Isolation, characterization and copper binding of Gaeumannomyces graminis var. graminis melanin mutants

by Barbara Anne Frederick

A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Microbiology

Montana State University

© Copyright by Barbara Anne Frederick (1997)

Abstract:

Gaeumannomyces graminis var. graminis is a filamentous soil ascomycete that synthesizes 1,8-dihydroxynapthalene (DHN) melanin as a secondary metabolite. We isolated two mutant strains affected in the melanin biosynthetic pathway, using either chemical or UV mutagenesis. One of these mutants, JH4300, was unable to synthesize DHN-melanin. Because it accumulated 2-hydroxyjuglone, a melanin pathway shunt product, this mutant was most likely defective in the reductase that catalyzes the conversion of 1,3,8-trihydroxynapthalene to vermelone, the penultimate reaction in DHN synthesis. Genetic crosses with our wild-type strain indicated that this deficiency was the result of a single mutation. Another slow-growing mutant, JH4301, constitutively synthesized DHN-melanin and produced more mucilage surrounding the cell wall than our wild-type strain. Genetic crosses with our wild-type strain suggested that the heavily melanized mutant had a single mutation responsible for its phenotype.

The melanized wild-type and dark mutant strain JH4301 were more resistant to the lytic enzymes chitanase and glucanase, and to UV damage than the unmelanized mutant. The heavily melanized mutant JH4301 secreted fewer lytic enzymes, and tricyclazole inhibition of melanin restored its secretory ability. Both mutants were unaltered in pathogenicity to rice compared to the wild-type strain, but the wild-type was a better competitor in mixed rhizosphere communities of rice.

The light mutant JH4300 absorbed less copper than our wild-type as determined by inductively-coupled plasma atomic emission spectroscopy analysis and silver staining, while the dark mutant bound significantly more copper when grown with copper or when exposed to copper following growth. The heavily melanized mutant was more sensitive to the toxic effects of copper than either the wild-type or unmelanized strains. All three strains bound significant amounts of copper, lead, zinc and iron from tailings material. Inoculation of range grass rhizospheres with the three strains did not affect the uptake of copper, iron or zinc from tailings into plant tissue. However, melanized strains increased the uptake of lead from tailings into shoot tissue. These results suggest that melanized G. graminis var. graminis may have application in phytoremediation of lead-contaminated sites.

ISOLATION, CHARACTERIZATION AND COPPER BINDING OF *Gaeumannomyces graminis* var. *graminis* MELANIN MUTANTS

by

Barbara Anne Frederick

A thesis submitted in partial fulfillment
of the requirements for the degree

of

Doctor of Philosophy

in

Microbiology

MONTANA STATE UNIVERSITY-BOZEMAN
Bozeman, Montana

September 1997

D378
F8726

APPROVAL

of a thesis submitted by

Barbara Anne Frederick

This thesis has been read by each member of the thesis committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style, and consistency, and is ready for submission to the College of Graduate Studies.

Joan M. Henson Joan M. Henson 10-9-97
(Signature) Date

Approved for the Department of Microbiology

Keith Cooksey Keith E. Cooksey 9 OCT 97
(Signature) Date

Approved for the College of Graduate Studies

Joseph Fedock Joseph J. Fedock 10/15/97
(Signature) Date

STATEMENT OF PERMISSION TO USE

In presenting this thesis in partial fulfillment of the requirement for a doctoral degree at Montana State University-Bozeman, I agree that the Library shall make it available to borrowers under rules of the Library. I further agree that copying of this thesis is allowable only for scholarly purposes, consistent with "fair use" as prescribed in the U.S. Copyright Law. Requests for extensive copying or reproduction of this thesis should be referred to University Microfilms International, 300 North Zeeb Road, Ann Arbor, Michigan 48106, to whom I have granted "the exclusive right to reproduce and distribute my dissertation in and from microform along with the non-exclusive right to reproduce and distribute my abstract in any format in whole or in part."

Signature Barbara A. Frederick

Date 9/26/97

ACKNOWLEDGMENTS

I thank my thesis advisor, Dr. Joan Henson, and committee members Drs. TheCan Caesar, Cliff Bond, Tim McDermott, Dave Dooley and Ellen Kreighbaum. I also thank Dr. Greg Olsen of Little Bear Labs for the ICP-AES analysis, Dr. Mike Wheeler for the TLC analysis and Dr. Caesar for the beautiful electron micrographs. The support of a Patricia Roberts-Harris fellowship is gratefully acknowledged, and I thank Adele Pittendrigh and Diane Cattrell for guiding me through the complexities of the fellowship.

Thanks to Tresa Goins for her advice, support and the coffee breaks. Without the friendship of fellow lab rats Deanna Nash and Anastassia Livintseva, my lab time would have been much less enjoyable. Thanks also to fellow graduate students Scott Kobayashi, Wendy Cochran, Robin Williams, Tom Foubert, Maria Kohler, and Kathy Billings-Sandoval for their tolerance and support.

I reserve a special thank you for my husband Tom, and children, Rachel and Andrew, for their patience, love and understanding.

TABLE OF CONTENTS

Page

ACKNOWLEGMENTS. iv
LIST OF TABLES vii
LIST OF FIGURES ix
ABSTRACT xi
CHAPTER

1. INTRODUCTION 1
   Competition. 2
      Exploitation Competition 2
      Interference Competition. 4
   Metal Accumulation in Fungal Biomass 6
   Environmental Metal Contamination. 10
      Historical Overview of the Anaconda Tailings Ponds. 10
      Phytoremediation of Metalliferous Sites. 12
      The Rhizosphere and Metal Cycling. 15
   Hypotheses 17
   Literature Cited 19

2. ISOLATION AND CHARACTERIZATION OF *Gaeumannomyces graminis* var.*graminis* MELANIN MUTANTS. 27

   Introduction 27
   Materials and Methods. 30
      Strains, Media and Growth Conditions 30
      Mutant Isolation 31
      Strain Crosses. 33
      Electron Microscopy 33
      Melanin Quantitation 34
      Novozyme Digestion 35
      UV Protection 36
      Enzyme Secretion 37

TABLE OF CONTENTS - (Continued)

Page

Pathogenicity 37
Results 38
Mutant Characterization 38
UV Light Sensitivity 44
Lytic Enzyme Protection 44
Enzyme Secretion 46
Pathogenicity 46
Discussion 50
Literature Cited 55

3. COPPER BINDING AND LOCALIZATION OF CuS BY SILVER STAINING OF *Gaeumannomyces graminis* var. *graminis* MELANIN MUTANTS 60

Introduction 60
Materials and Methods 63
Fungal Strains and Growth Conditions 63
Growth Curves in Minimal Medium with Copper Amendment 63
Copper Binding and Accumulation 64
Inductively Coupled Plasma-Atomic Emission Spectroscopy (ICP-AES) 64
Silver Staining 65
Results 65
G. graminis var. *graminis* Growth with Copper 65
Copper Binding to Hyphae 67
Silver Enhancement of Copper Sulfide 67
Discussion 69
Literature Cited 71

4. CONCLUSIONS 76

TABLE OF CONTENTS - (Continued)

Page

APPENDICES 78

APPENDIX A--RHIZOSPHERE COMPETITIVENESS OF *Gaeumannomyces graminis* var. *graminis* MELANIN MUTANTS 79
Materials and Methods 82
Competition Assay 82
Rhizosphere DNA Extraction and PCR Amplification 84
Results 85
Competitiveness 85
PCR 85
Discussion 86
Literature Cited 91

APPENDIX B--PHYTOREMEDIATION APPLICATION OF *Gaeumannomyces graminis* var. *graminis* MELANIN MUTANTS 93
Materials and Methods 95
Metal Sorption of Tailings Material toBiomass 95
Metal Uptake by Plant Biomass 96
Rhizosphere Hyphal Lengths 98
Results 99
Tailings Sorption to Biomass 99
Tailings Metal Uptake by Plants Inoculated with *G. graminis* var. *graminis* 99
Discussion 102
Literature Cited 105

LIST OF TABLES

Table Page

2.1. Genetic crosses 40

2.2. Enzyme secretion of *G. graminis* var. *graminis* wild-type and melanin mutant strains grown with or without tricyclazole 49

2.3. Pathogenicity of wild-type and mutant strains of *G. graminis* var. *graminis* to rice. 49

3.1. Cu accumulation of wild-type *G. graminis* var. *graminis* and two melanin mutant strains following 14 days growth in minimal medium with Cu 68

3.2. Copper bound to 14 day-old cultures of *G. graminis* var. *graminis* and two melanin mutants following 1 h exposure of hyphae to 40 mM $CuSO_4$ after 14 days in minimal medium with trace Cu 68

B.1 Metal concentrations of tailings pond material sorbed to wild-type (JH2033) and melanin mutant strains of *G. graminis* var. *graminis*. 100

B.2. Significant treatment effects of plant metal uptake experiment. 101

B.3. Significant main effect of plant species on root and and shoot biomass 102

LIST OF FIGURES

Figure Page

1.1. Anaconda tailings pond system 13

2.1. 1,8-dihydroxynaphthalene (DHN) melanin pathway and shunt products produced by mutations in the pathway 30

2.2. Electron micrographs of wild-type and melanin mutant cell walls 42

2.3. Hyphal tips of wild-type and melanin mutant strains 45

2.4. Hyphopodia of wild-type and melanin mutant strains 46

2.5. Growth curves and melanin concentrations of *G. graminis* var. *graminis* wild-type and melanin mutant strains 47

2.6. Biomass of *G. graminis* var. *graminis* wild-type (JH2033) and melanin mutant strains following treatment with various levels of UV light 48

2.7. N-acetylglucosamine released from wild-type or melanin mutant strains of *G. graminis* var. *graminis* with or without Novozyme 234 digestion 48

3.1. *G. graminis* var. *graminis* wild-type and melanin mutant growth in minimal medium containing varying concentrations of $CuSO_4$ 66

LIST OF FIGURES - Continued

Figure Page

3.2. Silver-stained hyphae showing localization of CuS 69

A.1 Internal transcribed spacer (ITS) region of ribosomal DNA 81

A.2. Significant interactive effect between strain inoculation and rhizosphere inoculum on root and shoot dry weight 88

A.3. PCR- amplified ITS regions of DNA extracted from rhizosphere material with or without rhizosphere inoculum 89

A.4. PCR-amplified ITS regions of JH2033, rhizosphere inoculum extract and rhizosphere inoculum spiked with JH2033 90

B.1. Interactive treatment effect of plant species and tailings addition on iron uptake into shoot tissue 103

B.2. Significant interaction between strain inoculum and tailings addition on lead concentration in shoot biomass 105

ABSTRACT

Gaeumannomyces graminis var. *graminis* is a filamentous soil ascomycete that synthesizes 1,8-dihydroxynapthalene (DHN) melanin as a secondary metabolite. We isolated two mutant strains affected in the melanin biosynthetic pathway, using either chemical or UV mutagenesis. One of these mutants, JH4300, was unable to synthesize DHN-melanin. Because it accumulated 2-hydroxyjuglone, a melanin pathway shunt product, this mutant was most likely defective in the reductase that catalyzes the conversion of 1,3,8-trihydroxynapthalene to vermelone, the penultimate reaction in DHN synthesis. Genetic crosses with our wild-type strain indicated that this deficiency was the result of a single mutation. Another slow-growing mutant, JH4301, constitutively synthesized DHN-melanin and produced more mucilage surrounding the cell wall than our wild-type strain. Genetic crosses with our wild-type strain suggested that the heavily melanized mutant had a single mutation responsible for its phenotype.

The melanized wild-type and dark mutant strain JH4301 were more resistant to the lytic enzymes chitanase and glucanase, and to UV damage than the unmelanized mutant. The heavily melanized mutant JH4301 secreted fewer lytic enzymes, and tricyclazole inhibition of melanin restored its secretory ability. Both mutants were unaltered in pathogenicity to rice compared to the wild-type strain, but the wild-type was a better competitor in mixed rhizosphere communities of rice.

The light mutant JH4300 absorbed less copper than our wild-type as determined by inductively-coupled plasma atomic emission spectroscopy analysis and silver staining, while the dark mutant bound significantly more copper when grown with copper or when exposed to copper following growth. The heavily melanized mutant was more sensitive to the toxic effects of copper than either the wild-type or unmelanized strains. All three strains bound significant amounts of copper, lead, zinc and iron from tailings material. Inoculation of range grass rhizospheres with the three strains did not affect the uptake of copper, iron or zinc from tailings into plant tissue. However, melanized strains increased the uptake of lead from tailings into shoot tissue. These results suggest that melanized *G. graminis* var. *graminis* may have application in phytoremediation of lead-contaminated sites.

CHAPTER 1

INTRODUCTION

Melanins are pigments synthesized by many fungi as secondary metabolites. These pigments are not required for growth, but have been implicated in enhanced survival in the environment (reviewed in ref. 4). Melanins are heterogeneous polymers of dihydroxyphenylalanine (DOPA), γ-glutaminyl-4-hydroxybenzene (GHB), catechol, or dihydroxynaphthalene (DHN) that probably contribute to the competitive fitness of fungi by providing a barrier to environmental stressors. However, melanin protection against enzymatic degradation, UV light damage and oxidizing agents have only been studied with non-isogenic strains. Melanins have also been implicated in metal binding in solution, but most emphasis has focused on synthetic or extracted L-DOPA melanin. The focus of this review will be on the role of melanin in saprophytic competition and metal binding in the rhizosphere, with reference to phytoremediation of metalliferous sites.

