 The development of a real-time quantitative PCR assay for the detection of *Gaeumannomyces graminis*.

1. The avenacinase gene sequence from Gga and avenacinase-like gene sequence from Ggt were >97% identical. However, Ggg sequences were only 85% homologous to those of Gga and Ggt. These results indicate Gga and Ggt are more closely related to each other than to Ggg.

2. The putative active sites containing aspartic acid residues within the avenacinase amino acid sequence were conserved in the avenacinase-like sequence from Ggt indicating this gene may be functional in Ggt and it may be the precursor to the avenacinase gene in Gga isolates.

3. The real-time PCR assay could not be optimized to generate amplicons with only the Gga genomic DNA. However, SYBR green analysis in conjunction with the Gga:AV3 primer set holds promise based on previous results using Gga:AV3.

APPENDIX A

RESULTS OF POLYMERASE CHAIN REACTIONS PRESENTED IN TABLE 2.1.

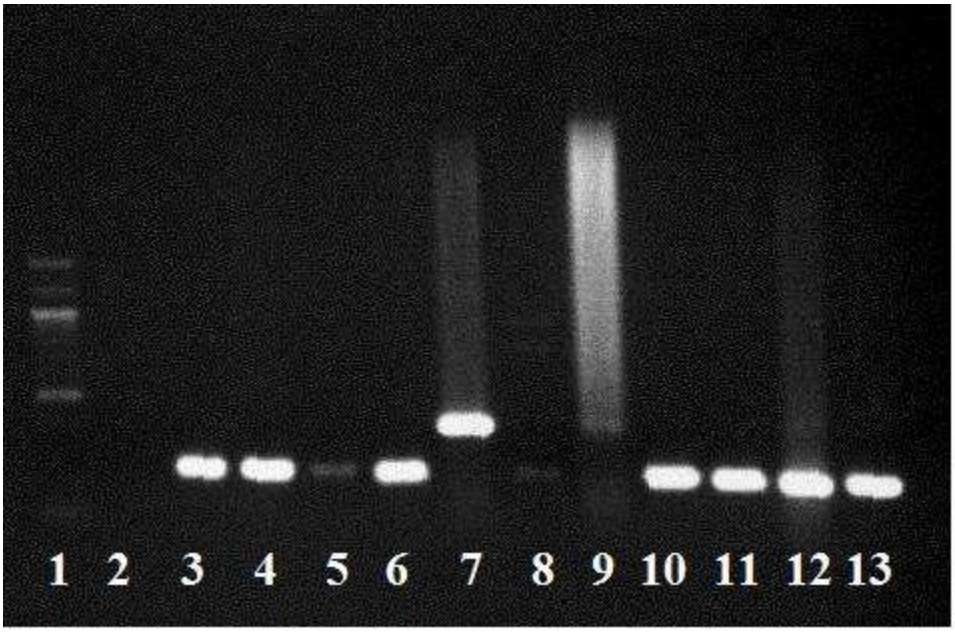

Figure A.1. Amplicons generated from PCR reactions with genomic DNA from Gga isolates containing the primer set NS5:GgtRP. Lane 1 contains a 100 bp ladder. The remaining lanes represent amplifications containing 10 ng genomic DNA from isolates: 2) TE (negative control); 3) MB 013; 4) MB 002; 5) MB 003; 6) MB 004; 7) MB 005; 8) MB 011; 9) MB 012; 10) MB 001; 11) MB 014; 12) MB 017; and 13) MB 019.

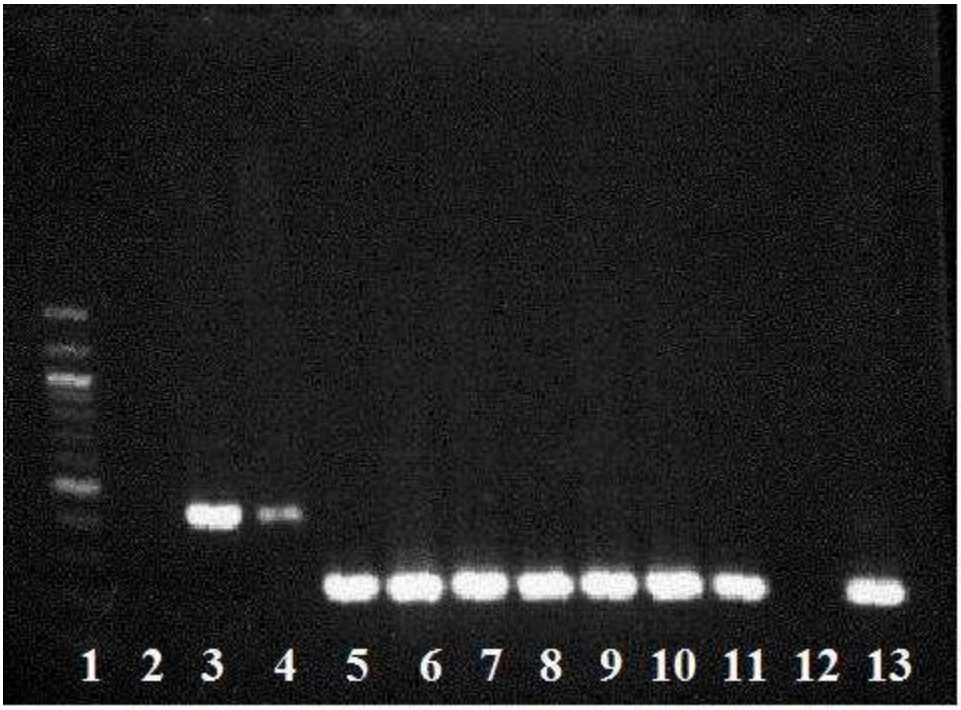

Figure A.2. Amplicons generated from PCR reactions containing the primer set NS5:GgtRP and genomic DNA from Gga isolates. Lane 1 contains a 100 bp ladder. The remaining lanes represent amplifications containing 10 ng genomic DNA from isolates: 2) TE (negative control); 3) MB 0111; 4) MB 0112; 5) MB 0114; 6) MB 0115; 7) MB 0116; 8) MB 021; 9) MB 022; 10) MB 023; 11) MB 0215; 12) MB 0217; and 13) MB 032.

