Project: Pathogenicity and Biological Control of Gaeumannomyces-like Fungi

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At least five 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, spring dead spot and bermudagrass decline of bermudagrass and take-all patch of bentgrass. The causal agents of these diseases are Magnaporthe poae, Leptosphaeria korrae, L. narmari, Ophiosphaerella herpotricha, Gaeumannomyces graminis var. graminis, and G. graminis var. avenae. DHN (1,8-dihydroxynaphthalene) melanin is the most common fungal cell wall melanin. Inhibition of the production of DHN melanin has been demonstrated to be a disease control method, primarily with the plant pathogens Pyricularia oryzae and Colletotrichum spp. In addition, melanin deficient mutants of these species are capable of colonizing plant tissue but can not penetrate the plant tissue.

The two objectives of this project were to: 1) develop a model system for determining the relationship between melanization of fungal structures and pathogenicity (ability to cause disease) of Gaeumannomyces species and related fungi; and 2) determine the biological control potential of non-pathogenic mutant strains of Gaeumannomyces fungi for control of turfgrass patch diseases.

Compounds which inhibit DHN melanin were evaluated in the laboratory for their ability to inhibit the growth of the fungi and to inhibit their ability to cause disease. The results indicate that the melanin in G. graminis var. graminis, G. incrustans and Magnaporthe poae is DHN melanin. However, inhibition of melanin production does not appear to inhibit their ability to cause disease.

Seventy-five mutant strains of G. graminis var. graminis have been obtained. Twenty-nine have been evaluated for their ability to cause disease. All strains were still pathogenic. However, their ability to produce the sexual spores of this fungus and a structure called a hyphopodia were severely impaired. The remaining strains must be evaluated before we will know if this will be a viable method for obtaining biological control of patch diseases.

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INTRODUCTION

At least five 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, spring dead spot and bermudagrass decline of bermudagrass and take-all patch of bentgrass. The causal agents of these diseases are Magnaporthe poae, Leptosphaeria korrae, L. narmari, Ophiosphaerella herpotricha, Gaeumannomyces graminis var. graminis, and G. graminis var. avenae. The status of G. incrustans as a primary or secondary pathogen of patch diseases is still being evaluated.

DHN (1,8-dihydroxynaphthalene) melanin, synthesized from pentaketides, is the most common fungal cell wall melanin. Inhibition of the production of DHN melanin has been demonstrated to be a disease control method, primarily with the plant pathogens Pyricularia oryzae and Colletotrichum spp. In addition, melanin deficient mutants of these species are capable of colonizing plant tissue but can not penetrate the plant tissue.

The two objectives of this project were to: 1) develop a model system for determining the relationship between melanization of fungal structures and pathogenicity of Gaeumannomyces species and related fungi; and 2) determine the biological control potential of non-pathogenic mutant strains of Gaeumannomyces fungi for control of turfgrass patch diseases.

METHODS, RESULTS and DISCUSSION

I. In Vitro Effect of DOPA and DHN Melanin Inhibitors

DHN melanin is the most likely fungal melanin to be associated with the cell walls of Gaeumannomyces species. DOPA (dihydroxyphenylalanine) melanin would be the next possible type of melanin, although it has normally been associated with extracellular components. Compounds which inhibit formation of DOPA melanin are tyrosinase enzyme inhibitors. Four of these inhibitors were evaluated for in vitro melanin inhibition - kojic acid, tropolone, 2-mercaptobenzothiazole and diethyldithiocarbamic acid. These compounds were incorporated into potato-dextrose agar supplemented with 100 ug/ml streptomycin sulfate (PDAS) at the rates of 1, 10 and 100 ug/ml. A 5-mm-diameter mycelial agar plug of each isolate was placed on each medium with three replicates per isolate. The control was non-supplemented PDAS. Plates were incubated at 28 C. Radial growth from the plug was determined after 5 and 10 days. Also at 10 days, the color of the

mycelia associated with each treatment was compared to the control. The experiment was repeated two times. Results are reported in Table 1.

Compounds which inhibit formation of DHN melanin include tricyclazole, pyroquilon, chlobenthiazone and fthalide. These compounds are currently used commercially in Japan for control of rice blast caused by P. oryzae. A preliminary experiment with four fungal isolates used 1, 10 and 100 ug/ml of each compound incorporated into PDAS. Only technical material, >95% active ingredient, was used in all experiments described. Based on those results, the first three compounds were tested further at only 1 and 10 ug/ml and fthalide was tested at all three levels. The control was non-supplemented PDAS. Inoculation, incubation and evaluation were conducted the same as for the tyrosinase inhibitors. The experiment was repeated three times. Results are reported in Tables 2 and 3.