Competition

In ecological terms, competition is comprised of two types: exploitation and interference. Exploitation competition is the depletion of resources by one organism without limiting the access of another organism, and interference competition includes mechanisms to reduce access to a resource (35).

Exploitation Competition

In general, resource limitation is the primary competitive strategy of exploitation. For example, wood-decomposing basidiomycetes with high foraging tendencies exclude other wood-decaying fungi from substrates (28). *Aspergillus flavus* colonization and aflatoxin B_1 formation in barley grain are affected by competition by other colonizing fungi (42), and nutrient competition with *Trichoderma harzianum* suppresses *Fusarium* wilt of melon and cotton (49). Antagonism of non-pathogenic *Fusarium oxysporum* against pathogenic *F. oxysporum* is associated with the competition for glucose (34).

Investigations of the biological control of root diseases using antagonistic microorganisms suggests that the interaction between the pathogen and the antagonist takes place in the rhizosphere

(35). Weakly pathogenic fungi with the same rhizosphere niche as stronger pathogens can offset pathogenicity. For example, *Phialophora graminicola* protects against *G. graminis* var. *tritici*, cause of take-all in wheat (13), and substrate competition with *Microdochium bolleyi* reduces take-all lesions (29). In addition, competition for thiamine by a sterile red fungus suppresses take-all (54). Another important determinant in substrate competitiveness in the rhizosphere is the ability to utilize cellulose (1, 22). Lockwood (1992) suggested that the correlation between rhizosphere competence and cellulolytic activity is because the mucilage overlay of the root surface is composed of cellulosic primary cell walls of epidermal cells.

Infection site competition by plant pathogenic fungi can also be considered a type of substrate competition. *Rhizoctonia solani* successfully outcompetes *Pythium ultimum* in the pea (*Pisum sativum*) rhizosphere because it is more aggressive at competing for infection sites (62). Binucleate *Rhizoctonia* spp. and hypovirulent *R. solani* are effective in biocontrol of *R. solani* by competing for invasion sites (26). Suppression of take-all and common root rot by plant growth promoting fungi (PGPF) is due to competitive root

colonization of PGPF, which blocks sites available for infection by pathogens (55).

Fungal protein secretion occurs exclusively at growing apices (60), and competitive ability is enhanced by efficient enzyme secretion for substrate acquisition. Melanins interfere with secreted enzymes, including cellulases, by inhibiting enzyme activity (10, 56) and thus may contribute to decreased competition for nutrients. However, because hyphal tips are unmelanized, autochthonous enzymes may not be inactivated, while melaninized fungal structures may inactivate enzymes from other sources. This would allow melanized fungi a competitive advantage in resource acquisition, by limiting access of fungal competitiors to substrates via enzyme inactivation.

Interference Competition

According to Wicklow (1992), interference competition is the result of a chemical or physical interaction between individuals before resource use. This phenomenon, also called antibiosis, is mediated by specific or non-specific metabolites, by lytic agents, enzymes, volatile compounds or toxic substances (17).

Soil microorganisms secrete lytic enzymes, such as chitanase and glucanases, that are active against fungi. Melanized fungi are more resistant to lysis by these enzymes (6, 31) and suggest that melanin acts as a protection against interference competition. Viable resting spores of the obligate pathogen *Synchytrium endobioticum* persist in soil for long periods (24), and resistance and protection provided by melanization of these spores is implicated in their longevity in soil (25).

Melanin may also contribute to competitive interference. Oxidized melanin precursors exhibit antibiosis (23, 51) that could potentially inhibit competing fungi. The accumulation of DHN-melanin metabolites flaviolin and 2-hydroxyjuglone results in suppression of *Magnaporthe grisea* sporulation (57).

In summary, it is evident that melanins do not significantly enhance exploitation competition in fungi. However, melanins contribute to interference competition by various mechanisms, including inactivation of enzymes from competing fungi as well as by blocking lytic enzymes such as chitinase and glucanases. Melanins and melanin precursors are antibiotic to other organisms and probably contribute to interference with other organisms in competition for the same substrates. In addition, melanins non-

specifically enhance competitive abilities by providing protection against environmental stressors such as UV light (59). Melanins also bind metals that may provide a toxic defense mechanism against other soil inhabitants (45).

Metal Accumulation in Fungal Biomass

Accumulation of heavy metals by microbial biomass is a well characterized phenomenon, and has received recent attention as a mechanism for environmental protection against metal toxicity and in the recovery of heavy metals (reviewed in ref. 58). While some metals (Fe^{3+}, Zn^{2+}, Cu^{2+} and Co^{2+}) are essential, an overabundance of these elements leads to cell toxicity. Metals can exhibit toxicity above metal-specific threshold concentrations. Toxic effects include functional group blockage of metabolically important molecules, denaturation of enzymes, and disruption of membrane integrity (20). Because metal ions frequently fluctuate in the environment, many microorganisms have developed mechanisms to control metal uptake and at the same time sequester elements for future use. Microbial metal uptake and detoxification processes include: uptake of metals into the cell by active or passive processes, biosorption to cell associated materials,

entrapment in extracellular capsules, precipitation, activation of metal-specific binding proteins and oxidation-reduction reactions (19).

Heavy metals enter the soil environment at levels toxic to microorganisms through mining, and industrial and agricultural discharges. High metal levels exert a selective pressure on microorganisms that necessitates expression of a metal detoxification mechanism to ensure survival (19). There is a strong correlation between Cu^{2+}, Cd^{2+} and Zn^{2+} in soil and the level of metal tolerance in soil microbial communities (14). Fungi are generally considered to be less sensitive to metal pollution than bacteria (15), and have been shown to be the dominant fraction of the microbial community in some metal contaminated soils (18).

Metal ions are actively transported into fungal cells, where they bind specific proteins. The metallothioneins (MT's) are small cysteine-rich polypeptides that bind essential heavy metals such as copper and zinc, and non-essential metals such as mercury and cadmium. Metallothionein expression is induced by the same metals that bind to that protein, thereby providing a mechanism of protection against toxicity. Other metals are taken up into fungal biomass by passive processes. Pb^{2+} uptake into *S. cerevisiae* is

energy-independent and the result of affecting the permeability of yeast cell membranes to K^+ and Mg^{2+} (58).

Fungal cell walls contain polysaccharides active in biosorption of metals. These polymers include glycans and cellulose with hydroxyl groups as potential metal binding sites, chitosan and chitin with metal binding potential at amino and hydroxyl groups, and polyuronide with carboxyl and hydroxyl groups (50). Negative surface charge on microbial cell walls caused by the dissociation of chemical groups, allow for cation exchange or coordination of metal cations (7). Metals are classified according to whether they preferentially form complexes with nonpolarizable ligands (hard) or polarizable (soft) ligands (41). Copper and zinc are borderline hard/soft metal ions and adsorption of these ions to biomass is correlated with complexation to soft anions (2). Biomass contains many of the softer binding sites such as carboxyl and amino groups that are involved in metal binding, suggesting that protein and carbohydrate fractions of cell wall bind cations (7).

Fungal pigments such as melanin bind metals and serve as a protective mechanism against metal toxicity. Melanins are high molecular weight polymers that enhance the survival of fungi in

adverse environmental conditions, including high concentrations of heavy metals. Melanin expression is induced in some fungi by Pb^{2+}, Fe^{2+}, Al^{3+}, Hg^{2+} (*Aureobasidium pullulans*, 21) and Cu^{2+} (*G. graminis* var. *graminis*,11; *Aureobasidium pullulans*, 21), suggesting that these pigments provide protection against metal toxicity. Melanins contain negatively charged hydroxyl groups that could potentially bind cations, as well as amino acid and carboxyl groups present in macromolecules trapped in the melanin polymer (47). The structure and characteristics of melanins are similar to weak cation exchangers, and metal ions and melanins probably interact by ion exchange (9). Metals can be desorbed from melanized fungal structures by the application of chelators or pH changes (36, 45) Attraction of metal ions to melanin increases with increasing valance, which is characteristic of cation exchange reactions. Thus, of the divalent metals Pb^{2+}, Cu^{2+}, Ni^{2+}, Co^{2+}, and Mn^{2+}, Pb^{2+} has the highest affinity for melanin, followed by Cu^{2+} (32). Melanized fungal biomass absorbs 2.5 to 4-fold more Ni, Cu, Zn Cd and Pb and at higher rates than unmelanized cultures (50). Fungal melanins also show a high absorptive capacity for Fe^{3+} (48).

Environmental Metal Contamination

Anthropogenic deposition of heavy metals has dramatically increased as a result of mining, industrial and agricultural practices, and fossil fuel consumption (2). Reclamation of metal-impacted soils remains one of the most difficult areas of bioremediation. Techniques in current use are generally based on immobilization by liming, or extraction of metals by acid-leaching or electro-osmosis (4). Some of the more spectacular mining-contaminated sites are the tailings ponds of Anaconda, Montana.

Historical Overview of the Anaconda Tailings Ponds

Large scale copper mining began in the Butte mining district during the early 1880's. Both mining and smelting of the Butte copper ores required a dependable water source, and companies typically located their processing facilities near or adjacent to the Silver Bow Creek. These companies deposited the waste material or tailings from their smelters into ponds near their plants or directly into Silver Bow Creek. Some of this material eventually washed into the Clark Fork River. The early smelters did not efficiently extract copper or other minerals from the ore and consequently pilings contained significant concentrations of metal.

In addition, early smelting operations sent mineral laden fumes into the air. By the early 1900's, smelting operations were moved to Anaconda (39).

In 1883, construction began on a smelting facility 26 miles west of Butte on Warm Springs Creek in the Deer Lodge Valley. Within two years, the Anaconda "Upper Works" was expanded and doubled the daily ore capacity. In 1887, an additional smelting facility (the "Lower Works") was constructed southeast of the first smelter. Finally the construction of the Anaconda Reduction Works south of Warm Springs Creek in 1902 resulted in the largest copper smelter in the world (39).

The Upper and Lower Works (collectively called the "Old Works") at Anaconda deposited waste into Warm Springs Creek, a tributary of the Clark Fork River, or into ponds adjacent to the creek. With the construction of the Anaconda Reduction Works smelter, the company began construction of a system of tailings ponds north and east of the smelter, near the Clark Fork River. During the early 1900's, portions of the tailings pond waste overflowed into the Clark Fork River (39).

The Anaconda Copper Company built five tailings ponds to catch the overflow in 1910. Then in 1917, the ponds B1, B2, C1

and C2 (Opportunity Ponds), and WS1 and WS2 (Warm Springs Ponds) were constructed. About 75% to 80% of the tailings were settled in the B and C ponds, the remainder into Silver Bow Creek and then into the Warm Springs Ponds (39).

In 1956, construction began on a new tailings pond that would cover the entire area of the A, B and C ponds, by raising dikes around the area to 90 feet. The area spanned 4200 acres and had the capacity to hold 400 million tons. The "New River Ponds" were completed around 1957 (Fig. 1.1).

The Anaconda tailings pond system is part of the largest U.S. E.P.A. Superfund in the United States, and contain significant levels of copper, arsenic, lead and zinc. Current efforts to reclaim the tailings site include stabilization by revegetation following liming and organic matter addition, and phytoremediation to extract the metals (F. Munshower, pers. comm.).

Phytoremediation of Metaliferous Sites

Phytoremediation is the use of green plants to remove pollutants from the environment or render them harmless (43). Aside from the relative inexpense of this approach, from an aesthetic viewpoint, revegetation of these sites is more desirable

than removal of contaminated soil for treatment off-site. Phytoremediation of soil can be divided into two processes: phytoextraction, in which metal-accumulating plants are used to transport and concentrate metals from soil into harvestable plant parts; and phytostabilization, in which heavy metal tolerant plants are used to reduce the mobility of heavy metals, thereby reducing the risk of leaching into ground water (46).

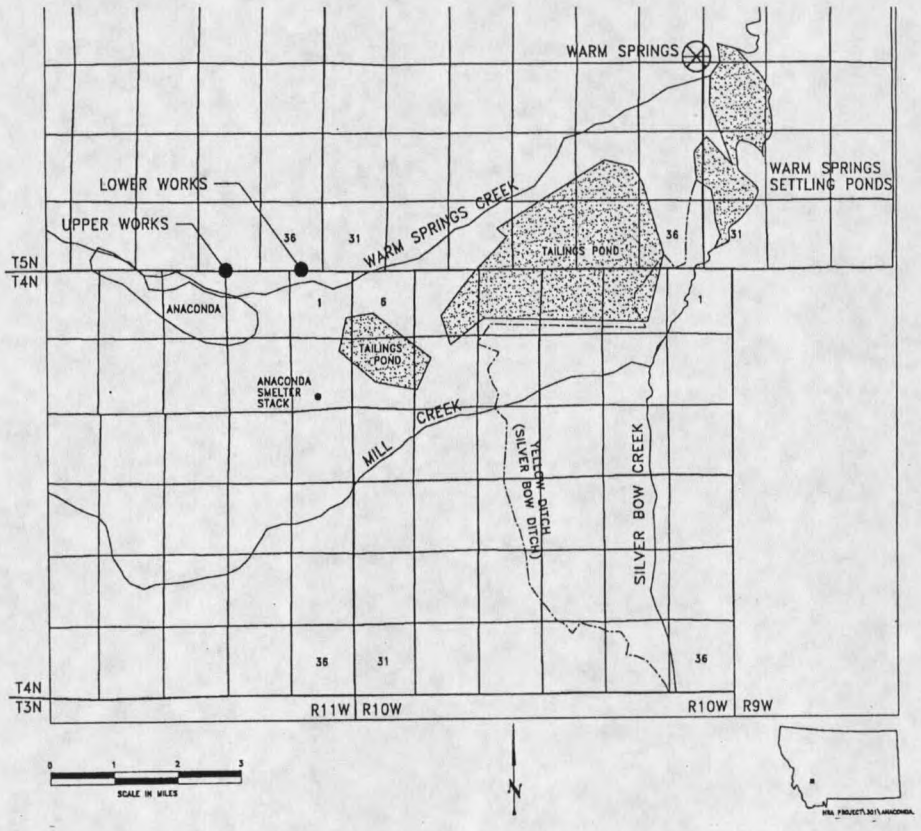


Figure 1.1. Anaconda tailings pond system (39).

