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TITLE: Pathogenicity and Biological Control of *Gaeumannomyces*-like Fungi

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PROJECT: Pathogenicity and Biological Control of Gaeumannomyces-like Fungi

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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 included 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. Gaeumannomyces graminis var. graminis is associated with the diseases on bermudagrass and St. Augustinegrass grown in the southern United States.

All of these fungi are ectomycorrhizal which means they colonize roots and therefore move with the roots. For vegetatively-propagated turfgrass, 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 prior to planting. 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 Gaeumannomyces graminis var. graminis for control of turfgrass patch diseases.

A total of 170 "presumed" mutants of parent strain G. g. graminis FL-39 have been obtained thus far, primarily using the mutagen N-methyl-N-nitro-N-nitrosoguanidine (MNNG). Concerning pathogenicity, 135 "mutants" were still as pathogenic in vitro as the parent FL-39 strain. Two "mutants" were not pathogenic, seven "mutants" were intermediate in pathogenicity and two "mutants" were slightly less pathogenic than the parent strain. All of the non-pathogenic and intermediate-pathogenic "mutants" had also lost the ability to consistently produce perithecia.

Fifteen "mutants" of FL-39 have been selected for testing in vitro. These are currently being grown on sterile ryegrass seed for use as inoculum sources. All fifteen isolates have been stable in storage and are growing as rapidly on the ryegrass seed as the parent strain. This inoculum will be used in three different methods for evaluation of biological control activity: 1) Simultaneous inoculation of sterilized topsoil mix with a "mutant" and a pathogenic strain of G. g. graminis prior to planting with a pathogen-free bermudagrass sprig; 2) Inoculation of sterilized topsoil mix with a "mutant" two weeks prior to infestation with a pathogenic strain and planting with a pathogen-free bermudagrass sprig; 3) Inoculation of sterilized topsoil mix with a "mutant" followed by planting G. g. graminis infected bermudagrass plants.

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INTRODUCTION

When this project first began, there were five turfgrass patch diseases caused by soilborne fungi with dark-pigmented (melanized) hyphae and an ectotrophic growth habit on roots. These diseases included summer patch and necrotic ring spot of Kentucky bluegrass, spring dead spot and bermudagrass decline of bermudagrass and take-all patch of bentgrass. A sixth disease was recently added to the list - take-all root rot of St. Augustinegrass. The cause of a patch disease on zoysiagrass in the Midwest may also be caused by this group of fungi. The causal agents of these diseases are Magnaporthe poae, Leptosphaeria korrae, L. narmari, 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.

These root rot diseases continue to be the most difficut turfgrass diseases to control. All of these fungi are ectomycorrhizal which means they colonize roots and therefore move with the roots. For vegetatively-propagated turfgrass, 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, athletic field, lawn, etc.), this provides the pathogen with the means of spreading quickly since natural antagonists (bacteria, fungi, amoeba, nematodes) have been eliminated by the 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 prior to planting. 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 Gaeumannomyces graminis var. 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 also learned this past year that we can separate this isolate from G. g. graminis isolates originating from bermudagrass based on an assay using the polymerase chain reaction. G. incrustans isolate FL-32 was also mutagenized a limited number of times.

Four methods are being used to obtain mutants. One method requires mutagenizing the culture and then obtaining and regenerating protoplasts. The G. g. graminis isolate is grown in Luria Bertani medium (LB) for 4 days with shaking (100 rpm) at room temperature (approximately 25 C). The mutagen, N-methyl-N-nitro-N-nitrosoguanidine (MNNG), is added to the broth culture and allowed to incubate for 3 more hours. Rates vary from 50 ug ml^{-1} to 3 ug ml^{-1}. The culture is then collected by centrifugation, washed in phosphate buffer and placed in fresh LB for growth overnight. Cultures are protoplasted the next morning and the protoplasts placed in a Czapeks broth, supplemented with yeast extract and sorbitol, for growth overnight at a very low shaker speed (40 rpm) at room temperature. Protoplasts are then collected and placed on a regeneration medium for Gaeumannomyces. Plates are incubated at 28 C in the dark. As soon as colonies develop, they are sub-cultured to potato-dextrose agar (PDA).

The second method simply requires obtaining and regenerating protoplasts of the isolate with no mutagen added at any point in the growth cycle. In a third method, the cultures are not protoplasted after mutagenizing but are homogenized, centrifuged and mycelia placed in fresh LB and then plated on regeneration medium the next morning. For both methods, colonies that develop are sub-cultured to PDA.