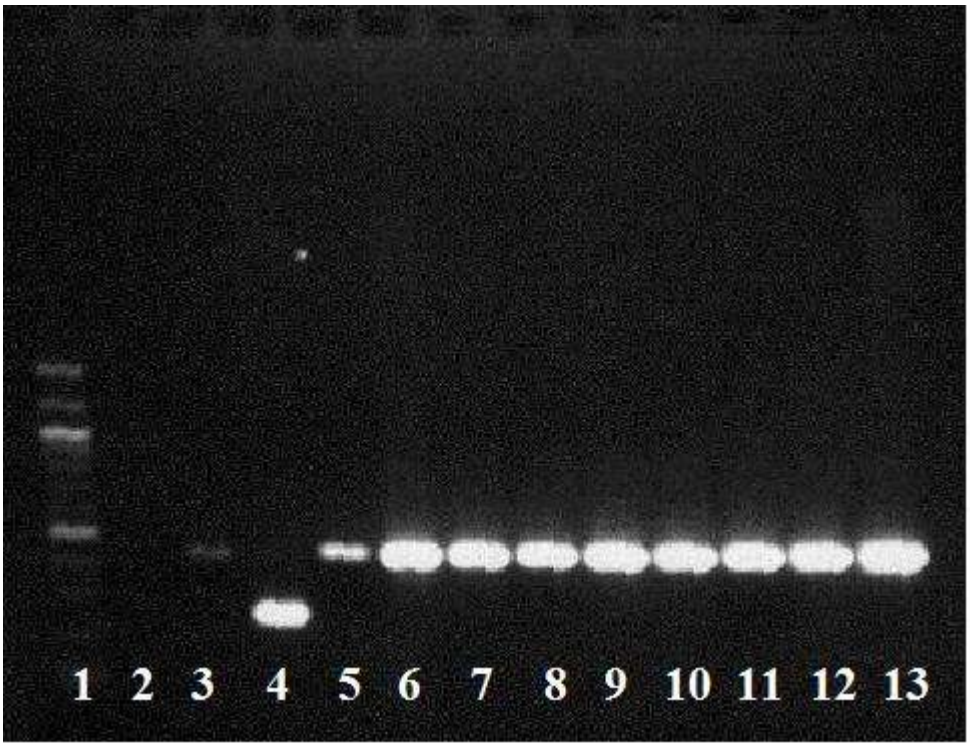

Figure A.3. Amplicons generated from PCR reactions containing the primer set NS5:GgtRP with genomic DNA from Gga and Ggt isolates. Lane 1 contains a 100 bp ladder. The remaining lanes represent amplifications containing 10 ng genomic DNA from isolates: 2) TE (negative control); 3) MB 033; 4) MB 0317; 5) MB 0319; 6) MB 0126; 7) MB 025; 8) MB 026; 9) MB 029; 10) MB 0212; 11) MB 0213; 12) MB 0313; and 13) MB 0314.

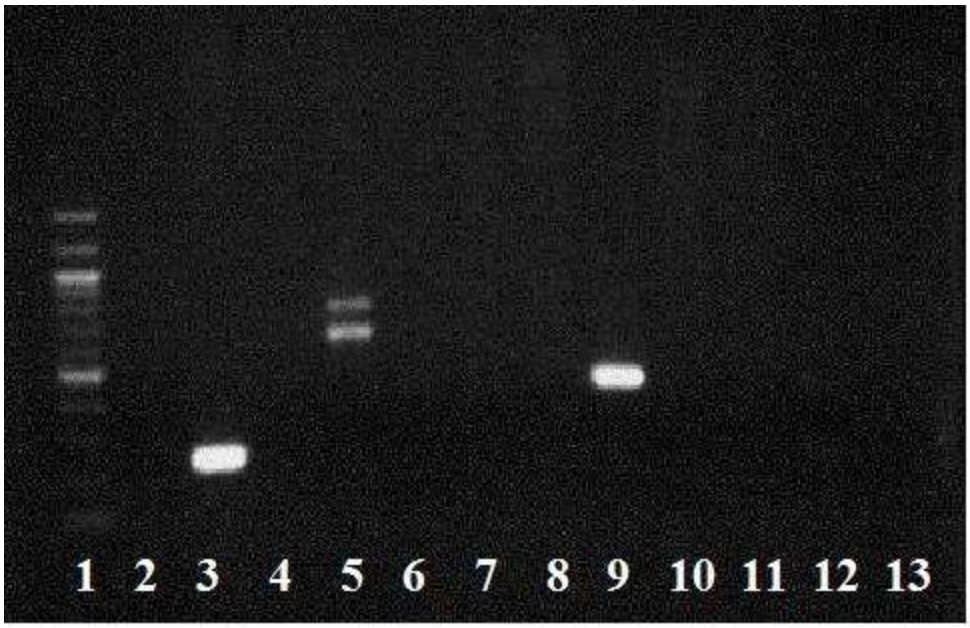

Figure A.4. Amplicons generated from PCR reactions containing the primer set NS5:GgtRP with genomic DNA from Gga and *G. graminis*-like isolates. Lane 1 contains a 100 bp ladder. The remaining lanes represent amplifications containing 10 ng genomic DNA from isolates: 2) TE (negative control); 3) MB 001; 4) MB 018; 5) MB 0110; 6) MB 0117; 7) MB 0118; 8) MB 0119; 9) MB 0120; 10) MB 0121; 11) MB 0122; 12) MB 0123; and 13) MB 0124.

Figure A.5. Amplicons generated from PCR reactions containing the primer set NS5:GgtRP with genomic DNA from Gga, Ggt, and Ggg isolates. Lane 1 contains a 100 bp ladder. The remaining lanes represent amplifications containing 10 ng genomic DNA from isolates: 2) TE (negative control); 3) MB 0125; 4) MB 0216; 5) MB 0113; 6) MB 0321; 7) TE; 8) MB 027; 9) MB 028; 10) MB 0210; 11) MB 0315; 12) MB 015; and 13) MB 016.