Recent reviews have discussed the use of plants as a strategy for the reclamation of soil environments contaminated with toxic metals (43, 46). On heavy metal-contaminated sites, the status of heavy metal content determines the degree and nature of heavy metal tolerance in plants (8); plants with high lead tolerance, for example, are found on sites with high lead content. Metal tolerant plants employ three strategies for growth on metalliferous soils. Metal excluders prevent metal from entering their aerial parts. Non-excluders accumulate metals in above-ground tissues. Metal non-excluders can be divided into two groups termed indicators and hyperaccumulators. Metal concentration in tissues of indicator species reflect metal levels in the soil. Hyperaccumulators concentrate metals in aboveground tissues (43). Species of metal tolerant plants include *Agrostis stolonifera, A. tenuis, Festuca rubra* (47), *F. ovina* (8) and *Minuaria hirsuta* (41). Revegetation of metalliferous soils with non-tolerant species requires the control of metal uptake by plant roots. The major factor governing the availability of metals to plants in soils is the solubility and thermodynamic activity of the uncomplexed ion, since in order for root uptake to occur, a soluble species must be present adjacent to the root interface (12). Therefore,

characteristics of the root zone, or rhizosphere will determine plant metal uptake.

The Rhizosphere and Metal Cycling

Hiltner originally introduced the concept of the rhizosphere as a zone of enhanced bacterial growth around roots of the Leguminoseae (27). Since then, the term "rhizosphere" has been broadened to include the environment inhabited by microorganisms as influenced by plant roots, through exudation or leakage of substances that affect microbial activity. The release of organic compounds by roots include exudates, which are low molecular weight substances that leak out of roots; secretions, whose release is mediated by biological activity; and mucilages, mucigel and lysates. Exudates of range grasses include carboxylic acids, carbohydrates, and amino acids (30).

The most important feature of the rhizosphere with respect to metal cycling is the modification of its chemical properties by the root. Rhizosphere processes mediate changes in pH, ionic strength, and composition of the soil solution. The soil solution pH in the rhizosphere changes by ionic uptake followed by charge balance correction in the root. Protons must be excreted if excess cations are absorbed, and hydroxyl or bicarbonate ions must be

excreted if excess anionic charge has been taken up. The rhizosphere pH of active roots most often increases as the result of anionic uptake (53). This increase could lead to enhanced metal complexation by solids such as organic matter with carboxyl or hydroxyl groups on their surfaces. In addition, the release of organic compounds in the rhizosphere can influence mobility of metals in the soil solution. Metal complexes that absorb weakly to root surfaces will have greater mobility and less likely to be taken up by roots. Metal bioavailability will increase if exudation includes compounds that form strongly absorbed metal complexes (53). However, only soluble metal complexes are absorbed by roots. In addition, plant roots are embedded in a mucilaginous layer that binds trace metals Pb^{2+} and Cu^{2+}, and can result in a decrease of Pb and Cu transport to roots (38).

Hydrated, soluble cations ions are considered to be the metal species absorbed by plants. To a large extent, metal chelation by organic and inorganic soil components controls the metal solubility and availability to plants (33). Clay minerals can adsorb heavy metal cations, but the influence of clays on total absorption capacity in soil is negligible compared to the contribution of organic matter (37). Metals sorbed to clay minerals such as oxide

surfaces are insoluble, while sorption to organics results in soluble complexes. Chelating agents such as humic acid enhance metal solubility but inhibit absorption by plants, and dissociation of these metal complexes is a prerequisite for uptake (33).

Fungal melanins contribute to soil organic matter and share many characteristics with humic acids (5, 16). Fungi such as *G. graminis* var. *graminis* produce melanin in response to copper (11), and the organic ligands provided by melanin probably bind metals as a detoxification mechanism. In the rhizosphere, melanin production could provide similar opportunities for metal chelation, thereby increasing complex formation and decreasing metal uptake by plants. However, melanins have been implicated in mobilizing metal ions from soil minerals into biological systems (63) and thus increases in plant uptake. It is not known what role fungal melanogenesis plays in metal availability for plant absorption.

Hypotheses

My first hypothesis is that wild-type melanized *G. graminis* var. *graminis* strains will be more saprophytically competitive than heavily- or non-melanized strains, and thus more pathogenic to rice in mixed rhizosphere communities. In studies using non-

isogenic strains, melanin provides protection from environmental stress factors such as lytic enzymes and UV light. Using isogenic strains, melanized fungi should be able to successfully compete with other rhizosphere inhabitants. However, melanin also inactivates enzymes necessary for nutrient acquisition, so an overproduction of these pigments will decrease the competitive ability for substrates.

My second hypothesis is that melanized *G. graminis* var. *graminis* strains will absorb more copper in liquid medium containing $CuSO_4$ than unmelanized strains. Previous work in our laboratory has shown that by treating *G. graminis* var. *graminis* with tricyclazole, an inhibitor specific to DHN-melanin synthesis, Cu^{2+} absorption to biomass decreases (11). Isolation of *G. graminis* var. *graminis* strains with mutations in the DHN-melanin pathway having either no melanogenesis or over-expression of melanin, will add to our knowledge of the relationship between metal binding and melanin.

My final hypothesis is that melanized *G. graminis* var. *graminis* strains will absorb metals from mine tailings in range grass rhizospheres, resulting in reduced metal concentrations in plant tissue. Melanins are chemically and structurally similar to

humic acids found in soils, and humic acids provide ligands that bind heavy metals. It follows that melanized fungi will bind heavy metals in a similar fashion, preventing metal uptake into plants.

Literature Cited

1. Ahmad, J. S and R. Baker. 1987. Competitive saprophytic ability and cellulolytic activity of rhizosphere-competent mutants of *Trichoderma harzianum*. Phytopathology **77**:358-362.

2. Avery, S. V. And J. M. Tobin. 1993. Mechanism of adsorption of hard and soft metal ions to *Saccharomyces cerevisiae* and influence of hard and soft anions. Appl. Environ. Microbiol. **59**:2851-2856

3. Ayers, R. U. 1992. Toxic heavy metals: Materials cycle optimization. Proc. Natl. Acad. Sci. USA. **89**:815-820.

4. Baker, A. J. M., S. P. McGrath, C. M. D. Sidoli and R. D. Reeves. 1994. The possibility of *in situ* heavy metal decontamination of polluted soils using crops of metal-accumualting plants. Res. Conserv. Recyl. **11**:41-49.

5. Bell, A. A. and M. H. Wheeler. 1986. Biosynthesis and functions of fungal melanins. Ann. Rev. Phytopathol. **24**:411-51.

6. Bloomfield, B. J. andM. Alexander. 1967. Melanins and resistance of fungi to lysis. J. Bacteriol. **93**:1276-1280.

7. Brady, D. And J. R. Duncan. 1994. Bioaccumulation of metal cations by *Saccharomyces cerevisiae*. Appl. Microbiolo. Biotechnol. **41**:149-154.

8. Brown, G. and K. Brinkmann. 1992. Heavy metal tolerance in *Fectuca ovina* L. from contaminated sites in the Eifel mountains, Germany. Plant Soil **143**:239-247.

9. Bruenger, F. W. B. J. Stover and D. R. Atherton. 1967. The incorporation of various metal ions in *in vivo* and *in vitro*-produced melanin. Rad. Res. **32**:1-12.

10. Bull, A. T. 1970. Kinetics of cellullase inactivation by melanin. Eyzmologia **39**:1276-1280.

11. Caeasar-Tonthat, T.-C., F. van Ommen Kloeke. G. G. Geesey and J. M. Henson. 1995. Melanin production by a filamentous soil fungus in response to copper and localization of copper sulfide by sulfide-silver staining. Appl. Environ. Microbiol. **61**:1968-1975.

12. Cataldo, D. A. and R. E. Wildung. 1979. Soil and plant factors influencing the accumulation of heavy metals by plants. Environ. Health Perspect. **27**:149-159.

13. Deacon, J. W. 1988. Biocontrol of soil-borne plant pathogens with introduced inocula. Phil. Trans. Roy. Soc. London, Ser. B. **318**:249-264.

14. Díaz-Raviña, E. Bååth and Å. Frostegård. 1994. Multiple heavy metal tolrance of soil bacterial communities and its measurement by a thymidine incorporation technique. Appl. Environ. Microbiol. **60**:2238-2247.

15. Doelman, P. 1985. Resistance of soil microbial communites to heavy metals. *In* T. J. Jensen, A. Kjöller and L. H. Sörensen (ed.), Microbial communites in soil. Elsevier, London.

16. Filip, Z. J. Semotan and M. Kutilek. 1976. Thermal and spectrophotometric analysis of some melanins and soil humic compounds. Geoderma **15**:131-142.

17. Fravel, D. R. 1988. Role of antibiosis in the biocontrol of plant diseases. Ann. Rev. Phytopathol. **26**:75-91.

18. Frostegård, Å, A. Tunlid and E. Bååth. 1993. Phospholipid fatty acid composition, biomass and activity of microbial communities from two soil types experimentally exposed to different heavy metals. Appl. Environ. Microbiol. **59**:3605-3617.

19. Gadd, G. M. 1993. Interactions of fungi with toxic metals. Tansley review No. 47. New Phytol. **124**:25-60

20. Gadd, G. M. 1992. Metals and microorganisms: a problem of definition. FEMS Microbiol. Lett.. **100**:197-204.

21. Gadd, G. M. 1981. Mechanisms implicated in the ecological success of polymorphic fungi in metal-polluted habitats. Environ. Technol. Lett. **2**:531-536.

22. Garrett, S. D. 1978. Cellulolysis rate as a determinant of saprophytic longevity among isolates of *Cochliobolus sativus*. Trans. Brit. Mycol. Soc. **81**:21-27.

23. Haars, A. and A. Hüttermann. 1980. Function of laccase in the white-rot fungus *Fomes annosus*. Arch. Microbiol. **125**:233-237.

24. Hampson. M. C. 1993. History, biology and control of potato wart disease in Canada. *Can. J. Pl. Pathol.* **15**:223-244.

25. Hampson, M. C., R. Amarowitz and F. Shahidi. 1996. The presence of melanin in *Synchytrium endobioticum*. Mycologia **88**:647-650.

26. Herr, L. J. 1995. Biological control of *Rhizoctonia solani* by binucleate *Rhizoctonia* spp. and hypovirulent *R. solani* agents. Crop. Prot. **14**:179-186.

27. Hiltner, L. 1904. Üeber neuere Erfahrungen und Probleme auf Gegiete der Bodenkacteriologie unter besonberer Berücksichtgung der Gründügung und Brache. Arb. Deut. Landw. Ges. **98**:59-78.

28. Holmer, L. and J. Stenlid. 1996. Diffuse competition for heterogeneous substrate in soil among six species of wood-decomposing basidiomycetes. Oecologia. **106**:531-538.

29. Kirk, J. J. and J. W. Deacon. 1987. Control of the take-all fungus by *Microdochium bolleyi*, and interactions involving *M. Bolleyi, Phialophora graminicola* and *Periconia macrospinosa* on cereal roots. Plant Soil. **98**:231-237.

30. Klein, D. A., B. A. Frederick, M. Biondini and M. J. Trlica. 1988. Rhizosphere microorganism effects on soluble amino acids, sugars and organic acids in the root zone of *Agropyron cristatum, A. smithii* and *Bouteloua gracilis.* Plant Soil **110**:19-25.

31. Kuo, M.-J. and M. Alexander. 1967. Inhibition of the lysis of fungi by melanins. J. Bacteriol. **94**:624-629.

32. Larrson, B. and Tjälve. 1978. Studies in the melanin-affinity of metal ions. Acta. Physiol. Scand. **104**:479-484.

33. Laurie, S. H. and J. A. Manthey. 1994. The chemistry and role of metal ion chelation in plant uptake processes. *In* J. A. Manthey, D. E. Crowly and D. G. Luster (ed.), Biochemistry of metal micronutrients in the rhizosphere. CRC Press, Boca Raton, FL.

34. Lemanceau, P., P. A. H. M. Bakker, W. J. deKogel, C. Alabouvette, and B. Schippers. 1993. Antagonistic effect of nonpathogenic *Fusarium oxysporum* Fo47 and pseudobactin 358 upon pathogenic *Fusarium oxysporum* f. sp. *dianthi.* Appl. Environ. Microbiol. **59**:74-82.

35. Lockwood, J. L. 1992. Exploitation competition. *In* G. C. Carroll and D. T. Wicklow (ed.) The fungal community: its organization and role in the ecosystem., 2nd edition. Marcel Dekker, Inc., New York.

36. McDougall, D. N. and R. A. Blanchette. 1996. Metal ion adsorption by pseudosclerotial plates of *Phellinus weirii.* Mycologia **88**:98-103.