The fourth method requires growing the isolate in potato-dextrose broth for four days, homogenizing the growth and plating 0.1 ml of the homogenate on PDA supplemented with either 100 mg L^{-1} cycloheximide or 100 mg L^{-1} benomyl (a.i.). Plates are incubated at 28 C in the dark. As soon as colonies develop, they are sub-cultured to PDA.

After "mutants" have grown sufficiently on PDA, they are transferred to PDA slants for storage at 2-4 C and to 100% glycerol tubes for storage at -70 C. They are also transferred to three media for evaluation of growth - fresh PDA, Czapeks Agar, Czapeks Agar supplemented with yeast extract. Cultures are incubated at 28 C for 11 days and growth evaluated at that time. Parent strain FL-39 is always used as the check treatment.

"Mutants" are initially screened for pathogenicity using an in vitro method. Surface-sterilized wheat seeds (3 per plate) are germinated on water agar (1.5%). After 3 days, mycelial agar plugs of the "mutant" being evaluated are then placed next to the seedling (1 per seedling). The plates are then sealed and placed in a 25 C incubator with lights (12 hours) for 21 to 28 days. Plants are then evaluated for disease on a scale of 1 to 5 with 1 equal to a healthy green plant with white roots and 5 equal to a dead or nearly dead plant with rotted, black roots.

RESULTS AND DISCUSION

Our initial elation in quickly obtaining presumed "mutant" colonies was just as quickly doused when we developed a contamination problem that required almost two months to eliminate. The primary contamination source appeared to be the new glass wool used to separate mycelia from protoplasts in the last step of the procedure. By autoclaving the glass wool for 60-90 minutes rather than the normal 20 minutes, we were finally able to move forward with the experiments.

G. g. graminis

A total of 170 "presumed" mutants of parent strain FL-39 have been obtained thus far. The method using regrowth of homogenated mycelia on media with cycloheximide or benomyl has not produced any mutants. However, we have only recently started utilizing this method. Using the protoplast without mutagen method, 32 "mutants" have been obtained. The non-protoplast with mutagen method has produced 11 "mutants". With the protoplast plus mutagen (various rates) method, 127 "mutants" have been obtained. Only 5 "mutants" were obtained when the full mutagen rate of 50 ug ml^{-1} was used. Complete results from growth and pathogenicity tests are presented in Table 1.

Twenty-eight of the first 30 "mutants" produced were obtained by simply protoplasting the parent strain FL-39. None of the "mutants" produced perithecia and only two produced normal hyphopodia, but all were still pathogenic in vitro. The remaining "mutants" obtained, using the various methods, all had normal hyphopodia, although one produced a very limited number in comparison to the parent strain. We cannot explain why the loss of hyphopodia only occurred in our first few experiments.

Concerning pathogenicity, 135 "mutants" were still as pathogenic in vitro on wheat as the parent FL-39 strain (rating of 5). These mutants still produced perithecia, although three "mutants" had slightly abnormal perithecia. Two "mutants" were not pathogenic (rating of 1 or 2), seven "mutants" were intermediate with a rating of 3 and two "mutants" had pathogenicity ratings of 4. All of the "mutants" with pathogenicity ratings of 1, 2 or 3 and one of the "mutants" with a rating of 4 had also lost the ability to consistently produce perithecia. This inability to produce perithecia is a good one from an environmental standpoint if these strains are to be released in a natural environment. However, it will impede our ability to do genetic crosses to locate the gene(s) responsible for pathogenicity. The remaining "mutants" were not tested for pathogenicity because they were unstable in culture.

G. incrustans

Fifty "mutants" of parent strain FL-32 were obtained using the protoplast with mutagen method. None of the isolates appeared to be any

different than the parent strain. Thus, FL-32 will be used to evaluate its biological control potential rather than the "mutants".

Current Experiments

Fifteen "mutants" of FL-39 have been selected for testing in vitro. These are currently being grown on sterile ryegrass seed for use as inoculum sources. All fifteen isolates have been stable in storage and are growing as rapidly on the ryegrass seed as the parent strain. This inoculum will be used in three methods for evaluation of biological control activity. The pathogenic G. g. graminis isolate to be used in these experiments will be FL-19, an isolate obtained from bermudagrass which has been consistently pathogenic. This will allow us to separate the pathogen from the "mutant" based on the polymerase chain reaction assay that has been developed for G. graminis. We have not tested all the FL-39 "mutants", but those that have been tested have retained their characteristic DNA bands which are significantly different from the bands produced by FL-19.

The three biological control evaluation methods include:

1) Simultaneous inoculation of sterilized topsoil mix with a "mutant" and FL-19 prior to planting with a G. g. graminis-free bermudagrass sprig;

2) Inoculation of sterilized topsoil mix with a "mutant" two weeks prior to infestation with FL-19 and planting with a G. g. graminis-free bermudagrass sprig;

3) Inoculation of sterilized topsoil mix with a "mutant" and then planting G. g. graminis FL-19 infected bermudagrass plants.

