PROJECT: Pathogenicity and Biological Control of Gaeumannomyces-like Fungi

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Gaeumannomyces graminis var. graminis is the causal agent of bermudagrass decline on golf course putting greens in Florida. G. g. graminis is a dark-pigmented, ectomycorrhizal fungus that is a poor saprophyte. This means it actively colonizes roots and requires living roots to maintain adequate inoculum levels for disease development. For a vegetatively-propagated turfgrass like hybrid bermudagrass, the pathogen, if present in the sod fields, will be moved with the turfgrass to the new planting location. One of the best methods for control, in both economic and ecological terms, would be to introduce a biological control agent into the new planting location, both in the sod field and the golf course. Any organism that could occupy the same niche as the pathogen should be a viable candidate for biological control. One such group of organisms would be mutants of the pathogen that have been rendered non-pathogenic. The primary objective of this project was to develop and evaluate mutants of G. g. graminis for control of bermudagrass decline.

Characteristics of the G. g. graminis FL-39 parent isolate and the twelve strains (selected from 170 strains) obtained via protoplasting and chemical mutagenesis have been determined. There appears to be no distinct correlation between growth characteristics and pathogenicity on wheat, the model system used for screening. For example, isolates which never produced lobed hyphopodia were just as pathogenic as the parent strain. Also, fast-growing strains were both pathogenic and non-pathogenic. In general, strains that were pathogenic on wheat in the wheat/vermiculite assay had colonized the entire root system. Strains that appeared to be non-pathogenic were detected, using culture media, only in the root segment closest to the crown of the plant (0-2.5 cm). These results were surprising since two of these isolates were fast growing strains. We are now preparing to use the PCR assay (a DNA detection system) with root tissue to determine if these results are confirmed. We also need to determine if a longer growing period is necessary to obtain full colonization of the root system with these isolates.

The one test completed to date with the topsoil/bermudagrass assay was inconclusive due to the lack of consistent regeneration of the root system from the sprigs. This assay is being repeated using a different source of sprigs. We have also learned that viability of the inoculum must be checked immediately prior to its use in the assay as the ryegrass inoculum may lose its viability quickly, depending on the strain. Results from a strain storage experiment have indicated that viability is maintained the best on potato-dextrose agar slants stored at 28 C. Ryegrass inoculum was the next best storage system.

Further experiments will be conducted to confirm non-pathogenicity of specific strains, the mechanism(s) for this characteristic, and its utility in the golf course environment.

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INTRODUCTION

At least six turfgrass patch diseases are caused by soilborne fungi with dark-pigmented (melanized) hyphae and an ectotrophic growth habit on roots. These diseases include summer patch and necrotic ring spot of Kentucky bluegrass, take-all patch of bentgrass, spring dead spot and bermudagrass decline of bermudagrass, and take-all root rot of St. Augustinegrass. The causal agents of these diseases in the United States are Magnaporthe poae, Leptosphaeria korrae, Ophiosphaerella herpotricha, Gaeumannomyces graminis var. graminis, and G. graminis. var. avenae. Although G. incrustans is often isolated from the same symptomatic plant as the other fungi, it does not appear to be a primary pathogen. G. g. graminis is the pathogen associated with the root rot diseases on bermudagrass and St. Augustinegrass grown in the southern United States. These root rot diseases continue to be the most difficult turfgrass diseases to control.

All of these fungi are ectomycorrhizal and considered to be poor saprophytes. This means they actively colonize roots and require living roots to maintain adequate inoculum levels for disease development. For vegetatively-propagated turfgrass like hybrid bermudagrass, the pathogen, if present in the sod fields, will be moved with the turfgrass to the new planting location. If the new planting location is a recently fumigated area (putting green, tee, athletic field, etc.), this provides the pathogen with the means of spreading quickly since natural antagonists (bacteria, fungi, amoeba, nematodes) have been reduced significantly by fumigation.

One of the best methods for control, in both economic and ecological terms, would be to introduce a biological control agent into the new planting location, both in the sod field and the golf course. For areas already infested with the pathogen, the agents could be introduced in the material used for topdressing. Any organism that could occupy the same niche as the pathogen should be a viable candidate for biological control. One such group of organisms would be mutants of the pathogens that have been rendered non-pathogenic. The primary objective of this project was to develop and evaluate mutants of G. g. graminis for control of turfgrass patch diseases.