37. Merrington, G. and B. J. Alloway. 1994. The flux of Cd, Cu, Pb and Zn in mining polluted soils. Water Air Soil Pollut. **73**:333-344.

38. Morel, J., M. Mench and A. Guckert. 1986. Measurement of Pb, Cu, Cd binding with mucilage exudates from maize (*Zea mays* L.). Biol. Fertil. Soils. **2**:29-34.

39. Newell, A. S. 1995. A brief historical overview of Anaconda Copper Mining Company's principle mining and smelting facilities along Silver Bow and Warm Springs creeks, Montana. State of Montana Department of Justice, Natural Resource Damage Litigation Program.

40. Ouzouniduo, G. L., L. Symeonidis, D. Babalonas and S. Karataglis. 1994. Comparative responses of copper-tolerant and copper-sensitive populations of *Minuatia hirsuta* to copper toxicity. J. Plant Physiol. **144**:109-115.

41. Pearson, R. G. 1963. Hard and soft acids and bases. J Am. Chem. Soc. **85**:3533-3539.

42.. Ramakrishna, N., J. Lacey and J. E. Smith. 1996. *Aspergillus flavus* colonization and aflatoxin B_1 formation in barley grain during interaction with other fungi. Mycopathologia **136**:53-63.

43. Raskin, I., N. P. B. A. Kumar, S. Duchenkov and D. E. Salt. 1994. Bioconcentration of heavy metals in plants. Curr. Opin. Biotechnol. **5**:285-290.

44. Raskin, I., R. D. Smith and D. E. Salt. 1997. Phytoremediation of metals: Using plants to remove pollutants from the environment. Curr. Opin. Biotechnol. **8**:221-226.

45. Rizzo, D. M., R. A. Blanchette and M. A. Palmer. 1992. Biosorption of metal ions by *Armillaria* rhizomorphs. Can. J. Bot. **70**:1515-1520.

46. Salt, D. E., M. Blaylock, N. P. B. A. Kumar, V. Duchenkov B. D. Ensley and I. Chet. 1995. Phytoremediation: A novel strategy for the removal of toxic metals from the environment using plants. BioTechnology **13**:468-474.

47. Sarna, T., J. S. Hyde and H. M. Swartz. 1976. Ion exchange in melanin. An electron spin study with lantanide probes. Science **192**:1132-1134.

48. Senesi, N., G. Sposito and J. P. Martin. 1987. Copper (II) and iron (III) complexation by humic acid-like polymers (melanins) from soil fungi. Sci. Total Environ. **62**:241-252.

49. Sivan, A. and I. Chet. 1989. The possible role of competition between *Trichoderma hazianum* and *Fusarium oxysporum* on rhizosphere colonization. Phytopathology **79**:198-203.

50. Seigel, S. M., M. Galun and B. Z. Siegel. 1990. Filamentous fungi as metal biosorbants: A review. Water Air Soil Pollut. **53**:335-344.

51. Söderhäll, K. And R. Ajaxon. 1982. Effect of quinones and melanin on mycelial growth of *Aphanomyces* spp. and extracellular protease of *Aphanomyces astaci*, a parasite on crayfish. J. Invert. Pathol. **39**:105-109.

52. Smith, R. A. H., and A. D. Bradshaw. 1979. The use of metal tolreant plant populations for the reclamation of metalliferous wastes. J. Appl. Ecol. **16**:595-612.

53. Sposito, G. and A. L. Page. 1984. Cycling of metal ions in the soil environment. *In* H. Sigel (ed.) Metal ions in biological systems, vol. 18. Circulation of metals in the environment. Marcell Dekker, Inc., New York

54. Shankar, M., D. I. Kurtböke, L. M. J. Gillespie-Saae, C. Y. Rowland and K. Sivasithamparam. 1994. Possible roles of competition for thiamine, production of inhibitory compounds, and hyphal interactions in suppression of the take-all fungus by a sterile red fungus. Can. J. Microbiol. **40**:478-483.

55. Shivana, M. B., M. S. Meera, and M. Hyakumachi. 1996. Role of root colonization ability of plant growth promoting fungi in the suppression of take-all and common root rot of wheat. Crop Prot. **15**:4976-504.

56. Tshudi, S. and H. Kern. 1979. Specific lysis of the mycelium of *Gaeumannomyces graminis* by enzymes of *Streptococcus lavendulae.* Pp. 611-615. *In* B. Shippers and W. Gams (ed.), Soil-borne pathogens. Academic Press, London.

57. Uehara, T., S. Arase, Y. Honda, M. Nozu and K. Tsujimoto. 1995. Effect of pyroquilon, an inhibitor of melanin synthesis, on sporulation and secondary infection of *Magnaporthe grisea.* J. Phytopathol. **143**:573-576.

58. Volesky, B. 1990. Biosorption by fungal biomass. *In* B. Volesky (ed.), Biosorption of heavy metals. CRC Press, Boca Raton, FL.

59. Wang, Y. And A. Casadevell. 1994. Decreased susceptibility of melanized *Cryptococcus neoformans* to UV light. Appl. Environ. Microbiol. **60**:3864-3866.

60. Wessels, J. G. H., 1993. Wall growth, protein secretion and morphogenesis in fungi. Tansley Review No. 45. New Phytol.. **123**:397-413.

61. Wicklow, D. T. 1992. Interference competition. *In* G. C. Carroll and D. T. Wicklow (ed.) The fungal community: its organization and role in the ecosystem, 2nd Edition. Marcel Dekker, Inc. New York.

62. Xi, K., J. H. G. Stephens and S. F. Hwang. 1995. Dynamics of pea seed infection by *Pythium ultimum* and *Rhizoctonia solani*: effects of inoculum density and temperature on seed rot and pre-emergence damping off. Can. J. Plant Pathol. **17**:19-24.

63. Zunino, H. and J. P. Martin. 1977. Metal-binding organic macromolecules in soil: Hypothesis interpreting the role of soil organic matter in the translocation of metal ions from rocks to biological systems. Soil Sci. **123**:65-76.

CHAPTER 2

ISOLATION AND CHARACTERIZATION OF *Gaeumannomyces graminis* var. *graminis* MELANIN MUTANTS

Introduction

Facultative fungal pathogens are those fungi able to cause disease and live saprophytically in the absence of a host organism. These pathogens compete for nutrients with free-living decomposer populations. Garrett (1970) listed attributes contributing to the success of fungi as competitive saprophytes. They are: 1) rapid germination of propagules and rapid hyphal growth to reach substrates; 2) enzyme production to utilize substrates; 3) excretion of either bacteriostatic or fungistatic substances to reduce growth of competitors; and 4) tolerance to deleterious substances produced by other organisms.

Gaeumannomyces graminis var. *graminis* is a homothallic filamentous ascomycete that colonizes root and crown tissue of many members of the Poaceae plant family and survives saprophytically in soil or plant litter. Some strains invade host vascular tissue to cause root disease in rice and ornamental

turfgrasses (32, 36). *G. graminis* var. *graminis* produces melanin via the dihydroxynaphthalene (DHN) pathway (Fig. 2.1) in response to stress factors (7, 13). Melanin also accumulates in older hyphal cell wall and in sexual structures (perithecia) and adhesive infection cells (hyphopodia). Wang and Casadevall (1994) demonstrated that melanin serves as a protective layer against UV irradiation, by adding the melanin precursor L-dihydroxyphenylalanine (L-DOPA) to *Cryptococcus neoformans* cultures. Cultures grown with L-DOPA were more resistant to UV light damage than control cultures. At present, similar studies of DHN-melanin protection against UV irradiation, either by using melanin precusor addition or isogenic strains, have not been published.

Melanin content of fungal hyphae positively correlates with enhanced resistance to chitinase and glucanase degradation (25), and melanized *Rhizotonia solani* strains are less sensitive to lysis than unmelanized strains (23). In addition, when melanin-containing spicules of *Aspergillus phoencis* spores are removed, they are more sensitive to lytic activity (4). While these studies suggest a correlation between melanization and lytic enzyme protection, experiments using isogenic strains are lacking. In

summary, melanin serves as a protective layer against UV irradiation and lytic enzymes and may contribute to competitive success in a mixed microbial community.

Extracellular enzymes secreted by *G. graminis* are necessary for nutrient acquisition and implicated in the disease process (9). Infection requires penetration of host cells and involves the degradation of cell wall components by the pathogen. Lytic enzymes secreted at the growing apex aid in cell wall decomposition and subsequent pathogenesis. Previous studies (Goins and Henson, submitted) suggest that spontaneous melanin mutants of *G. graminis* var. *tritici* produce less extracellular protein and are less pathogenic than the lightly pigmented wild-type strain. In addition, melanin has been shown to provide structural rigidity required to maintain turgor pressure of appressorial infection structures (20, 21). *G. graminis* does not develop appressoria, but does produce melanized adhesion structures (hyphopodia) that are believed to serve a similar function as appressoria.

Because melanins are heterogeneous and difficult to characterize and quantitate, in this study we isolated and characterized *G. graminis* var. *graminis* mutant strains affected in

the melanin pathway to determine the effect of lack of melanin or overmelanization in protection from environmental stressors and the ability to produce disease in rice plants.

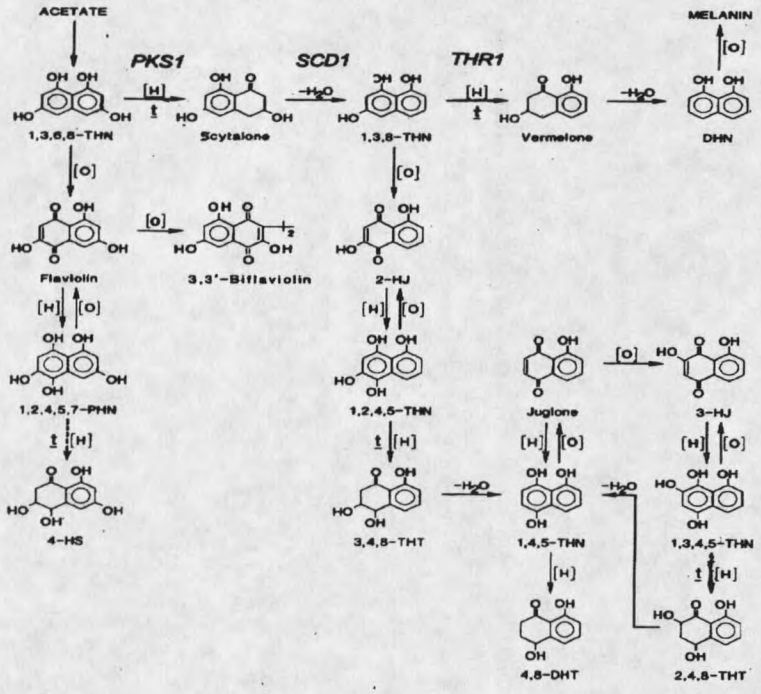


Figure 2.1. 1,8-dihydroxynaphthalene (DHN) melanin pathway and shunt products produced by mutations in the pathway (modified from reference 2). T = site of tricyclazole inhibition. Genetic nomenclature is from references 27 and 34. 2-HJ = 2-hydroxyjuglone; 1,3,8-THN = 1,3,8-trihydroxynaphthalene.

Materials and Methods

Strains, media and growth conditions

Our wild type *G. graminis* var. *graminis* strain, JH2033, is a single ascospore derivative of an isolate collected by Dr. Craig Rothrock from soybean (*Glycine max* L.).

Defined liquid minimal medium contained in g per liter: sucrose, 3; K_2HPO_4, 1; $MgSO_4$, 0.05; KCl, 0.05; $CaCl_2$, 0.1; $FeCl_3$, 0.0005; and trace elements [$ZnSO_4 \cdot H_2O$, 0.0125; $Fe(NH_4)(SO_4)_2 \cdot 6H_2O$, 0.0025; $CuSO_4 \cdot 5H_2O$, 2 x 10^{-6}; $MnSO_4 \cdot H_2O$, 0.0001; H_3BO_3, 0.0001; $NaMoO_4 \cdot H_2O$, 0.0001; biotin, 0.001 and thiamine, 0.001]. The stock trace element solution was filter sterilized and diluted 1:4000 into autoclaved medium. Stock solutions of $MgSO_4$, KCl and $CaCl_2$ were filter sterilized and diluted 1:1000 into autoclaved medium. Filter sterilized $FeCl_3$ solution was diluted 1:200000 into autoclaved medium. The pH was adjusted to 5.5 with HCl for both solid and liquid media before autoclaving. Other media used were potato dextrose agar (PDA; Difco, Detroit, Michigan), potato dextrose broth (PDB; Difco, Detroit, Michigan) and Luria-Bertani (LB) with or without 1.0 % agar (Meer Corp., New Bergen, NJ). Cultures were inoculated from LB agar into 200 ml liquid medium in 1 liter flasks, and shaken (50 cycles min^{-1}) at 25 C with a 12 h photoperiod and 3000 Lux.

Hyphopodia were grown by inoculating mylar (14C thickness, a gift from DuPont, Wilmington, DE) membranes that were ethanol-sterilized and placed on 1% water agar.

Mutant isolation

A five-day culture of wild-type hyphae grown in 100 ml LB broth was harvested by filtration through a Whatman #1 filter and resuspended in 75 ml protoplast buffer (PBI- 0.2 M NaH_2PO_4 and

0.6 M KCl, pH 5.8) with 0.375 g each of Novozyme 234 (InterSpex Products, Inc) and cellulase (Sigma C-8546). Hyphae were shaken slowly for 1.5 h at 25 C, filtered through glass wool, and protoplasts in the filtrate were washed twice with PBI and resuspended in 5 ml PBI.