Except for 2-mercaptobenzothiazole at 10 and 100 ug/ml and tropolone at 100 ug/ml, the tyrosinase inhibitors did not significantly effect growth of the fungal isolates evaluated. None of these compounds produced a change in pigment coloration. The DHN melanin inhibitors at 10 ug/ml had minimal effect on growth. Melanin production for all fungal isolates evaluated was inhibited at 10 ug/ml of tricyclazole, chlobenthiazone and pyroquilon. Fthalide at the same rate inhibited melanin production of G. graminis var. graminis and four of the six G. incrustans isolates but not M. poae. At 100 ug/ml, fthalide still did not inhibit melanin production of the M. poae isolates. Melanin inhibition was observed as a reddish-brown pigment instead of a black pigment indicating an accumulation of the intermediate metabolites in the DHN melanin biosynthesis pathway. Therefore, if would appear that the melanin in G. graminis var. graminis, G. incrustans and Magnaporthe poae is DHN melanin and not DOPA melanin.

II. In Vivo Effect of DHN Melanin Inhibitors

Since G. graminis var. graminis, G. incrustans and Magnaporthe poae are consistently pathogenic on wheat, the water agar-wheat assay procedure of Speakman (Trans. Br. Mycol. Soc., 79:350) was used to evaluate the effect of the DHN melanin inhibitors on pathogenicity rather than the more cumbersome and non-sterile container system with topsoil mix. The four DHN melanin inhibitors were incorporated into 1.5% water agar at 10 ug/ml. Check treatment was non-supplemented water agar.

Germinated wheat seed (3 seeds per plate; 2 plates per fungal isolate) were placed on the media and allowed to grow at room temperature in order to allow uptake of the compounds from the media. After 2 days, the plates were inoculated with the appropriate fungal isolate, except for plates with chlobenthiazone. This compound severely inhibited wheat growth. Therefore, it was not evaluated in this particular study. Plates were incubated for 23 days at 25 C with 12 hr light each day. The plants were then rated for disease, and fungal structures were examined microscopically. This experiment was conducted three times. Disease ratings are provided in Table 4.

Minimal differences were observed between treatments. The compounds

did not effect formation of hyphopodia of G. g. graminis nor the production of melanin in the hyphopodia. Perithecia and ascospores produced by isolates 561 and FL-39 were normal. Plates with DHN melanin inhibitors present in the water agar resulted in more consistent production of crusts and larger number of crusts on wheat tissue colonized by G. incrustans. This was more apparent for tricyclazole and pyroquilon than fthalide. In my laboratory, the formation of crusts, which is necessary for identification of this fungus, has not been very consistent. The DHN melanin compounds could be useful in the identification of this fungus and reduce the number of isolates which have often been classified previously as "unkown Gaeumannomyces-like fungus".

One experiment has been completed using tricyclazole, pyroquilon and fthalide at 100 ug/ml, but only with G. graminis var. graminis isolates. Wheat plants were stunted in growth by tricyclazole and pyroquilon but not fthalide. The fungal isolates were also inhibited in growth. In addition, FL-39, in contrast to the check plates, did not produce perithecia on the plates containing DHN melanin inhibitors. However, all six isolates were still pathogenic to the wheat plants.

III. Development of G. graminis var. graminis Mutants

Since the project proposal was written, a new method for obtaining mutants has been developed (R. V. Miller, personal communication) as have new media for regenerating protoplasts. Briefly, isolates of G. graminis var. graminis or G. incrustans are grown in Luria Bertani Medium (LB). The mutagenic compound is added to the broth culture after 4 to 5 days of growth and allowed to incubate for up to 3 hours. The culture is then collected by centrifugation, washed in phosphate buffer and placed in fresh LB for growth overnight. Cultures are protoplasted and protoplasts placed in a Czapeks broth, supplemented with yeast extract and sorbitol, for growth overnight at a very low speed (40 rpm). Protoplasts are then collected and placed on a regeneration medium (J. Phytopathology, 122:146). Regenerated protoplasts are sub-cultured to PDA, Czapeks Agar alone and Czapeks Agar supplemented with yeast extract. Growth is evaluated after 11 days at 28 C. This allows us to quickly characterize the mutant strains in regards to melanin production and nutrient requirements.

The mutagenic compound being utilized currently is N-methyl-N-nitro-N-nitrosoguanidine (MNNG). G. graminis var. graminis isolate FL-39 has been used as the parent isolate as it easily produces perithecia in the water agar-wheat assay. This would be a useful trait if genetic crosses are to be made later. A total of 73 strains have been obtained - 58 strains from protoplast treatment plus mutagen treatment, 11 from mutagen treatment alone and 4 from protoplast treatment alone. A recent paper has indicated that protoplasting alone may cause mutagenesis to occur. Thus, we are now selecting strains from our check plates also.