MATERIALS AND METHODS

G. g. graminis isolate FL-39 was selected for use in this project. It was originally isolated from a St. Agustinegrass sod production field in southern Florida but has been shown to be pathogenic on both bermudagrass and

rice. It is one of the few isolates that has consistently and readily produced perithecia (sexual stage) with viable ascospores in vitro. It also readily produces lobed hyphopodia in culture. We have determined that we can separate this isolate from G. g. graminis isolates originating from bermudagrass based on an assay using the polymerase chain reaction which amplifies fungal DNA.

Using methods described in previous reports, 170 "presumed" mutants, hereafter referred to as strains, were obtained. All strains were evaluated for growth on potato-dextrose agar (PDA), Czapeks agar and Czapeks agar supplemented with yeast extract. Czapeks agar is a defined growth medium with minimal growth components. Strains were also screened for pathogenicity using an in vitro method with wheat as the host.

The twelve strains selected for further evaluation (Table 1) were obtained using protoplasts. Two strains were not subjected to the chemical mutagen but the remaining ten were obtained after incubating protoplasts in 25 or 12.5 ug ml^{-1} methyl-N-nitro-N-nitrosoguanidine (MNNG) prior to regeneration. Regenerated colonies were immediatedly sub-cultured to PDA and then transferred to PDA slants for storage at 2-4 C and to 100% glycerol tubes for storage at -70 C.

The twelve strains selected and the parent FL-39 isolate have been subjected to the following tests thus far:

1) growth rate on PDA (4 tests)
2) water agar/wheat seedling assay (3-4 tests)
3) wheat/vermiculite assay (4 tests)
4) bermudagrass/topsoil assay (1 test)
5) storage evaluation

Growth rate on PDA: Agar plugs (5-mm diameter) colonized with the strains are transferred to PDA plates, 3 or 4 plates (replicates) per strain, and grown at 28 C. Radial growth is determined after 3, 4 and 5 days of growth.

Water agar/wheat seedling assay: Surface-sterilized wheat seeds are germinated on 1.5% water agar. After 3 days, agar plugs colonized with the strains are placed next to the germinated seed. At least six germinated seeds are inoculated per strain. Plates are sealed and incubated for 21 days at 25 C with 12 hours light. Plants are evaluated for disease using the following scale: 1=plants healthy with white roots; 2=plants healthy but roots discolored (tan not white); 3=majority of roots black in color, basal stem white, <50% chlorotic leaves; 4=all roots black in color, basal stem black, 50-75% chlorotic or necrotic leaves; 5=roots and basal stem black, >75% chlorotic or necrotic leaves.

Wheat/vermiculite assay: Wheat from surface-sterilized seed is grown in 2.5-cm by 18-cm tapered plastic Conetainers™. Cones are filled 75% full with horticultural grade vermiculite (nonsterile) and moistened with deionized water. A 20-mm diameter colonized agar plug is placed on top of the vermiculite, mycelia side up. Check treatment is a plain, uncolonized agar plug. Two wheat seeds are placed on the agar plug and then covered with more

vermiculite. Each treatement (strains and check) has four cones (replicates) and are arranged in a randomized complete block design in a cone rack. The rack is placed in the greenhouse (25-35 C) and plants provided with 10 ml deionized water every day. After 21 days, plants are harvested and roots evaluated for disease based on the following scale: 1=completely white roots; 2=<25% of roots with black lesions; 3=26-50% of roots with black lesions; 4=51-75% of roots with black lesions; 5=76-100% of roots with black lesions.

After evaluation, roots are then used for reisolation of strains using PDA supplemented with 100 ug ml^{-1} each streptomycin and rifampicin (PDASR). For selected strains, roots are cut into four 2.5-cm segments before placement on PDASR to determine location of strain on roots.