Protoplasts were subjected to chemical mutagenesis using 4-nitroquinolene oxide (NQO) or UV light. Approximately 10^8 protoplasts were incubated for 1 h at room temperature in 5 ml PBI containing 1 μg/ml NQO (3). NQO was neutralized by addition of an equal volume of 5% sodium thiosulfate in PBI, then washed twice with PBI and resuspended in 5 ml of PBI. For UV mutagenesis, 10^8 protoplasts in 5 ml PBI were exposed to 0.09 J per cm^2 in a Stratalinker® UV Crosslinker, Model 1800 (Stratagene, La Jolla, CA).

Treated protoplasts were plated onto regeneration medium (17) and incubated at room temperature in the dark for 7 days, and regenerated protoplasts were transferred to PDA with 1% sorbose. Sorbose inhibits hyphal spreading (16) so that more protoplasts could be screened on a 90 mm^2 petri plate. Mutants with pigment production different from our wild-type strain were transferred to PDA and LB agar. Two mutants were chosen for further characterization: JH4300, a mutant that did not produce melanin on PDA and JH 4301, that constitutively synthesized melanin.

Strain crosses

JH2028, a bleomycin-resistant transformant of JH2033 (28) was inoculated with JH4300 or JH4301 onto sterile, moist filter paper with a layer of toothpicks onto which autoclaved rice leaves were placed. Plates were incubated at 25 C with a 12 h photoperiod at 3000 Lux. Single, mature perithecia were squashed on slides under glass coverslips and spread onto LB agar. After 24 h germinated ascospores were transferred to PDA plates to detect pigmentation phenotypes. Ascospore derivatives were screened for $Phleo^R/Phleo^S$ on LB agar+2.5 g/ml Bleomycin (a gift from Bristol-Meyer, Evansville, IN).

Electron microscopy

Nine day-old cultures grown in minimal medium were collected for microscopy. Specimens were processed for freeze substitution by freezing in liquid propane at -190 C to -193 C, substituting in 2% osmium tetroxide and 0.05% uranyl acetate in acetone, and embedding between two microscope slides in Epon 812-Araldite 6005 epoxy resin (18, 19, 20). Cells were examined for freeze damage by phase contrast optics at 1000x. Undamaged cells were mounted onto epoxy stubs, thin-sectioned longitudinally, and stained with uranyl acetate and lead citrate. Specimens were examined with a Jeol JEM 100CX transmission microscope operated at 80 kV. At least 15-30 cell walls and one hyphal tip from each strain were viewed.

Melanin Quantitation

Melanin concentration of the three strains were estimated by the method of Butler and Lachance (1986). For preparation of melanin for a standard curve, 10 plugs of JH2033 from an LB agar plate were inoculated into 250 ml of LB broth. The culture was incubated for 7 days at room temperature with shaking (150 rpm). Hyphae were removed by filtration through a Whatman #1 filter and the supernate acidified to 4 N HCl with concentrated HCl. After allowing precipitation at 4° C for 4 days, melanin was recovered by centrifugation at 5000 x *g* for 15 minutes. The pellet was washed 3X with 0.1 N HCl followed by 5 washes with distilled water. The melanin was lyophilized overnight to remove residual water and stored at -20 °C in the dark. The standard melanin curve was prepared by weighing duplicate 250, 500 and 1000 µg samples into 15 ml Corex® tubes. Three ml of Azure A (Sigma) solution were added to each tube and allowed to stand for 30 minutes. Solutions were then filtered through a 0.45 µm syringe filter. Azure A solution was prepared from a stock solution of 0.1 g Azure A in 500 ml 0.2 N HCl that was diluted 1:30 in 0.2 N HCl. Melanin concentration was determined by a decrease in absorbance at 628 nm.

Melanization of wild-type, JH4300 and JH4301 was determined at 3, 4, 5, 6 and 7 days following inoculation of 3 plugs into 60 ml LB broth in 300 ml Erlenmeyer flasks. Flasks were shaken at 150 rpm at room temperature in the dark. At harvest, hyphae were collected by filtration through a Whatman #1 filter and lyophilized overnight. Triplicate 2 mg samples of dried hyphae were assayed as described for the standard melanin curve.

Novozyme Digestion

A standard N-acetylglucosamine standard curve was prepared from 1 mM N-acetylglucosamine in 0.033 M phosphate buffer (pH 6.1). N-acetylglucosamine was detected by the method of Reissieg et al. (1955). To tubes containing 500 µl of solution, 100 µl of potassium borate (49.64 g H_3BO_3 per liter, pH 9.1) were added, then heated in a boiling water bath for 3 minutes and cooled in a cold water bath. Three mls of dimethylaminobenzadehyde were added, the contents mixed, then heated at 37 °C for 20 minutes. After cooling in a cold water bath, absorbance was read at 544 and 585 nm.

Duplicate wild-type, JH4300 and JH4301 strains were grown for 7 days in 50 ml LB broth with shaking at room temperature.

Hyphae were harvested by filtration through a Whatman #1 filter, washed 2X with sterile phosphate buffer and transferred to sterile 250 ml Erlenmeyer flasks containing 50 mg Novozyme 234 in 50 ml phosphate buffer. Flasks were incubated with shaking at room temperature and aliquots of the supernate taken at 1, 2 and 3 hours. Supernates were analyzed for N-acetylglucosamine as described for the standard curve. Controls consisted of hyphae without Novozyme 234.

UV protection

Durapore type GV 0.22μm 47 mm diameter filters (Millipore cat no. GVMP 047 00) were autoclaved, weighed and placed on 90 mm diameter petri plates containing LB agar. One plug of JH2033, JH4300 or JH4301 from LB agar was inoculated onto the center of the filter and the plates incubated in the dark for 3 days at room temperature. Lids were removed from plates, placed in a UV Stratalinker, then exposed to 200, 400 or 600 μJ. Plate lids were replaced, and incubated for 3 days at room temperature in the dark. Following incubation, filters were removed, dried overnight on a lyophilizer and then weighed.

Enzyme Secretion

Polygalacturonase (PGU; EC 3.2.1.15) secretion was detected on solid medium containing 1% sodium polypectate (Sigma) at pH 6.5 in 10 mM sodium phosphate buffer with 0.1% yeast extract, 0.125% each of biotin and thiamin, and 2% Bacto agar. Clearing around fungal colonies, indicating enzyme secretion, was detected by flooding plate with 5 N HCl (40). Base medium for the detection of α-cellulose contained 2.5% Czapek's Dox broth (Difco), 0.125% each of biotin and thiamin, 1% α-cellulose (Sigma) and 2% Bacto agar. Hydrolysis was detected by flooding plates with Lugol's iodine and measuring the cleared area (1). Hydrolysis of skim milk was assessed on plates containing 2% skim milk, and 2% Bacto agar. Clearing around colonies indicated hydrolysis. Filter-sterilized tricyclazole from a 2.5 mg/ml 100 % EtOH stock was added at a concentration of 16 μg/ml to cooled media.

Pathogenicity

Hulled rice seeds (*Oryza sativa* Gulfport) were surface sterilized in 1% $AgNO_3$ for 7 min and rinsed 2X in sterile deionized water. Sterile vermiculite in 10 cm diameter pots was inoculated with hyphal on plugs from LB agar and seeds were placed next to

the plugs. Plants were grown for 28 days at 25 °C with a 12 h photoperiod at 3000 Lux and a watering schedule of 100 ml sterile water every 5 days. At harvest, roots and shoots were separated, lyophilized overnight and weighed.

Results

Mutant characterization

Mutant strain JH4300, isolated after treatment of wild-type protoplasts with 4-nitroquinolene, did not produce the dark green DHN-melanin pigment that is characteristic of the wild-type strain on minimal, LB or potato dextrose media. Mutant JH4300 was orange on PDA as the result of 2-hydroxyjuglone and flaviolin accumulation, which are dark orange and brown, respectively and confirmed by thin layer chromatography (TLC, data not shown, ref. 2). This result suggested that its melanin pathway was blocked at the reductase step that converts 1,3,8-trihydroxynaphthalene to vermelone (Fig. 2.1). Moreover, feeding experiments with scytalone did not restore the wild-type melanin phenotype (data not shown), an expected finding if the pathway was blocked downstream of the scytalone biosynthetic step (2).

When we crossed mutant 4300 with the wild-type strain, progeny displayed a 1:1 wild type: mutant melanin phenotype (Table 2.1), and suggested that this mutant phenotype resulted from a single mutation affecting the gene encoding trihydroxynaphthalene reductase, or the *THR1* gene (*Colletotrichum lagenarium* nomenclature, ref. 27). The bleomycin resistance marker did not segregate 1:1, a result consistent with other studies of *G. graminis* var. *graminis* transformant crosses in which heterologous, transformed sequences such as the *BLE* gene sometimes did not segregate in a 1:1 fashion (28).

We isolated JH4301 following treatment of wild-type protoplasts with UV light and it constitutively produced a dark green-black pigment on minimal, LB and potato dextrose media. Hyphae of this strain were more heavily pigmented than wild-type hyphae and accumulated a variety of melanin metabolites in PDA containing 8 and 30 μg/ml tricyclazole, a specific DHN-melanin pathway inhibitor (Fig. 2.1, ref. 2). In addition to flaviolin and 2-hydroxyjuglone, JH4301 accumulated 3,4,8-trihydroxytetralone and 4,8-dihydroxytetralone while tricyclazole-treated wild-type cultures accumulated flaviolin and 2-hydroxyjuglone, the oxidative products of 1,3,6,8-tetrahydroxynaphthalene and 1,3,8-

trihydroxynaphthalene, respectively. All shunt products were identified by TLC with flaviolin and 2-hydroxyjuglone as standards (data not shown, ref. 2).

Table 2.1. Genetic Crosses.

Cross	Total Perithecia	Hybrid Perithecia	Total Ascospores	Phenotype	Number Progeny
JH2028 X JH4300	26	3	262	Ble^S Thr1$^-$	92
				Ble^R Thr1$^-$	35
				Ble^S Thr1$^+$	86
				Ble^R Thr1$^+$	49
JH2028 X JH4301	3	1	233	Ble^S Moe$^-$	27
				Ble^R Moe$^-$	51
				Ble^S Moe$^+$	88
				Ble^R Moe$^+$	67
JH4302 X JH2033	9	1	69	Ble^S Moe$^-$	14
				Ble^R Moe$^-$	11
				Ble^S Moe$^+$	25
				Ble^R Moe$^+$	19

JH4301 did not produce mature ascospores when selfed, but the cross between wild-type and mutant JH4301 displayed 1:2 mutant: wild-type melanin phenotype (Table 2.1). Perhaps this ratio was skewed because JH4301 ascospores germinated more slowly than those of the wild-type and some viable JH4301 ascospores were possibly overgrown with hyphae from more rapidly germinating wild-type ascospores. A backcross of a $Phleo^R$ mutant JH4301 derived from this initial cross with the wild-type strain

gave approximately 1:1 wild-type:mutant progeny. Again, slow mutant ascospore germination possibly influenced this ratio. Progeny of the crossed perithecia with JH4301 melanin phenotype grew at a similar rate and produced similarly shaped hyphopodia as JH4301. These results suggested that JH4301 contained a single mutation (designated *moe*) affecting the melanin biosynthetic pathway.

Electron micrographs of freeze-substituted hyphae demonstrated cell wall differences between the wild-type strain and two melanin mutants. Nine day-old wild-type cultures grown in the presence of trace copper had only two layers: an electron dense outer mucilage layer and an electron translucent inner chitin layer (Fig. 2.2a.) previously found to bind wheat germ agglutinin (7). Strain JH4300 cell walls were composed of two layers: an inner translucent chitin layer and a thin dense mucilage outer layer (Fig. 2.2c). By comparison, dark mutant JH4301 had an intermediate melanized layer even when grown in trace copper and a dense outer layer with thicker mucilage closely associated with the cell wall (Fig. 2.2b).