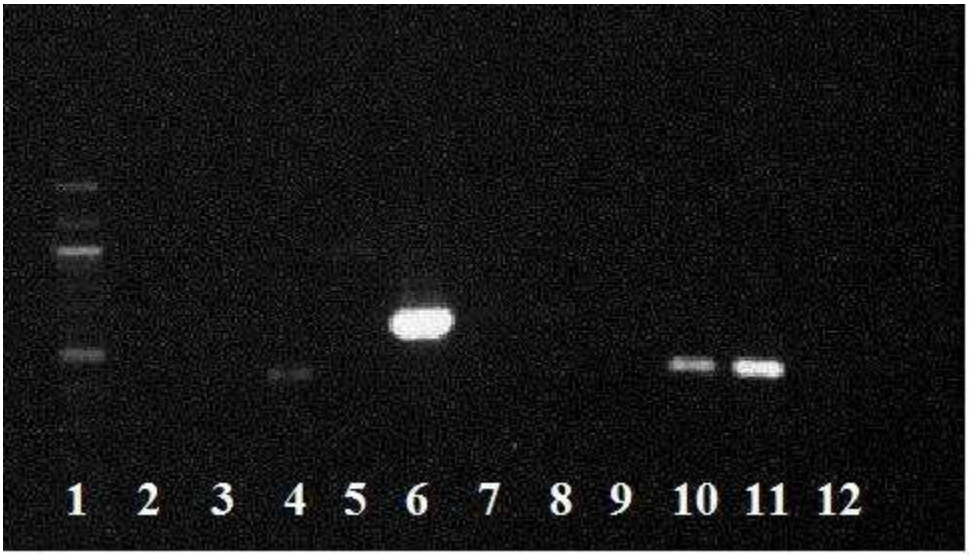

Figure A.6. Amplicons generated from PCR reactions containing the primer set NS5:GgtRP with genomic DNA from Ggg and *G. graminis*-like isolates. Lane 1 contains a 100 bp ladder. The remaining lanes represent amplifications containing 10 ng genomic DNA from isolates: 2) TE (negative control); 3) MB 0219; 4) MB 0220; 5) MB 031; 6) MB 034; 7) MB 036; 8) MB 037; 9) MB 0223; 10) MB 0316; 11) *Phialophora graminicola*; and 12) *Gaeumannomyces cylindrosporus*.

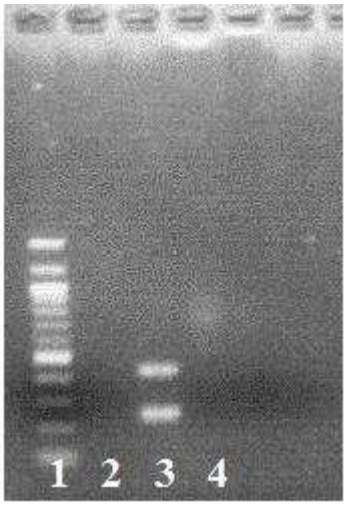

Figure A.7. Amplicons generated from PCR reactions containing the primer set NS5:GgtRP with genomic DNA from *Magnaporthe grisea* and *Microdochium nivale.* Lane 1 contains a 100 bp ladder. The remaining lanes represent amplifications containing 10 ng genomic DNA from isolates: 2) TE (negative control); 3) *Magnaporthe grisea*; and 4) *Microdochium nivale*.

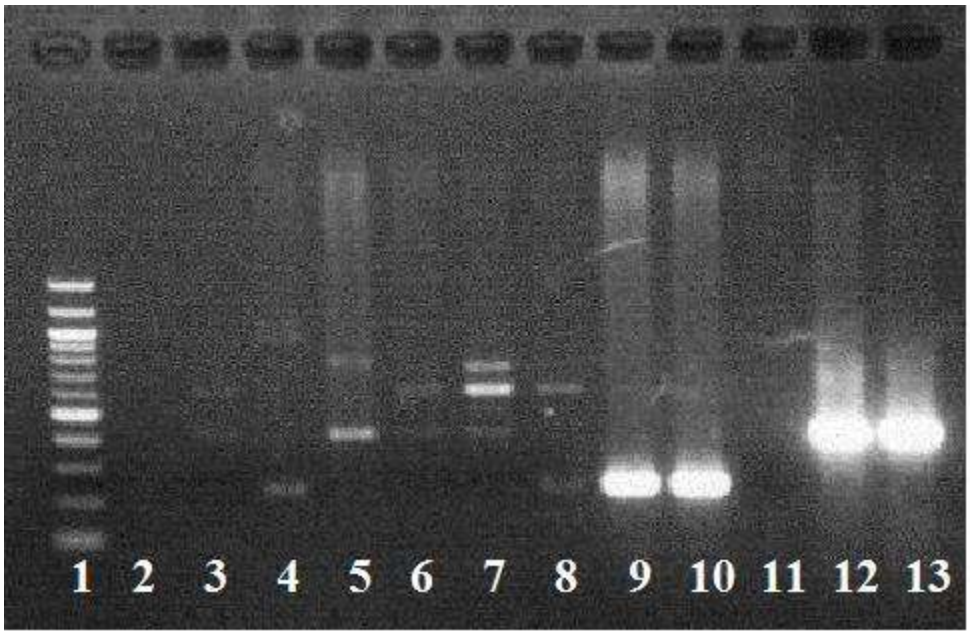

Figure A.8. Amplicons generated from PCR reactions containing the primer set NS5:GgtRP with genomic DNA from Gga and Ggt isolates. Lane 1 contains a 100 bp ladder. The remaining lanes represent amplifications containing 10 ng genomic DNA from isolates: 2) TE (negative control); 3) MB 0217; 4) MB 011; 5) MB 012; 6) MB 018; 7) MB 0110; 8) MB 0113; 9) MB 0214; 10) MB 0318; 11) MB 0321; 12) MB 0218; and 13) MB 0211.

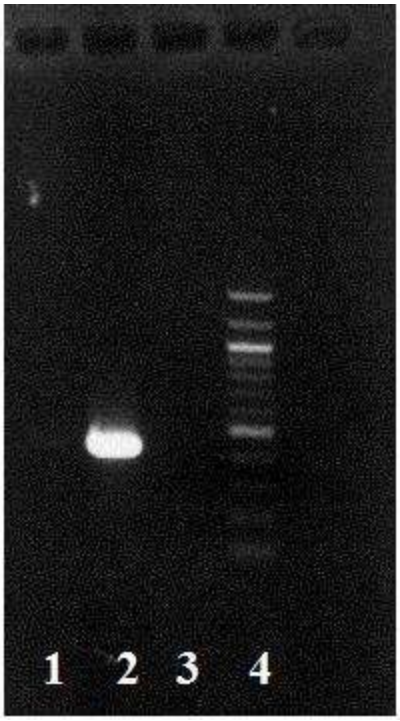

Figure A.9. Amplicons generated from PCR reactions containing the primer set NS5:GgtRP with genomic DNA from Ggt isolate MB 0315 and Ggg isolates MB 024 and MB 035. Lane 4 contains a 100 bp ladder. The remaining lanes represent amplifications containing 10 ng genomic DNA from isolates: 1) MB 0315; 2) MB 024; and 3) MB 035.