All mutant strains exhibit reduced growth on the Czapek agar without the yeast extract supplement. This is not too surprising since even the parent strain grows quite slowly in the absence of biotin and thiamine vitamins. On Czapek agar with yeast extract, only 5 mutant strains are

equal in growth to the parent strain. Interestingly, no mutant strains exhibit melanin deficient characteristics.

The first 29 mutant strains have been evaluated for pathogenicity on wheat using our standard water agar assay. All were pathogenic. However, only one strain still produced normal perithecia. Five produced abnormal perithecia and the remaining strains produced no perithecia or perithecial initials at all. Also, the majority of the mutant strains either no longer produced hyphopodia or produced abnormal hyphopodia. Only three strains produced normal hyphopodia. Mutant strain FL-39-29 was the only strain that produced normal perithecia and normal hyphopodia.

The remaining strains will be evaluated for pathogenicity on wheat. In addition, all strains will be evaluated for their response to manganese sulfate when incorporated into the water agar. Preliminary research by Dr. Don Huber at Purdue University indicates that the oxidation and reduction of manganese by G. graminis varieties is correlated with pathogenicity. If this is the case, screening could be greatly simplified. Biological control evaluations will be conducted after all mutant strains have been evaluated in vivo for pathogenicity.

Experiments are currently underway using G. incrustans isolate FL-32 and the mutagen MNNG. In addition, we have obtained a mutagenic compound used in the Institute for Cancer Research program called ICR-170. Its properties are considerably different from those of MNNG and may provide different strain types than have been obtained thus far with G. graminis var. graminis isolate FL-39.

SUMMARY

As is usually the case, more time and funds are required to evaluate all aspects of an evolving research project. However, we have confirmed that DHN melanin is the melanin type present in Gaeumannomyces-like fungi. DHN melanin inhibitors currently used commercially for disease control in rice did not demonstrate disease control under the testing procedure described herein. It would also appear that the hyphopodia of G. graminis var. graminis are not necessary for pathogenicity. Since we have only evaluated 29 mutant strains for pathogenicity, it is not possible to make any conclusions concerning the use of these strains for biological control.

Table 1. *In vitro* growth effect of DOPA melanin (tyrosinase) inhibitors on *Gaeumannomyces*-like species.

	Percentage of Check Growth after 10 Days[a]											
	Kojic Acid			Tropolone			2-mercaptobenthiazole			Diethyldithiocarbamic Acid		
Fungus/Isolate	1[b]	10	100	1	10	100	1	10	100	1	10	100
G. graminis var. *graminis*												
561	100±0[c]	100±0	100±0	100±0	100±0	---[d]	100±0	29±13	--	100±0	100±0	100±0
FL-19	100±0	100±0	100±0	100±0	86±10	---	91±6	7±5	--	100±0	100±0	93±5
FL-36	100±0	100±0	100±0	100±0	82±13	---	100±0	22±15	--	100±0	100±0	93±5
FL-39	100±0	100±0	100±0	100±0	42±0	---	46±29	23±16	--	100±0	100±0	98±2
FL-46	100±0	100±0	100±0	100±0	100±0	---	100±0	34±24	--	100±0	100±0	100±0
ATCC 64419	100±0	100±0	100±0	100±0	57±0	---	100±0	7±5	--	100±0	100±0	93±5
G. incrustans												
FL-28	100±0	100±0	100±0	100±0	100±0	---	100±0	29±20	--	100±0	100±0	100±0
FL-32	100±0	100±0	97±2	100±0	100±0	---	68±15	29±4	--	100±0	100±0	100±0
FL-38	100±0	100±0	100±0	100±0	100±0	---	97±2	23±16	--	100±0	100±0	100±0
ATCC 64416	100±0	100±0	98±1	100±0	77±16	---	94±5	9±6	--	100±0	100±0	100±0
ATCC 64417	100±0	100±0	100±0	100±0	100±0	---	100±0	13±9	--	100±0	100±0	100±0
ATCC 64418	100±0	100±0	100±0	100±0	100±0	---	77±16	13±0	--	100±0	100±0	100±0
Magnaporthe poae												
FL-4	100±0	100±0	100±0	100±0	100±0	---	88±9	38±27	--	100±0	100±0	100±0
ATCC 64413	100±0	100±0	100±0	100±0	100±0	---	98±1	45±31	--	100±0	100±0	100±0

[a] Radial mycelial growth was determined for amended PDAS and those values compared with unamended PDAS, i.e. check growth. Values are an average of the results from two trials of this experiment.
[b] Amount of active ingredient (ug/ml) added to PDAS.
[c] Mean ± standard error.
[d] No growth occurred at this rate.

Table 2. *In vitro* growth effect of melanin inhibitors on *Gaeumannomyces*-like species.