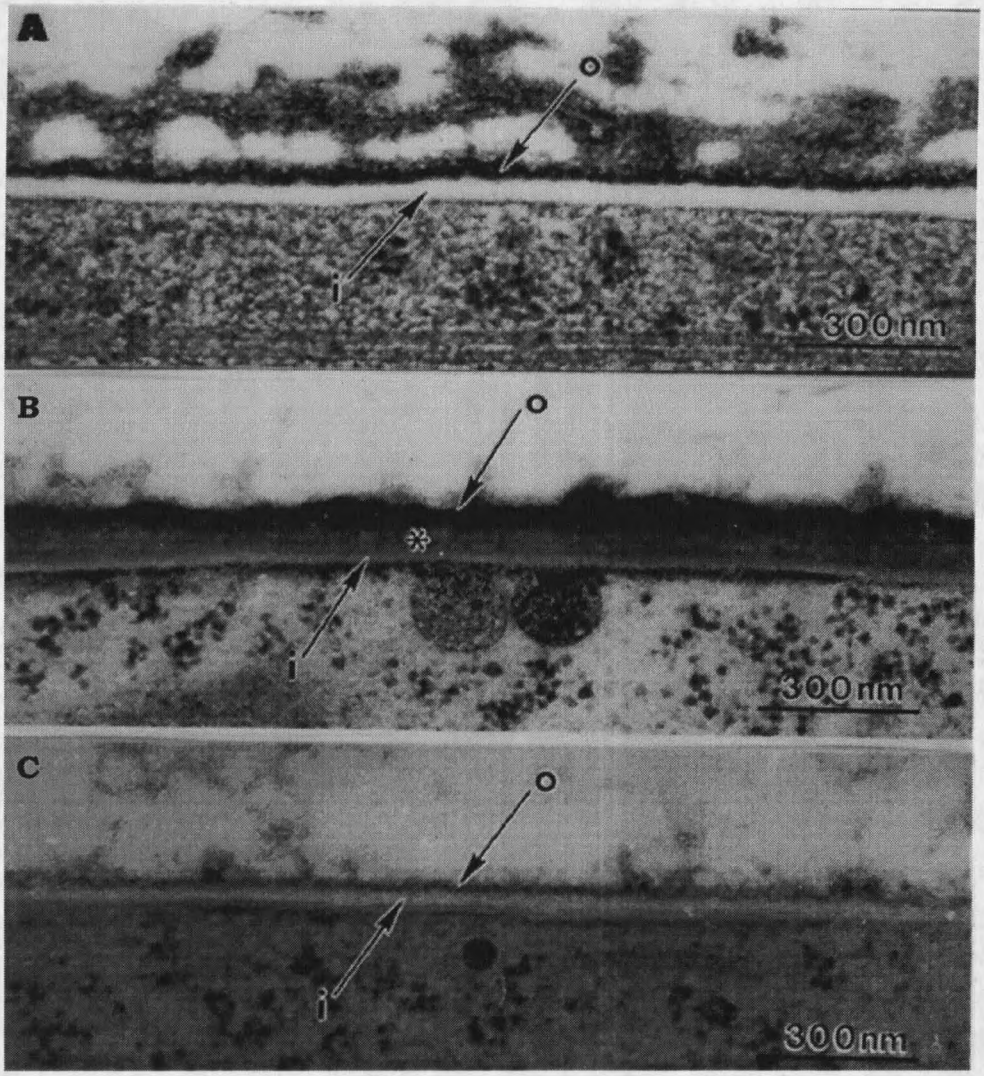


Figure 2.2. Electron micrographs of wild-type and melanin mutant cell walls. A. Wild-type JH2033, B. Dark mutant JH4301, C. Light mutant JH4300. * is the intermediate melanin layer, i is the inner chtin layer and 0 is the outer mucilage layer. Magnification = 66,000X.

Thin sections of hyphal tips also showed differences in mucilage between the three strains (Fig. 2.3). While the wild-type displayed an electron dense mucilage layer closely associated with

the apical region (Fig. 2.3a.), the dark mutant showed a denser mucilage that extended to the apical tip (Fig. 2.3b). The light mutant exhibited a loose layer of mucilage associated with the cell (Fig. 2.3c).

Hyphopodia of the three strains differed in pigmentation or shape (Fig. 2.4). Wild-type hyphopodia on mylar were melanized and lobed (Fig. 2.4a.) The light mutant produced brownish-orange lobed hyphopodia (Fig. 2.4c.) while the dark mutant developed highly pigmented, round structures (Fig. 2.4b). Perithecia of self-crossed mutant JH4300 were orange-light brown and under these conditions dark mutant JH4301 produced immature perithecia without mature ascospores unless crossed with the wild-type (data not shown).

Our wild-type strain reached the stationary growth phase by day 6 when grown in LB (Fig. 2.5b), with melanin concentration roughly increasing in parallel with biomass. The light mutant JH4300 had not reached stationary phase by the end of 7 days of growth (Fig. 2.5a), and melanin concentrations remained low throughout the growth curve. The dark mutant biomass leveled off between days 6 and 7 (Fig. 2.5c), while melanin concentration was

highest at day 5. Except for day 7, the melanin concentration of JH4301was significantly higher than our wild-type strain.

UV Light Sensitivity

The heavily melanized JH4301 strain was less sensitive to UV light than either our wild-type strain or the light JH4300 strain (Fig. 2.6). UV exposure reduced JH4300 biomass by 55-62% and was not dose-dependent. At low exposure (200 and 400 $\mu J/cm^2$) the wild-type biomass was reduced by approximately 40%, and 54% at the 600 $\mu J/cm^2$ exposure. UV exposure did not significantly reduce the dark mutant biomass.

Lytic Enzyme Protection

Supernates from the dark melanin mutant had higher N-acetylglucosamine initially, and following 1 hour digestion with Novozyme 234, than either our wild-type strain or JH4300 (Fig. 2.7). However, significantly more N-acetylglucosamine was released from the light melanin mutant than the melanized strains after 2 or 3 hours of incubation with Novozyme 234. After 3 hours of digestion, the amount of N-acetylglucosamine released from JH4301 in the presence of Novozyme was not significantly different from the JH4301 control.

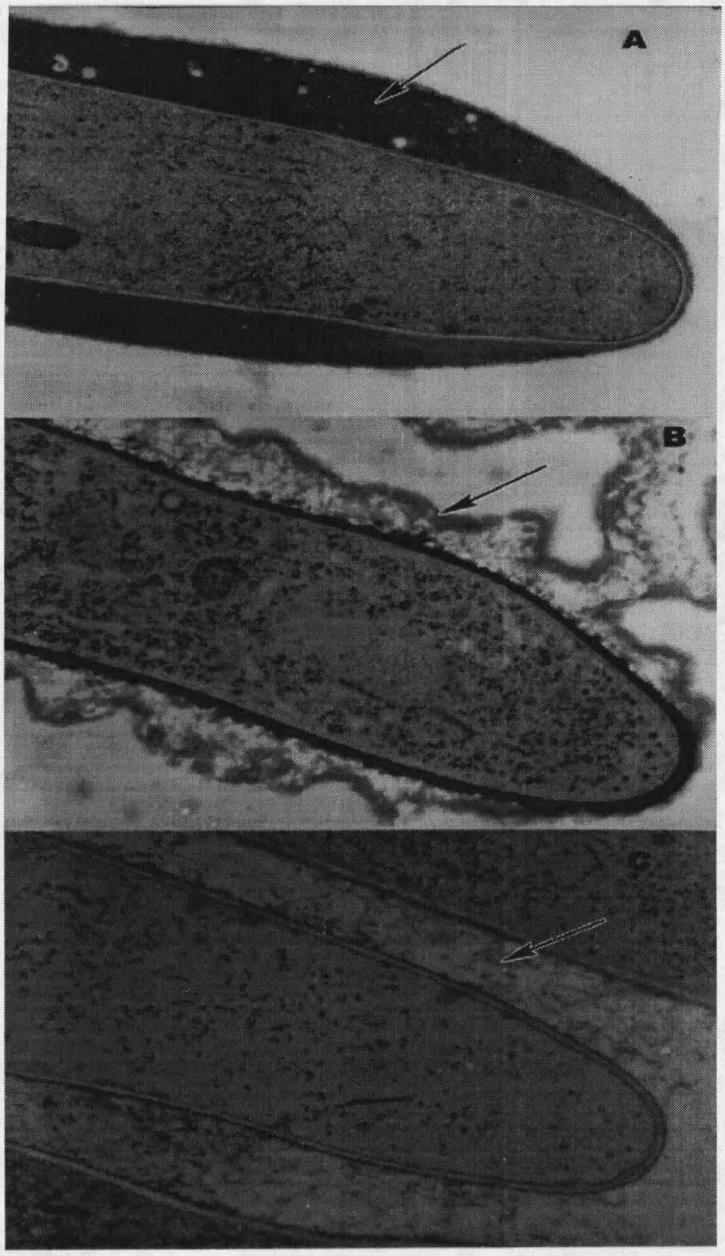


Figure 2.3. Hyphal tips of wild-type and melanin mutants strains. A) Wild-type, B) Dark mutant JH4301, and C) Light mutant JH4300. Arrows indicate mucilage. Magnification = 33,000X.

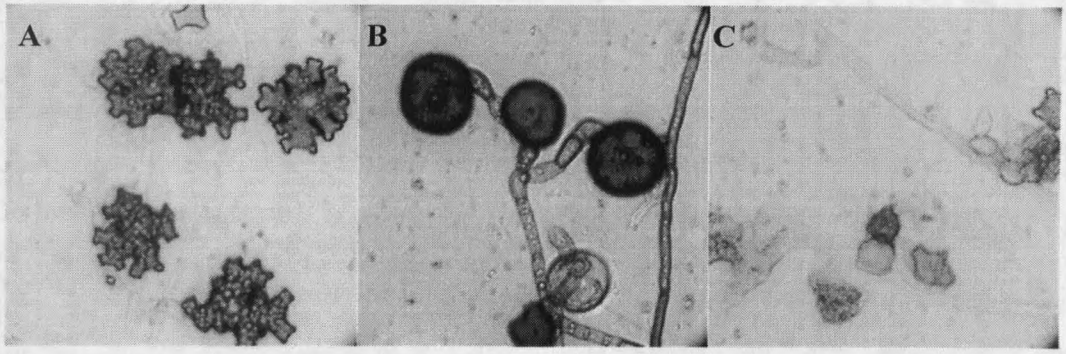


Figure 2.4. Hyphopodia of wild-type and melanin mutant strains. A) Wild-type JH2033, B). Dark mutant JH4301, C) Light mutant JH43011. Magnification = 400X.

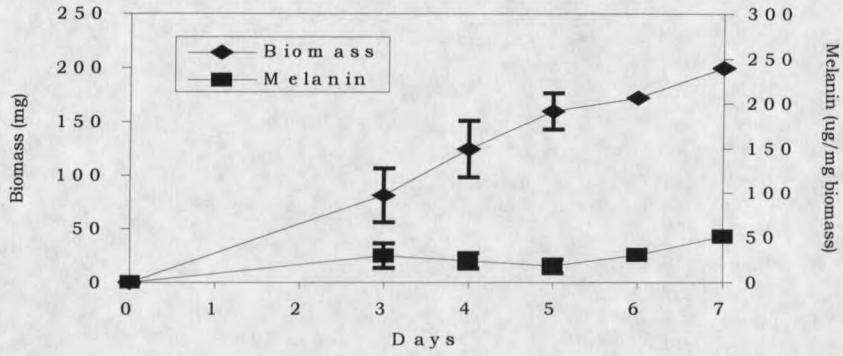
Enzyme Secretion

The light mutant strain JH4300 secreted more enzymes than the melanized strains when grown without tricyclazole, while the dark mutant showed very little enzyme secretion (Table 2.2). Tricyclazole enhanced enzyme secretion of the two melanized strains, but did not affect the light mutant.

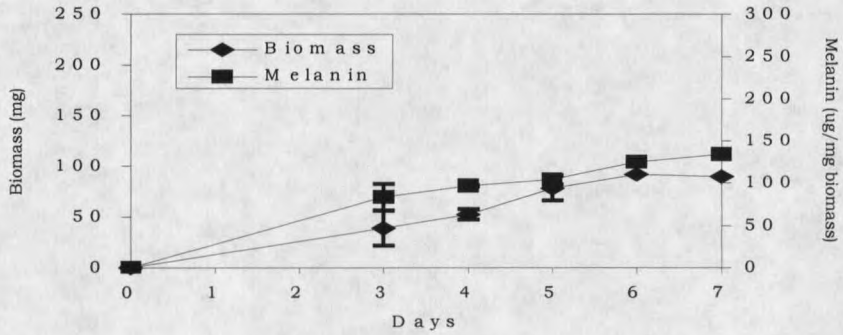
Pathogenicity

All three strains decreased the percent of rice seeds germinated (Table 2.3), and JH4300 reduced shoot lengths and biomass equivalent to the wild-type strain. The dark mutant significantly reduced shoot lengths compared to the control treatment, but less than the wild-type or light mutant, and decreased shoot biomass to an intermediate weight between control and wild-type treatments.

A. JH4300



B. JH2033



C. JH4301

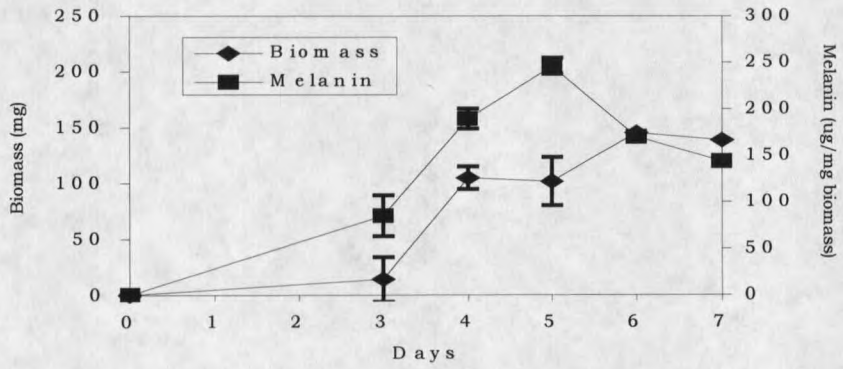


Figure 2.5. Growth curves and melanin concentrations of *G. graminis* var. *graminis* wild-type and melanin mutant strains. Biomass is total biomass in 60 ml culture, and error bars are ± standard error. Each point is the average of triplicate cultures.