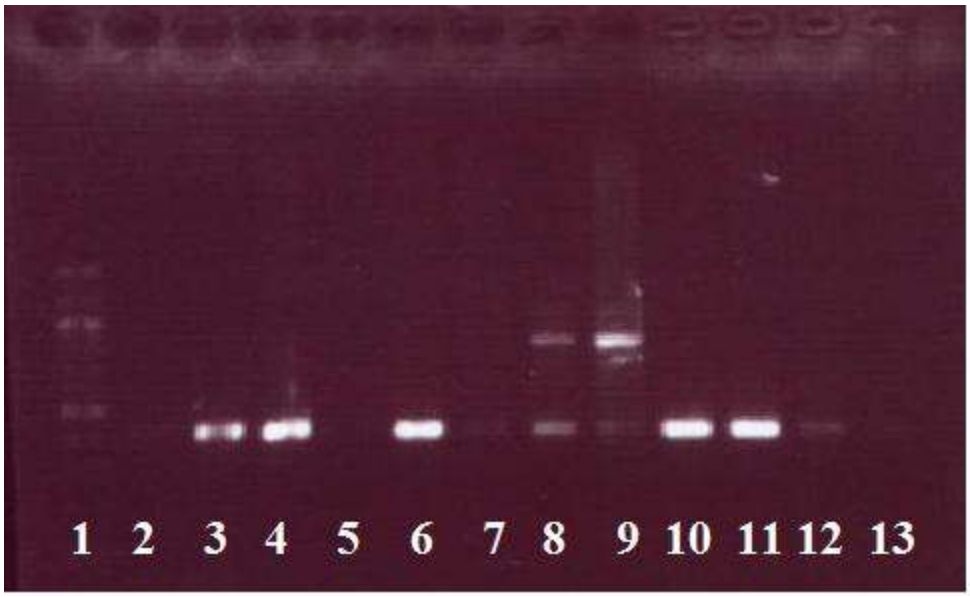

Figure A.10. Amplicons generated from PCR reactions containing the primer set NS5:GgaRP with genomic DNA from Gga and *G. graminis*-like isolates. Lane 1 contains a 100 bp ladder. The remaining lanes represent amplifications containing 10 ng genomic DNA from isolates: 2) TE (negative control); 3) MB 001; 4) MB 002; 5) MB 003; 6) MB 004; 7) MB 005; 8) MB 011; 9) MB 012; 10) MB 013; 11) MB 014; 12) MB 017; and 13) MB 018.

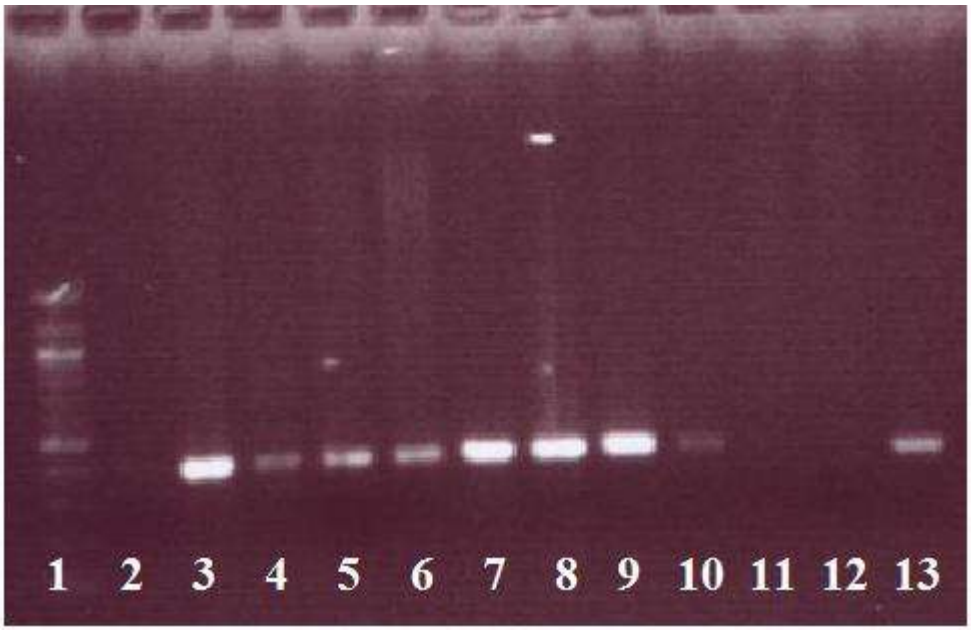

Figure A.11. Amplicons generated from PCR reactions containing the primer set NS5:GgaRP with genomic DNA from Gga and *G. graminis*-like isolates. Lane 1 contains a 100 bp ladder. The remaining lanes represent amplifications containing 10 ng genomic DNA from isolates: 2) TE (negative control); 3) MB 019; 4) MB 0110; 5) MB 0111; 6) MB 0112; 7) MB 0114; 8) MB 0115; 9) MB 0116; 10) MB 0117; 11) MB 0118; 12) MB 0119; and 13) MB 0120.