Bermudagrass/topsoil assay: Ryegrass inoculum is made using perennial ryegrass and deionized water (2:1/v:v) mixed in a flask and autoclaved on two consecutive days for 90 min each day. Flasks are inoculated with appropriate strain and incubated 4 weeks. Infested ryegrass is then dried for 5-7 days, assayed for viability, placed in a self-seal plastic bag and then placed in a heat-sealed bag stored at room temperature. This is the only way we can keep fungal mites from invading our inoculum!

Topsoil mix, composed of 80% sand and 20% Canadian peat moss (pH 5.8), is autoclaved for 90 min prior to filling Conetainers described previously. A 2-cm deep layer of ryegrass inoculum of the appropriate strain is placed in each cone and covered with more topsoil. Check treatment is autoclaved, non-infested ryegrass. Each cone is planted with one sprig of Tifgreen (Tif 328) hybrid bermudagrass. Each sprig has a minimum of one node and 3-6 leaves. There are no roots as we are using aerial sprigs - i.e. sprigs are derived from runners growing over the side of 4-inch pots planted with Tifgreen.

Storage evaluation: As stated previously, when the strains were first obtained from regeneration plates, they were stored on PDA slants at 2-4 C and in 100% glycerol at -70 C. When we began to evaluate the twelve strains listed in Table 1, fresh material was obtained from storage. We could not recover all strains from the PDA slants. Those not recovered from PDA slants were recovered from the glycerol tubes. Thus, a preliminary experiment was established to determine the best way to store these strains. In February 1993, strains were stored on new PDA slants at 2-4 C, PDA slants at 28 C, PDA slants covered with sterile mineral oil (after the strain had grown over the slant) at 2-4 C and as ryegrass inoculum at room temperature. After six months, transfers were made from each slant, the ryegrass inoculum and the glycerol tubes to determine viability under each of these conditions.

RESULTS AND DISCUSION

Characteristics of the G. g. graminis FL-39 parent isolate and the twelve strains obtained via protoplasting and/or chemical mutagenesis are summarized in Table 1. There appears to be no distinct correlation between growth characteristics and pathogenicity on wheat. Isolates which never produced lobed hyphopodia were just as pathogenic as the parent strain. Fl-39-106 and FL-39-150 were fast growing strains but were non-pathogenic in both wheat assays. In general, strains that were pathogenic on wheat in the

wheat/vermiculite assay had colonized the entire root system. Apparently non-pathogenic strains (FL-39-106, -111 and -150 were detected (on PDASR media) only in the root segment closest to the crown of the plant (0-2.5 cm). We are now preparing to use the PCR assay with root tissue to determine if these results are confirmed. The results with PDASR were surprising since two of these isolates were fast growing strains. We also need to determine if a longer growing period is necessary to obtain full colonization of the root system with these isolates.

The one test completed with the topsoil/bermudagrass assay was inconclusive due to the lack of consistent regeneration of the root system from the sprigs. This assay is being repeated using a different source of Tifgreen sprigs. We have also learned that viability of the inoculum must be checked immediately prior to its use in the assay as the ryegrass inoculum may lose its viability quickly, depending on the strain.

Results from the storage experiment is presented in Table 2. The best storage conditions were PDA slants maintained at 28 C and ryegrass inoculum stored at room temperature. Based on these results, we are using the strains recovered in August 1993 as our known strains and repeating all assays. Strains will be stored with ten different methods and assayed every six months for two years to determine the best storage method. Since they all have slightly different morphological and physiological characteristics, we can also determine the effects of storage on these strains.

SUMMARY

Experiments will continue to determine if strains FL-39-106, -111 and -150 are truly non-pathogenic and why they are non-pathogenic. If they are non-pathogenic because they do not colonize roots (even after a long incubation period), then there may be no need to continue the study. If they are truly non-pathogenic and do colonize roots, then studies will begin to determine if they can be used as biological control agents. Remember that the goal of this project was to develop a non-pathogen from a pathogen in the belief that the non-pathogen would occupy the same niche as the pathogen and thereby exclude the pathogen from the root system. One method for their utilization would be to inoculate soil prior to planting, but that would require producing large amounts of inoculum. It is highly unlikely this could be commercialized.