Fungus/Isolate	Percentage of Check Growth after 10 Days[a]							
	Tricyclazole		Chlobenthiazone		Pyroquilon		Fthalide	
	1[b]	10	1	10	1	10	1	10
G. graminis var. *graminis*								
561	100±0	100±0	100±0	83±3	100±0	100±0	100±0	100±0
FL-19	100±0	100±0	100±0	70±9	100±0	100±0	100±0	100±0
FL-36	100±0	100±0	100±0	95±4	100±0	100±0	100±0	100±0
FL-39	95±1	95±4	95±4	95±4	95±4	91±6	95±4	95±4
FL-46	100±0	100±0	100±0	100±0	100±0	100±0	100±0	100±0
ATCC 64419	100±0	100±0	100±0	100±0	100±0	100±0	100±0	100±0
G. incrustans								
FL-28	100±0	100±0	100±0	77±7	100±0	100±0	100±0	100±0
FL-32	100±0	100±0	100±0	86±12	100±0	100±0	100±0	100±0
FL-38	100±0	100±0	100±0	97±4	100±0	100±0	100±0	100±0
ATCC 64416	100±0	100±0	100±0	80±4	100±0	100±0	100±0	100±0
ATCC 64417	100±0	100±0	100±0	95±9	100±0	100±0	100±0	100±0
ATCC 64418	100±0	100±0	100±0	76±0	100±0	100±0	100±0	100±0
Magnaporthe poae								
FL-4	100±0	100±0	100±0	100±0	100±0	100±0	100±0	100±0
ATCC 64413	100±0	100±0	100±0	100±0	100±0	100±0	100±0	100±0

[a]Radial mycelial growth was determined for amended PDAS and those values compared with unamended PDAS, i.e. check growth. Values are an average of the results from three trials of this experiment.
[b]Amount of active ingredient (ug/ml) added to PDAS.
[c]Mean ± standard error.

Table 3. *In vitro* effect of DHN melanin inhibitors on *Gaeumannomyces*-like species.

Fungus/Isolate	Inhibition of Melanin Pigment Production[a]							
	Tricyclazole		Chlobenthiazone		Pyroquilon		Fthalide	
	1[b]	10	1	10	1	10	1	10
G. graminis var. *graminis*								
561	Y	Y	N	Y	Y	Y	N	Y
FL-19	Y	Y	Y	Y	Y	Y	N	Y
FL-36	Y	Y	Y	Y	Y	Y	N	Y
FL-39	Y	Y	Y	Y	Y	Y	N	Y
FL-46	Y	Y	Y	Y	Y	Y	N	Y
ATCC 64419	Y	Y	Y	Y	Y	Y	N	Y
G. incrustans								
FL-28	Y	Y	Y	Y	Y	Y	N	Y
FL-32	Y	Y	N	Y	Y	Y	N	Y
FL-38	Y	Y	N	Y	Y	Y	N	Y
ATCC 64416	Y	Y	N	Y	N	Y	N	N
ATCC 64417	Y	Y	N	Y	Y	Y	N	N
ATCC 64118	Y	Y	Y	Y	Y	Y	N	Y
Magnaporthe poae								
FL-4	Y	Y	N	Y	N	Y	N	N
ATCC 64413	Y	Y	N	Y	N	Y	N	N

[a]N=no change in pigment; Y=reddish-brown pigment observed on medium rather than black pigment observed on PDAS check medium. Results presented are an average from three trials of this experiment.
[b]Amount of active ingredient (ug/ml) added to PDAS.

Table 4. Effect of DHN melanin inhibitors on pathogenicity of *Gaeumannomyces*-like species.

Fungus/Isolate	Disease Rating[a]			
	Check	Tricylazole[b]	Pyroquilon[b]	Fthalide[b]
G. graminis var. *graminis*				
561	5.0	5.0	5.0	5.0
FL-19	4.9	5.0	4.9	5.0
FL-36	4.9	4.8	4.9	4.8
FL-39	5.0	5.0	5.0	5.0
FL-46	5.0	5.0	5.0	5.0
ATCC 64419	4.9	4.7	4.8	4.9
G. incrustans				
FL-28	4.2	4.4	4.4	4.7
FL-32	4.4	4.5	4.6	4.5
FL-38	4.4	4.2	4.8	4.3
ATCC 64416	4.2	4.3	4.3	4.1
ATCC 64417	4.7	4.4	4.5	4.3
ATCC 64418	4.4	4.5	4.9	4.6
Magnaporthe poae				
FL-4	5.0	4.7	4.7	4.8
ATCC 64413	5.0	4.9	4.9	5.0

[a]Based on a scale of 1 to 5, with 1=healthy plant and 5=dead plant. Values are average rating of 3 experiments.
[b]Compounds incorported into 1.5% water agar (check) at 10 ug/ml.