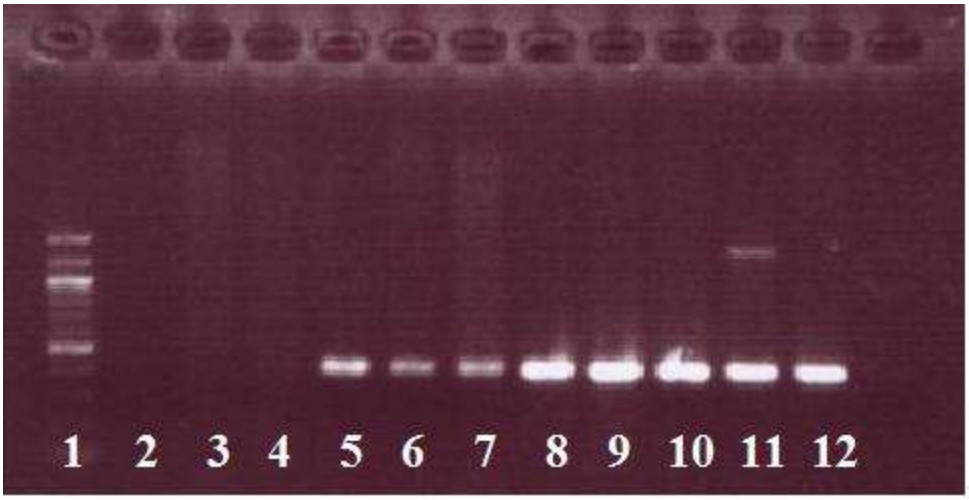

Figure A.12. Amplicons generated from PCR reactions containing the primer set NS5:GgaRP with genomic DNA from G. graminis-like and Gga isolates. Lane 1 contains a 100 bp ladder. The remaining lanes represent amplifications containing 10 ng genomic DNA from isolates: 2) TE (negative control); 3) MB 0121; 4) MB 0122; 5) MB 0123; 6) MB 0124; 7) MB 0125; 8) MB 021; 9) MB 022; 10) MB 023; 11) MB 0215; and 12) MB 0216.

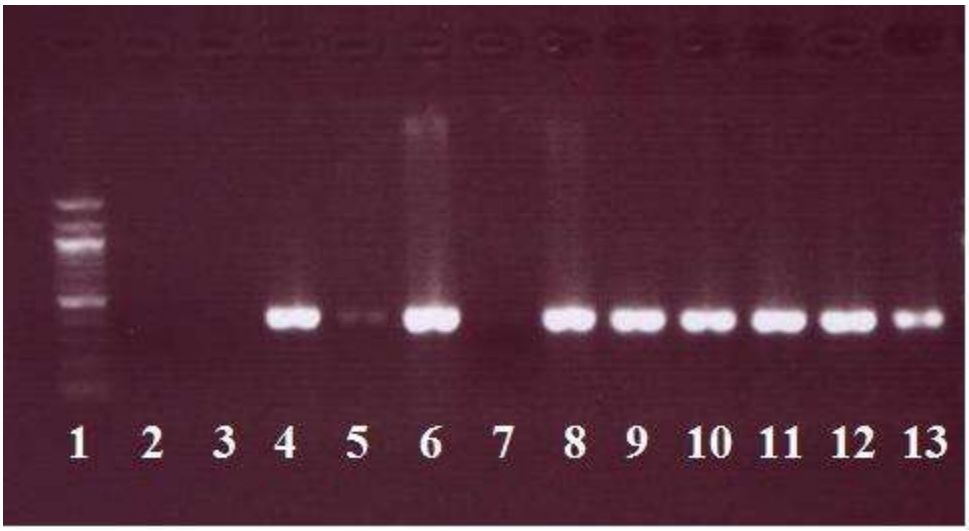

Figure A.13. Amplicons generated from PCR reactions containing the primer set NS5:GgaRP with genomic DNA from Gga and Ggt isolates. Lane 1 contains a 100 bp ladder. The remaining lanes represent amplifications containing 10 ng genomic DNA from isolates: 2) TE (negative control); 3) MB 0217; 4) MB 032; 5) MB 033; 6) MB 0317; 7) MB 0319; 8) MB 0126; 9) MB 025; 10) MB 026; 11) MB 027; 12) MB 028; and 13) MB 029.

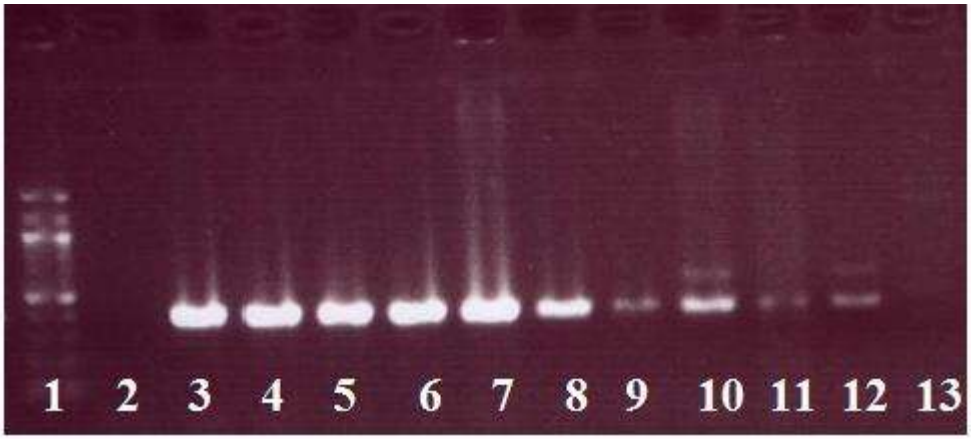

Figure A.14. Amplicons generated from PCR reactions containing the primer set NS5:GgaRP with genomic DNA from Ggt and Ggg isolates. Lane 1 contains a 100 bp ladder. The remaining lanes represent amplifications containing 10 ng genomic DNA from isolates: 2) TE (negative control); 3) MB 0210; 4) MB 0212; 5) MB 0213; 6) MB 0313; 7) MB 0314; 8) MB 0315; 9) MB 015; 10) MB 016; 11) MB 0219; 12) MB 0220; and 13) MB 031.