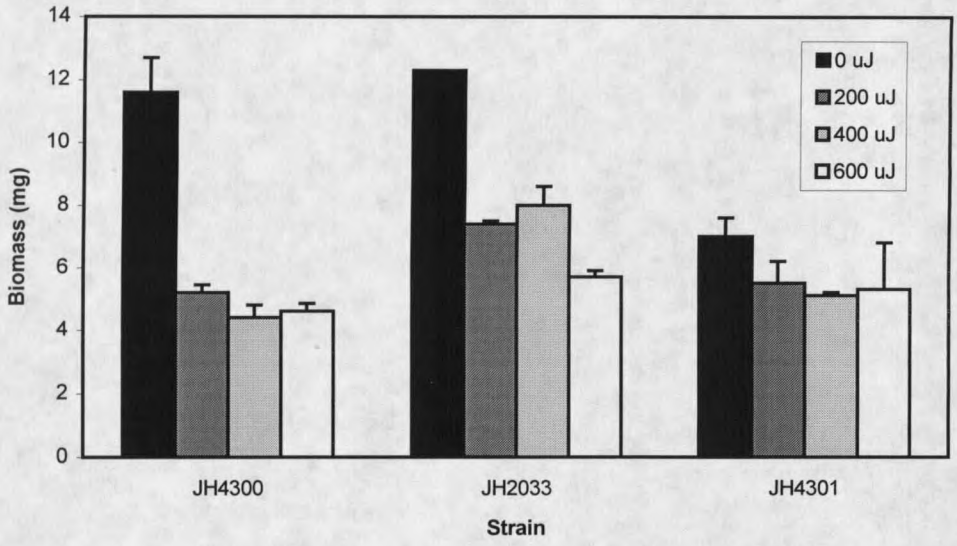


Figure 2.6. Biomass of *G. graminis* var. *graminis* wild-type (JH2033) and melanin mutant strains following treatment with various levels of UV light. Average of triplicate plates, error bars are ± standard error.

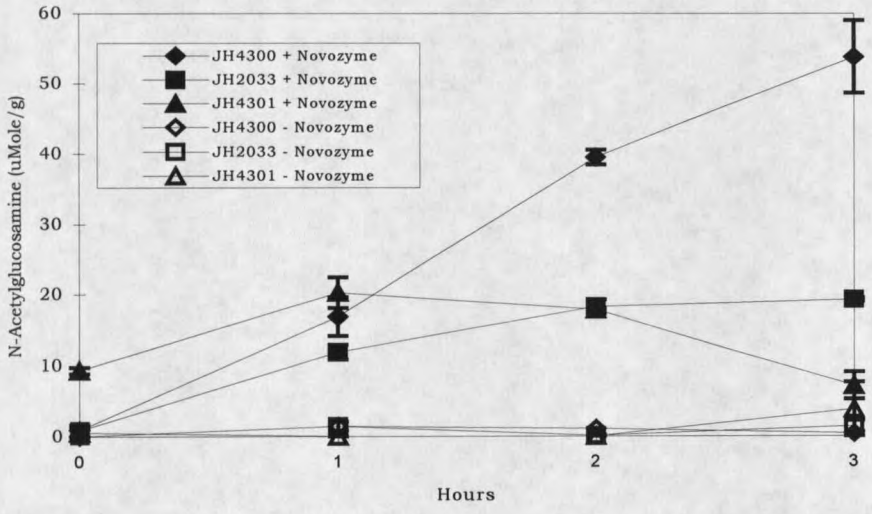


Figure 2.7. N-Acetylglucosamine released from wild-type or mutant strains of *G. graminis* var. *graminis* with or without Novozyme 234 digestion. Each point is the average of duplicate treatments, and error bars are ± standard error.

Table 2.2. Enzyme secretion of *G. graminis* var. *graminis* wild-type and melanin mutant strains grown with or without tricyclazole. - = no enzyme activity, + = enzyme secretion only beneath hyphae, ++ = enzyme secretion beyond hyphal growth.

	JH4300		JH2033		JH4301	
Tricyclazole (μg/ml)	0	16	0	16	0	16
α- Cellulase	++	++	++	++	+	++
PGU	++	++	-	++	-	+
Milk Protease	++	++	++	++	-	+

Table 2.3. Pathogenicity of wild-type and mutant strains of *G. graminis* var. *graminis* to rice. Parameter means followed by different letters are significantly different at $P < 0.05$ (Tukey's Studentized Range test, HSD), average of five replicates.

	Strain			
Parameter	Control	JH2033	JH4300	JH4301
% seeds germinated	82 b	64 a	65 a	67 a
Shoot Lengths[1]	16.5 c	7.8 a	8.8 a	12.4 b
Shoot Weight[2]	7.3 b	5.3 a	5.3 a	6.1 ab
Root Weight[2]	5.0 a	4.3 a	4.0 a	4.2 a

[1]average per plant (cm)
[2]Average dry weight per plant (mg)

Discussion

We isolated *G. graminis* var. *graminis* mutants affected in their melanin biosynthetic pathway to examine the role of melanin in plant pathogenicity. The two mutants described here differed from the wild-type strain in several aspects of their pigmentation, cell wall structure and hyphopodial morphology. Our light mutant JH4300 accumulated 2-hydroxyjuglone and flaviolin, probably as the result of a single mutation in the *THR1* gene. The dark mutant JH4301 constitutively produced dark pigmentation on all media without tricyclazole, whereas our wild-type strain was heavily pigmented on PDA and lightly melanized on LB and minimal media. Our genetic crosses with JH4301 and the wild-type suggested that the JH4301 phenotype is the result of a single mutation; this mutation may be in a regulatory gene associated with the melanin synthesis pathway rather than a structural gene, since this strain constitutively synthesized melanin.

Microscopic examination of the three strains revealed differences in hyphopodia, perithecia morphology and mucilage. *G. graminis* var. *graminis* produces brown, lobed hyphopodia on mylar, while the light mutant had orange-brown, lobed hyphopodia, which suggested that polymerized DHN was not required for initial hyphopodial development. Our heavily-melanized mutant produced dark hyphopodia with little or no

lobing on mylar membranes. Tricyclazole-treated mutant JH4301 hyphopodia were lobed and orange with little melanization. Perhaps mature, untreated JH4301 hyphopodia accumulated more melanin than the wild-type as they aged, and the structural rigidity and consequent turgor increase associated with melanin (19, 20) caused the lobes to unfold. The dark mutant appeared to either secrete or retain more mucilage around its cell walls than the wild-type strain, and the light mutant displayed more fibrillar mucilage. Mucilage is usually present on young hyphae, synthesized at or near the hyphal tip (14, 26, reviewed in 31), and typically consists of protein or glycoprotein (26). Perhaps the mucilage in these strains was of similar composition and amount, but differences in cell wall melanin affected its appearance and/or retention to the cell wall. Ellis and Griffiths (1975) observed melanin granules within cell walls of *Phomopsis*, but also found melanin deposition within mucilage. However, while the composition of *G. graminis* var. *graminis* mucilage is unknown, its accumulation by the dark mutant may have contributed to its slow growth. In addition to differences in hyphopodia and cell wall structure between strains, our light mutant had orange-brown perithecia but produced mature ascospores when selfed, while the heavily pigmented

mutant developed very small, dark, round perithecia without mature ascospores, and required the presence of wild-type to produce viable ascospores.

Melanin serves as a protection against a number of environmental stressors. Melanized spores are more resistant to UV irradiation (10), and melanized *Cryptococcus neoformans* yeast cells are less susceptible to UV light (37). Aromatics such as naphthalene are known to absorb UV light, and DHN-melanin probably provides protection to hyphae, thereby enhancing survivability in the soil environment. Our heavily melanized mutant was more resistant to UV irradiation than the wild-type strain, and the light mutant was highly susceptible to UV damage. UV damage enhances melanogenesis in mammalian cells (11), which synthesize L-DOPA melanin. We observed a similar increase in DHN-melanin production of our melanin-containing strains (wild-type and dark mutant JH4301, data not shown).

Melanins in fungi are important for resistance to microbial attack by lytic enzymes. Melanized structures, including sclerotia, conidia, and hyphae, are less susceptible to lysis than hyaline structures (4, 23, 25, 29). Attempts to digest *G. graminis* var. *graminis* with chitinase (Sigma C7809) were unsuccessful (data not

shown), perhaps because chitin was associated with glucan. We digested our wild-type and melanin mutant strains with Novozyme 234, which is a mixture of chitinase and glucanases, and found a similar correlation between melanization of *G. graminis* var. *graminis* strains and resistance to lytic attack. While our heavily melanized mutant initially had significantly more N-acetylglucosamine at the onset of lysis by Novozyme 234, after three hours of exposure very little chitin degradation was detected. This result suggested that chitin was released in the absence of Novozyme, and the enzymes had little effect on chitin degradation. By comparison, Novozyme treatment increased N-acetylglucosamine release from chitin from our light mutant, and to a lesser extent from the wild-type, indicating that melanin serves as a protective layer surrounding chitin.

Lytic enzymes are secreted by vesicles that fuse with the plasma membrane at the growing apex (38), and it is believed that protein secretion is restricted to hyphal tips (31). Because hyphal tips are unmelanized, it is unlikely that melanin acts as a physical barrier to enzyme secretion. Enzymes such as cellulase and polygalacturonase can be inhibited by phenols like those found in melanins (5), so perhaps melanin acts as an enzyme inactivator.

Melanin extracted from *G. graminis* inhibits lytic enzyme activity of *Streptomyces lavendulae* (35). Tricyclazole inhibition of our melanized strains resulted in increased enzyme activity, suggesting that phenolic polymers, rather than phenols such as 2-hydroxyjuglone and flaviolin, are responsible for enzyme inhibition. Alternatively, tricyclazole may directly enhance enzyme secretion by some unknown mechanism.

Pathogenicity is associated with melanin synthesis for a number of plant pathogenic fungi. Melanization of *Magnaporthe grisea* appressoria (similar to hyphopodia) is required to sustain the turgor pressure necessary to penetrate leaves via infection pegs that protrude from the adhesive surface of the appressorium (8, 19, 20). A similar melanin function is suggested for *Colletotrichum lagenerium* (24) and *C. gloesporiodes* (22) appressoria. In contrast to these fungal pathogens, our results do not suggest that melanin is required for *G. graminis* var. *graminis* pathogenicity to rice, at least in laboratory infections. Hyphopodia, unlike appressoria, are not required for plant penetration, as *G. graminis* hyphae can invade root tissue of host plants via enzymatic digestion (32). Other studies also demonstrated that compounds that inhibit DHN-melanin synthesis (tricyclazole, pyroquilon and fthalide) do

not alter *G. graminis* var. *graminis* pathogenicity to laboratory-infected wheat seedlings (13).

In the field, melanin may contribute to pathogenicity by protecting the fungus from environmental stressors and predation. We have shown that melanized *G. graminis* var. *graminis* hyphae are protected from lytic enzymes and UV light. However, melanized strains also have less lytic enzyme activity, and enzymatic lysis is essential for root penetration which sets the stage for pathogenesis. Therefore, to be a successful pathogenic competitor in a mixed microbial community, *G. graminis* var. *graminis* must synthesize enough melanin for protection without compromising its ability to secrete active lytic enzymes. Based on this argument, while melanin synthesis may not directly enhance *G. graminis* var. *graminis* pathogenicity, moderately melanized strains are more likely to survive saprophytically and become pathogenic should a suitable host be available.

Literature Cited

1. Addleman, K., T. Dumonceaux, M. G. Paice, R. Bourbonnais and F. S. Archibald. 1995. Production and characterization of *Trametes versicolor* mutants unable to bleach hardwood kraft pulp. Appl. Environ. Microbiol. **61**:3687-3694.

2. Bell, A. A. and M. H. Wheeler. 1986. Biosynthesis and functions of fungal melanins. Ann. Rev. Phytopathol. **24**:411-451.

3. Bowyer, P., R. Musker, A. E. Osborne, B. Clark, C. Caten and M. J. Daniels. 1992. Production of mutants of *Gaeumannomyces graminis* var. *tritici* and var. *avenae* by 4-nitroquinolene-oxide treatment of protoplasts. Fungal Genet. Newslett. **39**:13-14.

4. Bloomfield, B. J. and M. Alexander. 1967. Melanins and resistance of fungi to lysis. J. Bacteriol. **93**:1276-1280.

5. Bull, A. T. 1970. Kinetics of cellulase inactivation by melanin. Enzymologia **39**:333-347.

6. Butler, M. J. and M.-A. Lachance. 1986. Quantitative binding of Azure A to melanin of the black yeast *Phaecoccomyces*. Exp. Mycol. **10**:166-170.

7. Caesar-TonThat, T. C., F. van Ommen Kloeke, G. G. Geesey and J. M. Henson. 1995. Melanin production by a filamentous soil fungus in response to copper and localization of copper sulfide by silver-staining. Appl. Environ. Microbiol. **61**:1968-1975.

8. Chumley, F. G. and B. Valent. 1990. Genetic analysis of melanin-deficient, non-pathogenic mutants of *Magnaporthe grisea*. Mol. Plant Microbe Interact. **3**:135-143.

9. Dopi, S., Z. Solel and I. Barash. 1995. Cell wall-degrading enzymes produced by *Gaeumannomyces graminis* var.*tritici in vitro* and *in vivo*. Physiol. Mol. Plant Pathol. **46**:189-198.

10. Durrell, L. W. 1964. The composition and structure of dark fungus spores. Mycopathol. Mycol. Appl. **23**:339-45.

11. Eller, M. S. K. Ostrom and B. A. Gilchrest. 1996. DNA damage enhances melanogenesis. Proc. Natl. Acad. Sci. USA. **93**:1087-1092.

12. Ellis, D. H. And D. A. Griffiths. 1975. Melanin deposition in the hyphae of a species of *Phomopsis*. Can. J. Microbiol. **21**:442-452.