These experiments will also be repeated using G. incrustans isolate FL-32.

SUMMARY

Presumed "mutants" of G. g. graminis were obtained using three of the four methods described. From the 170 "mutants" obtained, 15 have been selected for evaluation as biological control agents against pathogenic strains of G. g. graminis. Those experiments are currently in progress.

Table 1. Characteristics of presumed "mutants" of Gaeumannomyces graminis var. graminis isolate FL-39.

"Mutant"	Mutagen[a] Rate	Growth on Czapeks[b] + YE	Growth on Czapeks[b] - YE	Disease[c] Rating	Hyphopodia[d]	Perithecia[e]
1a	Full	4	4	5	+	-
1b	0	4	2.5	5	-	-
2a	Full	4	4	5	+	-
2b	0	4	2.5	5	-	-
3	0	4	2.5	5	+?	-
4	0	4	4	5	+	+?
5	0	4	4	5	-	-
6	0	4	4	5	-	-
7	0	4	4	5	-	-
8	0	4	4	5	-	-
9	0	4	4	5	+	-
10	0	4	4	5	+?	+?
11	0	4	4	5	-	-
12	0	4	4	5	+?	-
13	0	4	4	5	-	-
14	0	4	4	5	+?	-
15	0	4	4	5	-	-
16	0	4	4	5	-	-
17	0	4	4	5	-	-
18	0	4	4	5	-	-
19	0	4	4	5	-	+?
20	0	4	4	5	+?	-
21	0	4	4	5	-	-
22	0	4	4	5	-	-
23	0	4	4	5	-	-
24	0	2.5	2.5	5	-	-
25	0	2.5	2.5	5	-	-
26	0	2.5	2.5	5	-	-
27	0	2.5	2.5	5	-	+?
28	0	2.5	2.5	5	-	-
29	Full	2/3	2/3	5	+	+
30	1/16	4	2	5	+	+
31	1/16	3	2	5	+	+
32	1/16	3	2	5	+	+
33	1/16	3	2	5	+	+
34	1/16	4	2	5	+	+
35	1/16	3	2	NT[f]	NT	NT
36	1/16	4	2	NT	NT	NT
37	1/16	3	2	5	+	+
38	1/16	4	2	NT	NT	NT
39	1/16	4	3	5	+	+
40	1/16	3	2	NT	NT	NT
41	1/16	3	3	5	+	+
42	1/16	4	2	NT	NT	NT
43	1/16	3	2	5	+	+
44	1/16	3	2	5	+	+

Table 1. Continued

"Mutant"	Mutagen[a] Rate	Growth on Czapeks[b] + YE	Growth on Czapeks[b] - YE	Disease[c] Rating	Hyphopodia[d]	Perithecia[e]
45	1/16	4	2	5	+	+
46	1/16	5	4	5	+	+
47	0	3	1	NT	NT	NT
48	0	2	1	3	+	-
49	0	3	2	NT	NT	NT
50	0	2	0	3	+	-
51	1/4 NP	2	3	5	+	+
52	1/4 NP	4	2	5	+	+
53	1/4 NP	5	2	NT	NT	NT
54	1/8 NP	4	2	5	+	+
55	1/8 NP	4	2	NT	NT	NT
56	1/8 NP	2	2	NT	NT	
57	1/8 NP	3	2	NT	NT	NT
58	1/16 NP	3	1	NT	NT	NT
59	1/16 NP	2	1	NT	NT	NT
60	1/16 NP	2	1	NT	NT	NT
61	1/16 NP	2	1	5	+	+
62	1/16	4	3	5	+	+
63	1/16	4	4	5	+	+
64	1/16	5	4	NT	NT	NT
65	1/16	4	4	NT	NT	NT
66	1/16	5	3	NT	NT	NT
67	1/16	4	4	NT	NT	NT
68	1/16	4	4	NT	NT	NT
69	1/16	4	4	5	+	+
70	1/2	4	2	3.5	+	-
71	1/4	4	3	4	+	+
72	1/4	4	2	NT	NT	NT
73	1/4	4	1	NT	NT	NT
74	1/4	4	1	5	+	+
75	1/4	4	1	5	+	+
76	1/4	4	1	5	+	+
77	1/4	4	2	5	+	+
78	1/4	4	1	5	+	+
79	1/4	4	1	5	+	+
80	1/8	4	1	5	+	+
81	1/8	4	1	5	+	+
82	1/8	4	1	5	+	+
83	1/8	4	1	5	+	+
84	1/8	4	1	5	+	+
85	1/4	4	1	5	+	+
86	1/4	4	1	5	+	+
87	1/4	3	2	5	+	+
88	1/4	4	1	5	+	+
89	1/4	4	1	5	+	+
90	1/4	4	1	5	+	-