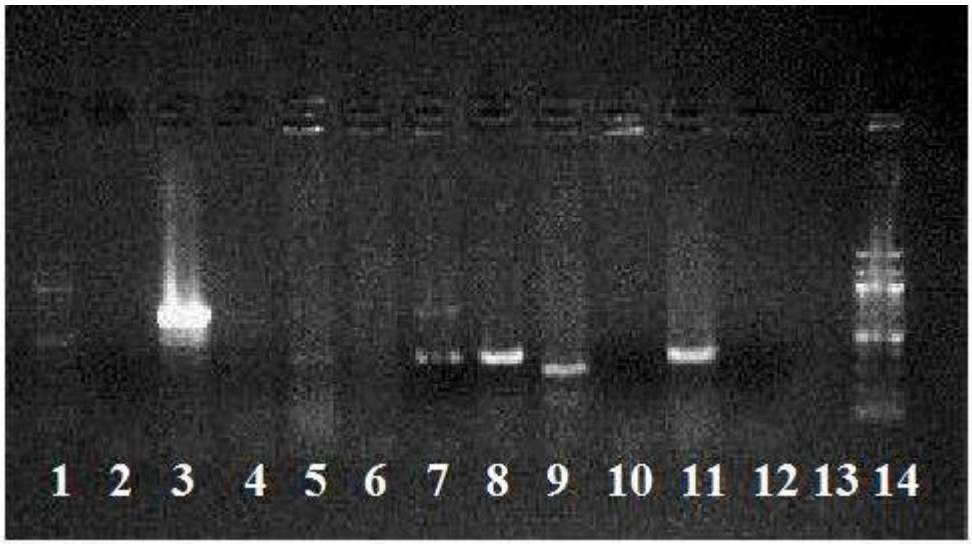

Figure A.15. Amplicons generated from PCR reactions containing the primer set NS5:GgaRP with genomic DNA from Ggg, *G. graminis*-like and known fungal isolates. Lanes 1 and 14 contain a 100 bp ladder. The remaining lanes represent amplifications containing 10 ng genomic DNA from isolates: 2) TE (negative control); 3) MB 034; 4) MB 036; 5) MB 037; 6) MB 0223; 7) MB 0316; 8) *Phialophora graminicola*; 9) *Gaeumannomyces cylindrosporus*; 10) *Microdochium nivale*; 11) *Magnaporthe grisea*; 12) TE; and 13) Blank.

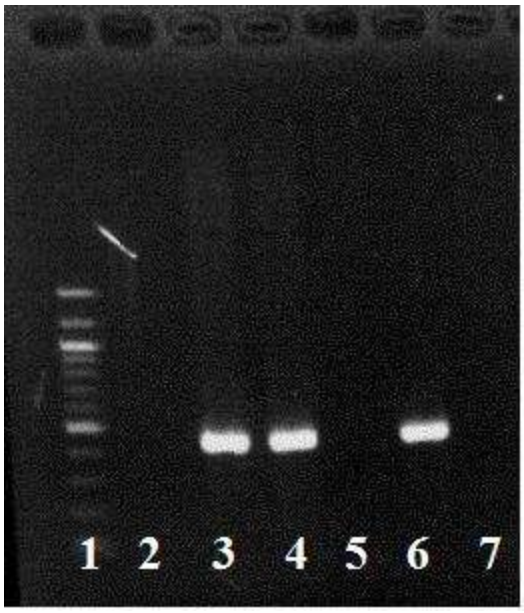

Figure A.16. Amplicons generated from PCR reactions containing the primer set NS5:GgaRP with genomic DNA from Ggt and Ggg isolates. Lane 1 contains a 100 bp ladder. The remaining lanes represent amplifications containing 10 ng genomic DNA from isolates: 2) TE (negative control); 3) MB 0218; 4) MB 0211; 5) MB 038; 6) MB 024; and 7) MB 035.

Figure A.17. Amplicons generated from PCR reactions containing the primer set Gga:AV3 with genomic DNA from Gga, Ggt and Ggg isolates. Lane 1 contains a 100 bp ladder. The remaining lanes represent amplifications containing 10 ng genomic DNA from isolates: 2) MB 013 (positive control); 3) MB 0217; 4) TE (negative control); 5) MB 015; 6) MB 026; 7) MB 001; 8) MB 004; 9) MB 024; 10) MB 0110; 11) MB 0113; 12) MB 0114; and 13) MB 021.

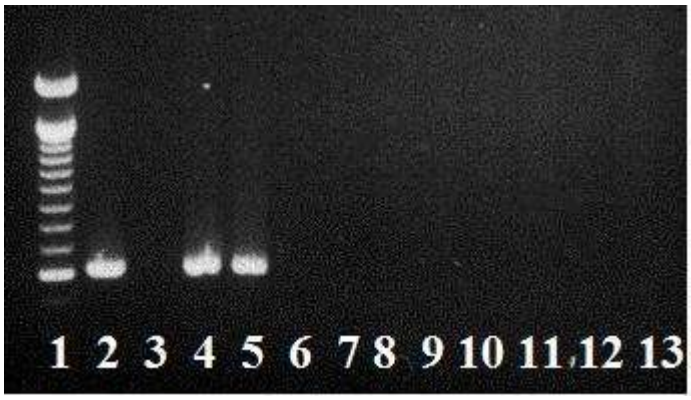

Figure A.18. Amplicons generated from PCR reactions containing the primer set Gga:AV3 with genomic DNA from Gga, Ggt and *G. graminis*-like isolates. Lane 1 contains a 100 bp ladder. The remaining lanes represent amplifications containing 10 ng genomic DNA from isolates: 2) MB 013 (positive control); 3) TE (negative control); 4) MB 0115; 5) MB 0116; 6) MB 0117; 7) MB 0118; 8) MB 0119; 9) MB 0121; 10) MB 0124; 11) MB 0125; 12) MB 016; and 13) MB 0126.

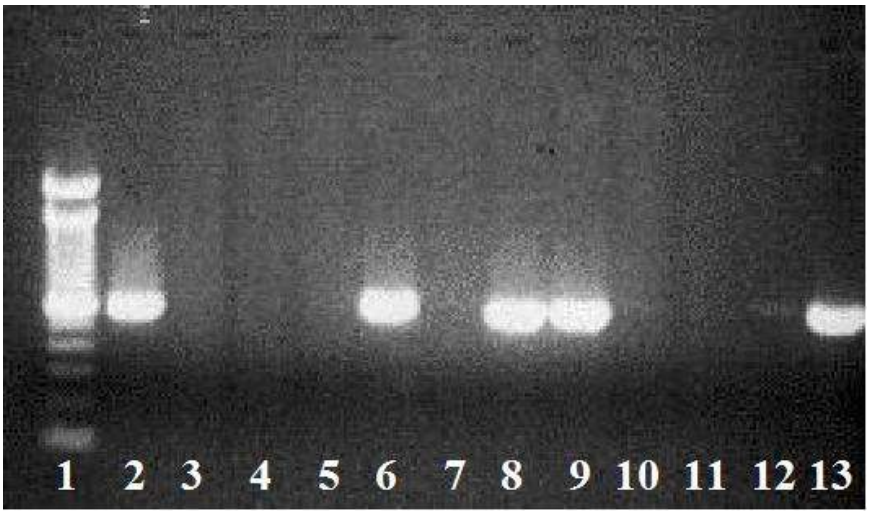

Figure A.19. Amplicons generated from PCR reactions containing the primer set Gga:AV3 with genomic DNA from Gga, Ggt and *G. graminis*-like isolates. Lane 1 contains a 100 bp ladder. The remaining lanes represent amplifications containing 10 ng genomic DNA from isolates: 2) MB 013 (positive control); 3) TE (negative control); 4) MB 026; 5) MB 027; 6) MB 003; 7) MB 025; 8) MB 014; 9) MB 019; 10) MB 0111; 11) MB 0120; 12) MB 0122 and 13) MB 012.