A more viable scenario would be to develop a 'cultivar' already colonized with the biological control agent. This would be similar to the use of endophyte-containing seed. In this manner, the biological control agent would already be intimately associated with the target host at the time of planting, both in the sod fields and the golf courses.

Table 1. Characteristics of Gaeumannomyces graminis var. graminis FL-39 strains.

Strain	Mutagen[a] Rate	Growth on Czapeks[b] +YE	Growth on Czapeks[b] -YE	Lobed Hyphopodia[c]	Perithecia[d]	Growth Rate on PDA (mm per day)[e]	Wheat Root Disease Rating water agar[f]	Wheat Root Disease Rating vermiculite[g]
FL-39 (parent strain)		4	4	+	+	5.5	4.6	3.9
FL-39-21	0	4	4	-	-	6.0	4.7	3.3
FL-39-26	0	3	3	-	-	5.4	4.7	3.5
FL-39-70	25.0	4	2	+	-	4.9	3.7	3.3
FL-39-71	12.5	4	3	+	+	5.1	4.5	4.4
FL-39-100	12.5	4	2	+	+	4.7	4.7	3.6
FL-39-106	12.5	4	2	+	-	5.8	1.7	1.1
FL-39-111	25.0	NT	NT	+	-	3.4	2.1	1.2
FL-39-114	25.0	NT	NT	+	-	2.1	2.9	3.2
FL-39-131	12.5	NT	NT	+	-	5.3	4.0	4.7
FL-39-137	12.5	4	2	+	+	2.7	3.4	3.3
FL-39-150	12.5	3	2	+	-	4.9	1.3	1.5
FL-39-154	12.5	4	2	+	-	4.6	4.6	2.2
Check	----	NA	NA	NA	NA	NA	1.0	1.0

[a]Mutagen was methyl-N-nitro-N-nitrosoquanidine (MNNG). Values are ug ml^{-1}.

[b]With (+YE) and without (-YE) yeast extract added to Czapeks medium; Rating: 1=20%, 2=40%, 3=60%, 4=80%, and 5=100% of parent FL-39 strain's growth on potato-dextrose agar.

[c]'+'=present; '-'=absent; FL-39-106 and -111 had odd-shaped hyphopodia; FL-39-111 also had few hyphopodia.

[d]'+'=present in at least one of the water agar/wheat assay tests; FL-39-100 had a very limited number of perithecia.

[e]Average across four tests with three or four replicates per strain per test.

[f]For each strain, average across three or four tests with minimum of six plants per test. See text for details on disease rating with range of 1=healthy plant and 5=dead plant.

[g]Average across four tests with four replicates per strain per test. See text for details on disease rating with range of 1=healthy roots and 5=black, rotted roots.

Table 2. Recovery from storage of Gaeumannomyces graminis var. graminis FL-39 strains in September 1993.

Strain	Mutagen Rate[a]	Original PDA Slants @ 2 C	PDA Slants @ 2 C[b] w/o Oil	PDA Slants @ 2 C[b] w/ Oil	PDA Slants[b] @ 28 C	Original Glycerol @ -70 C	Ryegrass Inoculum[c]
FL-39 (parent strain)		-	-	-	+	-	+
FL-39-21	0	-	+	+	+	+	+
FL-39-26	0	-	+	+	+	+	+
FL-39-70	25.0	-	-	-	+	+	-
FL-39-71	12.5	-	-	+	+	-	+
FL-39-100	12.5	-	-	-	+	-	+
FL-39-106	12.5	-	-	-	+	-	+
FL-39-111	25.0	-	-	+	+	-	+
FL-39-114	25.0	-	-	+	+	-	-
FL-39-131	12.5	-	-	+	+	+	-
FL-39-137	12.5	-	-	+	+	+	+
FL-39-150	12.5	-	-	+	+	-	-
FL-39-154	12.5	-	-	+	+	-	+

[a]Mutagen was methyl-N-nitro-N-nitrosoquanidine (MNNG). Values are ug ml^{-1}.

[b]Slants inoculated March 1993.

[c]Ryegrass inoculated March 1993, incubated for 4 weeks, dried and stored in sealed bags at room temperature. All inoculum was viable at the end of the 4 week incubation period.