Table 1. Continued

"Mutant"	Mutagen[a] Rate	Growth on Czapeks[b] + YE	Growth on Czapeks[b] - YE	Disease[c] Rating	Hyphopodia[d]	Perithecia[e]
91	1/4	4	1	5	+	+
92	1/4	4	2	5	+	+
93	1/4	4	1	5	+	+
94	1/4	4	2	5	+	+
95	1/4	4	1	5	+	+
96	1/4	4	1	5	+	+
97	1/4	4	1	5	+	+
98	1/4	4	1	5	+	+
99	1/4	4	2	5	+	+
100	1/2	4	2	5	+	+
101	1/2	4	2	5	+	+
102	1/4	4	2	5	+	-
103	1/4	4	2	5	+	+
104	1/4	4	2	5	+	+
105	1/4	4	2	5	+	+
106	1/4	4	2	2	+	-
107	1/2	4	2	5	+	+
108	1/2	4	2	5	+	+
109	1/2	4	2	5	+	+
110	1/2	3	2	5	+	+
111	1/2	NT	NT	3	+	-
112	1/2	3	2	5	+	+
113	1/2	4	2	5	+	+
114	1/2	NT	NT	3	+	-
115	1/2	3	2	5	+	+
116	1/2	4	2	5	+	+
117	1/2	4	1	5	+	+
118	1/2	NT	NT	5	+	+
119	1/2	4	2	5	+	+
120	1/2	3	2	5	+	+
121	1/2	4	2	5	+	+
122	1/2	3	2	5	+	+
123	1/2	4	2	5	+	+
124	1/2	4	2	5	+	+
125	1/2	3	2	5	+	+
126	1/2	NT	NT	5	+	+
127	1/2	NT	2	5	+	+
128	1/4	4	2	5	+	+
129	1/4	4	2	5	+	±
130	1/4	NT	NT	5	+	+
131	1/4	NT	NT	3	+	-
132	1/4	NT	NT	5	+	+
133	1/4	NT	NT	5	+	+
134	1/4	NT	NT	5	+	+
135	1/4	4	2	5	+	+
136	1/4	NT	NT	5	+	+

Table 1. Continued

"Mutant"	Mutagen[a] Rate	Growth on Czapeks[b] + YE	Growth on Czapeks[b] - YE	Disease[c] Rating	Hyphopodia[d]	Perithecia[e]
137	1/4	4	2	3	+	+
138	1/4	4	2	5	+	+
139	1/4	4	2	5	+	+
140	1/4	4	2	5	+	+
141	1/4	4	2	5	+	+
142	1/4	4	2	5	+	+
143	1/4	NT	NT	5	+	+
144	1/4	NT	NT	5	+	+
145	1/4	NT	NT	5	+	+
146	1/4	4	2	5	+	-
147	1/4	4	2	5	+	+
148	1/4	NT	NT	5	+	-
149	1/4	4	2	5	+	+
150	1/4	3	2	1	+	-
151	1/4	3	2	5	+	+?
152	1/4	3	2	5	+	+
153	1/4	3	2	5	+	+
154	1/4	4	2	4	+	-
155	1/4	4	2	5	+	+
156	1/4	3	1	5	+	+
157	1/4	3	2	5	+	+
158	1/4	4	2	5	+	+
159	1/4	4	2	5	+	+
160	1/2	4	2	5	+	+
161	1/2	NT	NT	5	+	+
162	1/2	NT	NT	5	+	+
163	1/2	3	2	5	+	+
164	1/2	NT	NT	5	+	+
165	1/2	NT	NT	5	+	+
166	Full	4	2	NT	NT	NT
167	Full	4	2	NT	NT	NT
168	Full	4	2	NT	NT	NT

[a]Full mutagen rate equals 50 ug ml^{-1}; NP = mycelia not protoplasted.
[b]With (+YE) and without (-YE) yeast extract added to basal Czapeks medium; Rating: 1 = 20%; 2 = 40%; 3 = 60%; 4 = 80% and 5 = 100% of parent strain's growth on potato-dextrose agar.
[c]Disease Rating: 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.
[d]"+" = present; "-" = absent; "+?" = unclear, appear to be abnormal in shape.
[e]"+" = present; "-" = absent; "+?" = appear abnormal.
[f]NT = Not tested.