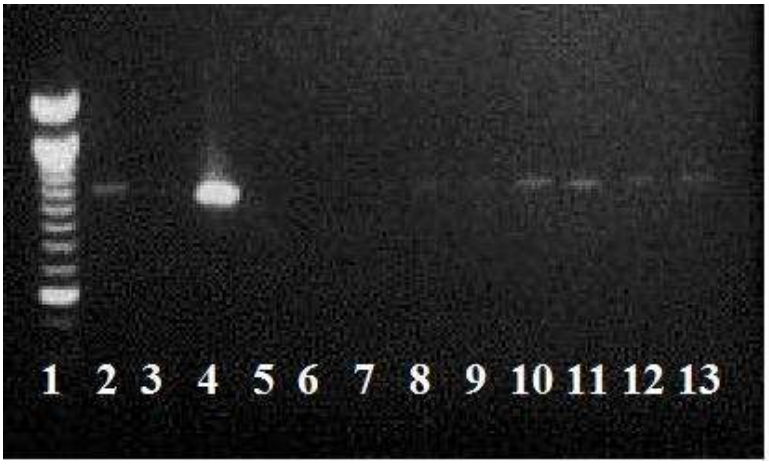

Figure A.20. Amplicons generated from PCR reactions containing the primer set Ggg:AV3 with genomic DNA from Gga and Ggg isolates. Lane 1 contains a 100 bp ladder. The remaining lanes represent amplifications containing 10 ng genomic DNA from isolates: 2) MB 013; 3) TE (negative control); 4) MB 024 (positive control); 5) MB 015; 6) MB 026; 7) MB 001; 8) MB 004; 9) MB 011; 10) MB 0110; 11) MB 0113; 12) MB 0114; and 13) MB 021.

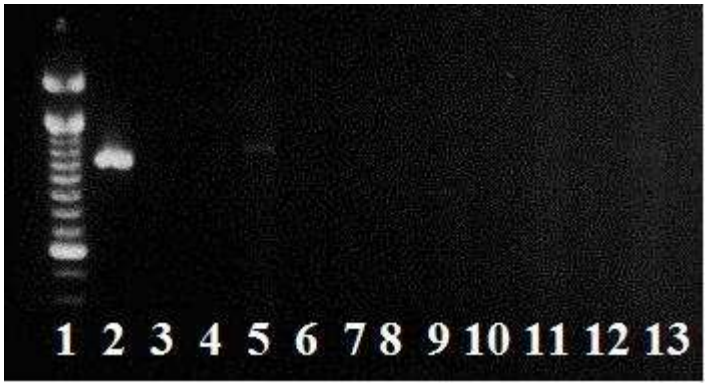

Figure A.21. Amplicons generated from PCR reactions containing the primer set Ggg:AV3 with genomic DNA from Gga, Ggg, Ggt and *G. graminis*-like isolates. Lane 1 contains a 100 bp ladder. The remaining lanes represent amplifications containing 10 ng genomic DNA from isolates: 2) MB 024 (positive control); 3) TE (negative control); 4) MB 0115; 5) MB 0116; 6) MB 0117; 7) MB 0118; 8) MB 0119; 9) MB 0121; 10) MB 0124; 11) MB 0125; 12) MB 016; and 13) MB 0126.

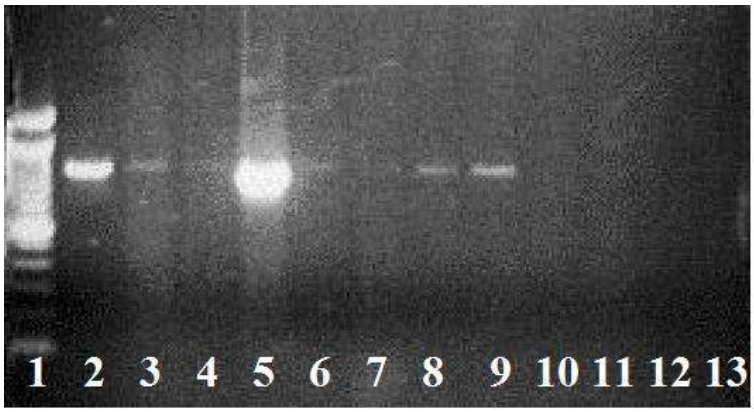

Figure A.22. Amplicons generated from PCR reactions containing the primer set Ggg:AV3 with genomic DNA from Gga, Ggt and *G. graminis*-like isolates. Lane 1 contains a 100 bp ladder. The remaining lanes represent amplifications containing 10 ng genomic DNA from isolates: 2) MB 024 (positive control); 3) MB 025; 4) MB 026; 5) MB 0218; 6) MB 003; 7) MB 012; 8) MB 014; 9) MB 019; 10) MB 0111; 11) MB 0120; 12) MB 0122; and 13) TE (negative control).

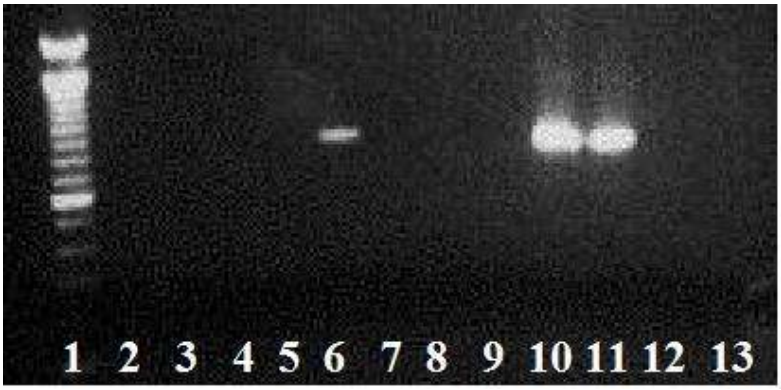

Figure A.23. Amplicons generated from PCR reactions containing the primer set Ggt:AV3 with genomic DNA from Gga and Ggg isolates. Lane 1 contains a 100 bp ladder. The remaining lanes represent amplifications containing 10 ng genomic DNA from isolates: 2) MB 013; 3) TE (negative control); 4) MB 024; 5) MB 015; 6) MB 026 (positive control); 7) MB 001; 8) MB 004; 9) MB 011; 10) MB 0110; 11) MB 0113; 12) MB 0114; and 13) MB 021.

Figure A.24. Amplicons generated from PCR reactions containing the primer set Ggt:AV3 with genomic DNA from Gga, Ggt, Ggg and *G. graminis*-like isolates. Lane 1 contains a 100 bp ladder. The remaining lanes represent amplifications containing 10 ng genomic DNA from isolates: 2) MB 026 (positive control); 3) TE (negative control); 4) MB 0115; 5) MB 0116; 6) MB 0117; 7) MB 0118; 8) MB 0119; 9) MB 0121; 10) MB 0124; 11) MB 0125; 12) MB 016; and 13) MB 0126.

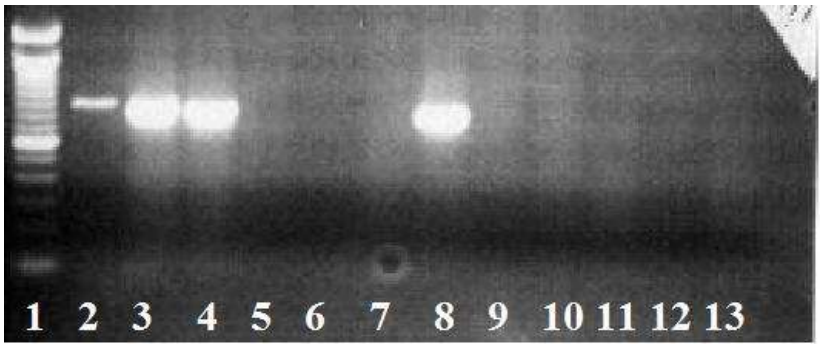

Figure A.25. Amplicons generated from PCR reactions containing the primer set Ggt:AV3 with genomic DNA from Gga, Ggt and *G. graminis*-like isolates. Lane 1 contains a 100 bp ladder. The remaining lanes represent amplifications containing 10 ng genomic DNA from isolates: 2) MB 0126; 3) MB 025; 4) MB 026; 5) MB 0218; 6) MB 003; 7) MB 012; 8) MB 014; 9) MB 019; 10) MB 0111; 11) MB 0120; 12) MB 0122; and 13) TE (negative control).

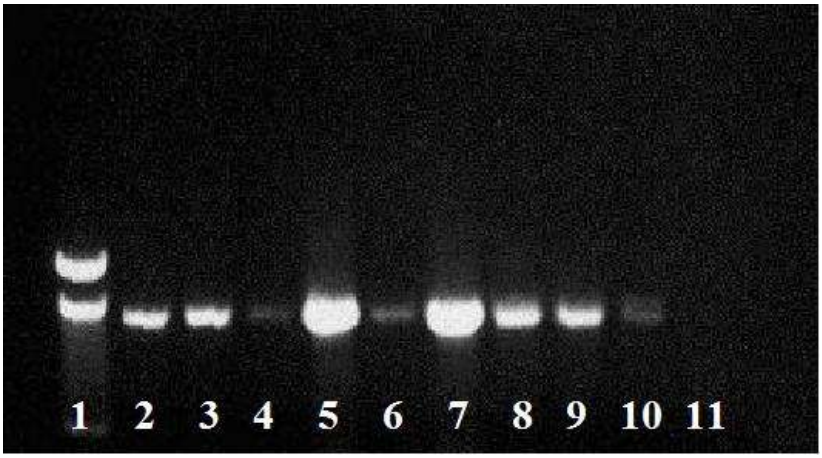

Figure A.26. Amplicons generated from PCR reactions containing the primer set AV1:AV3 with genomic DNA from Ggt isolates. Lane 1 contains a 100 bp ladder. The remaining lanes represent amplifications containing 10 ng genomic DNA from isolates: 2) MB 0217 (positive control); 3) MB 024 (positive control); 4) MB 026 (positive control); 5) MB 0126; 6) MB 025; 7) MB 0218; 8) MB 027; 9) MB 028; 10) MB 029; and 11) TE (negative control).

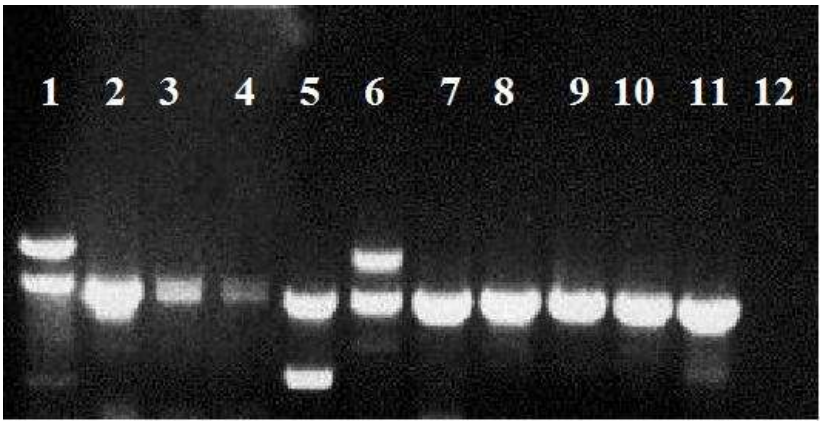

Figure A.27. Amplicons generated from PCR reactions containing the primer set AV1:AV3 with genomic DNA from Gga, Ggt, and Ggg isolates. Lane 1 contains a 100 bp ladder. The remaining lanes represent amplifications containing 10 ng genomic DNA from isolates: 2) MB 0211; 3) MB 0212; 4) MB 0213; 5) MB 015; 6) MB 016; 7) MB 0219; 8) MB 001; 9) MB 002; 10) MB 003; 11) MB 004; and 12) TE (negative control).

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