13. Elliott, M. L. 1995. Effect of melanin biosynthesis inhibiting compounds on *Gaeumannomyces* species. Mycologia. **87**:370-374.

14. Evans, R. C., Stempen, H., and P. Frasca. 1981. Development of hyphal sheaths in *Bipolaris maydis* race T. Can. J. Bot. **59**:453-459.

15. Garrett, S. D. 1970. Pathogenic root-infecting fungi. Cambridge Univ. Press. London.

16. Gold, M. H. And T. M. Cheng. 1978. Induction of colonial growth and replica plating of the white rot Basidiomycete *Phanaerochaete chrysosporium*. Appl. Environ. Microbiol. **35**:1223-1225.

17. Harling, R., L. Kenyon, B. G. Lewis, R. P. Oliver, J. G. Turner and A. Coddington. 1988. Conditions for efficient isolation and regeneration of protoplasts from *Fulvia fulva*. J. Phytopath. **122**:143-146.

18. Hoch, H. C. 1986. Freeze substitution of fungi, p. 183-212. *In* H. C. Aldrich and W. J. Todd (ed.), Ultrastructure techniques of microorganisms. Plenum Press, New York.

19. Howard, R. J. and M. A. Ferrari. 1989. Role of melanin in appressorium function. Exp. Mycol. **13**:403-418.

20. Howard, R. J., M. A. Ferrari, D. H. Roach, and N. P. Money. 1991. Penetration of hard substrates by a fungus employing enormous turgor pressure. Proc. Natl. Acad. Sci. USA **88**:11281-11284.

21. Howard, R. J. and K. L. O'Donnell. 1987. Freeze substitution of fungi for cytological analysis. Exp. Mycol. **11**:250-269.

22. Hwang, C.-S., M. A. Fliashman and P. E. Kolattukudy. 1995. Cloning of a gene expressed during appressorium formation by *Colletotrichum gloeosporiodes* and a marked decrease in virulence by disruption of this gene. Plant Cell **7**:183-193.

23. Hyakumachi, M. K. Yokoyama and T. Ui. 1987. Role of melanin in susceptibility and resistance of *Rhizoctonia solani* to microbial lysis. Trans. Br. Mycol. Soc. **89**:27-33.

22. Kubo, Y. and I. Furusawa. 1991. Melanin biosynthesis, p. 205-218. *In* G. T. Cole and H. C. Hoch (ed.), The fungal spore and disease initiaition in plants and animals. Plenum Press, New York.

25. Kuo, M.-J. and M. Alexander. 1967. Inhibition of the lysis of fungi by melanins. J. Bacteriol. **94**:624-629

26. Nicholson, R. L. and L. Epstein. 1991. Adhesion of fungi to the plant surface, p.3-23. *In* G. T. Cole and H. C. Hoch (ed.), The fungal spore and disease initiation in plants and animals. Plenum Press, New York.

27. Perpetua, N. S., Y. Kubo, N. Yasuda, Y. Takano and I. Furusawa. 1996. Cloning and characterization of a melanin biosynthetic *THR1* reductase gene essential for appressorial penetration of *Colletotrichum lagenarium.* Mol. Plant-Microbe Interact. **9**:323-329.

28. Pilgram, A. L., T. Goins and J. M. Henson. 1993. The fate of integrated DNA in *Gaeumannomyces graminis* transformants. FEMS Microbiol. Let. **113**:309-314.

29. Potgeiter, H. J. and M. Alexander. 1966. Susceptibility and resistance of several fungi to microbial lysis. J. Bacteriol. **91**:1526-1532.

30. Reissig, J. L., J. L. Strominger, and L. F. Leeloir. 1955. A modified colorimetric method for the estimation of *N*-acetylamino sugars. J. Biol. Chem. **217**:929-966.

31. Sietsma, J. H., H. A. B. Wösten and J. G. H. Wessels. 1995. Cell wall growth and protein secretion in fungi. Can J. Bot. **73**:S388-S395.

32. Skou, J. P. 1981. Morphology and cytology of the infection process. p. 175-198. *In* M. J. C. Asher and P. J. Shipton (ed.), Biology and control of take-all. Academic Press, London.

33. Smiley, R. W., P. H. Dernoeden, and B. B. Clarke. 1992. Compendium of turfgrass diseases, 2nd. Ed. American Phytopathological Society, St. Paul Minn.

34. Takano, Y., Y. Kubo, K. Shimizu, K. Mise, T. Okuno and I. Furusawa. 1995. Structural analysis of *PKS1*, a polyketide synthase gene involved in melanin biosynthesis in *Colletotrichum lagenarium.* Mol. Gen. Genet. **249**:162-167.

35. Tschudi, S. and H. Kern. 1979. Specific lysis of the mycelium of *Gaeumannomyces graminis* by enzymes of *Streptococcus lavendulae.* Pp. 611-615. *In* B. Schippers and W. Gams (ed.), Soil-borne plant pathogens. Academic Press, London.

36. Walker, J. 1981. Taxonomy of take-all fungi and related genera and species, p. 15-74. *In*M. J. C. Asher and P. J. Shipton (ed.), Biology and control of take-all. Academic Press, London.

37. Wang, Y. And A. Casadevall. 1994. Decreased susceptibility of melanized *Crytococcus neoformans* to UV light. Appl. Environ. Microbiol. **60**:3864-3866.

38. Wessels, J. G. H. 1993. Wall growth, protein secretion and morphogenesis in fungi. Tansley Review No. 45. New Phytol. **123**:397-413.

39. Weste, G. 1970. Extracellular enzyme production by various isolates of *Ophiobolus graminis* and *Ophiobolus avenae* I. Enzymes produced in culture. Phytopath. Zeit. **67**:189-204.

CHAPTER 3

COPPER BINDING AND LOCALIZATION OF CuS BY SILVER STAINING OF *Gauemannomyces graminis* var. *graminis* MELANIN MUTANTS

Introduction

Heavy metal concentrations in soil are increasing as a result of fossil fuel combustion, and mining, industrial and agricultural practices (1). Although some metallic elements are essential for microbial growth, their overabundance leads to excessive cellular accumulation, with subsequent toxic effects (reviewed in reference 16). Environmental metal concentrations frequently fluctuate and many fungi have developed mechanisms to cope with higher, more toxic levels of metals they encounter. Wall and membrane layers of eukaryotic microbes provide the major barrier to the influx and efflux of metal ions (25). Metals excluded by various metal tolerant microbes include lead, mercury, copper, cobalt, zinc, cadmium and manganese (12, 18, 19). Fungi also produce metallothioneins, small cysteine-rich proteins that bind and sequester heavy metals intracellularly (15, 32). *Saccharomyces cerevisiae* generates H_2S

that traps metal ions as insoluble metal sulfides either extra- or intracellularly (8, 24). Citric acid is also produced by many fungi and chelates copper and other metal ions (21).

Fungi also produce cell wall materials and extracellular substances, including phenolic polymers, glucans, mannans, melanins, chitins and chitosans that may remove or bind heavy metals from solution (23). In metal binding studies with *S. cerevisae* cell wall polymers, mannans were the most significant accumulators of metals, particularly copper (5). Other studies demonstrate that metal binds to carboxyl and amino groups, which suggests that protein and carbohydrate fractions are involved in metal binding (3).

Fungal pigments such as melanin are also implicated in metal binding and may serve as a protective mechanism against metal toxicity (reviewed in reference 16). Melanins are heterogenous, high molecular weight pigments produced by many fungi and often they are made in response to stress (reviewed in reference 2). These darkly pigmented polymers may serve as a protective barrier against adverse environmental conditions such as UV light, lytic enzymes, plant defense mechanisms, predation, desiccation, and metals. Fungi produce different types of melanin

including polymers of L-dihydroxyphenylalanine (L-DOPA), catechol, dihydroxybenezene, glutaminyl-3,4-dihydroxybenzene and 1,8-dihydroxynaphthalene (DHN). Because of its negatively charged hydroxyl groups, DHN-melanin could potentially bind cations and exclude them from cells. In addition, amino acids and carboxyl groups that act as binding sites for positively charged ions may be present in other macromolecules that are trapped in the melanin polymer (30). Both the reaction characteristics and chemical structures of melanin and of weak metal cation exchangers are similar and suggest that metal ions and melanin interact by ion exchange (6) *In vivo* and *in vitro* studies demonstrate that fungal melanins are efficient metal biosorbants (17, 18, 20, 26, 28), and suggest that this metal accumulation by fungal biomass may be exploited for heavy metal removal from contaminated soils or water (3, 4, 13, 14).

G. graminis var. *graminis* is a homothallic filamentous ascomycete that produces melanin via the dihydroxynaphthalene (DHN) pathway. We previously showed that cultures of *G. graminis* var. *graminis* grown in the presence of tricyclazole, a specific inhibitor of DHN-melanin synthesis (2), bound significantly less copper than cultures grown without tricyclazole, and silver

staining demonstrated localization of CuS within the melanin layer (7). In the present study, we examined the role of melanized fungi in copper binding using isogenic *G. graminis* var. *graminis* melanin mutants.

Materials and Methods

Fungal Strains and Growth Conditions

Generation, growth and characterization of *G. graminis* var. *graminis* melanin was previously described in Chapter 2. The three strains chosen for study were JH2033 (wild-type), JH4300 (a mutant derived from JH2033 that does not synthesize DHN-melanin), and JH4301 (a mutant derived from JH2033 that overproduces melanin).

Growth Curves in Minimal Medium with Copper Amendment

Two hundred mls of minimal medium were supplemented with trace (80 nM Cu), 20 μM Cu, 40 μM Cu or 80 μM Cu from a 1% (w/v) $CuSO_4$ stock solution. Five LB agar plugs were added to triplicate flasks, which were shaken at 50 rpm and harvested at 9,

14, 18 and 24 days. Hyphae were recovered by filtration through a Whatman #1 filter, lyophilized overnight and weighed.

Copper Binding and Accumulation

Fourteen day-old cultures grown in minimal medium were harvested by filtration through a Whatman #1 filter. Hyphae were resuspended in 100 ml 40 mM $CuSO_4$, shaken at 50 rpm for 1 h at 25 C, washed three times with 200 ml distilled water and frozen at -20 C until ICP-AES analysis of copper binding.

Copper accumulation by fungal biomass was also determined by ICP-AES following 14 days of growth in minimal medium containing trace (8 nM), 20, 40, 60 or 80 μM $CuSO_4$. Cultures were harvested by filtration through a Whatman #1 filter, washed 3X with distilled H_2O and then lyophilized overnight and frozen at -20 C until analysis.

Inductively Coupled Plasma
Atomic Emission
Spectroscopy (ICP-AES)

Samples were dried at 105 C for 2 h, weighed (16-104 mg) into glass vials and digested in 1 ml of 1:1 nitric acid at 70 C. After 2 h, 1 ml of 1:1 HCl was added and heating continued for 10 min. Samples were diluted to 10 ml with distilled water and analyzed on a Leeman Labs PS950 ICP spectrometer with background correction. Samples were diluted as needed in 2% HNO_3 prior to

ICP measurements. ICP analysis were performed at Little Bear Laboratories by Dr. Greg Olson.

Silver Staining

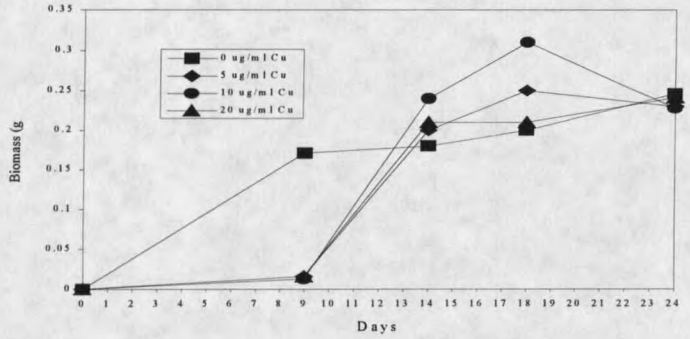
14 day-old cultures were harvested from minimal medium containing 40 μM $CuSO_4$. Hyphae were fixed and stained for 10 min as described elsewhere (7) following manufacturer's protocol (HQ SILVER, Nanoprobes).

Results

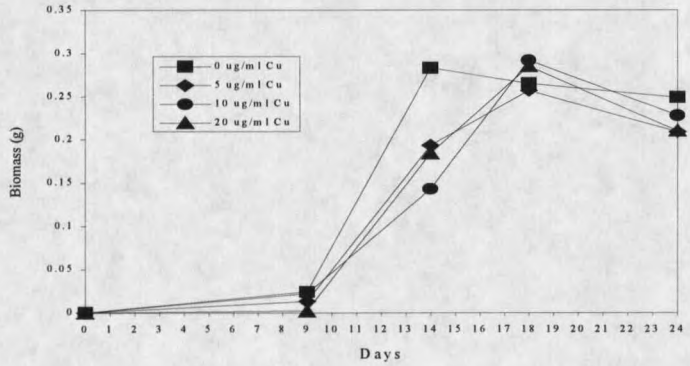
G. graminis var. *graminis* Growth with Copper

The wild-type strain and the light mutant JH4300 were not inhibited by copper concentrations up to 80 μM; after 24 days of growth, these two strains had approximately equivalent biomass with trace or higher copper in the medium (Fig. 3.1a and 3.1b). Cultures of the dark mutant JH4301 were inhibited by all concentrations of copper (Fig. 3.1c). At the higher copper concentrations (60 and 80 μM $CuSO_4$), growth of this mutant was almost completely inhibited. This strain also grew at a slower rate in minimal medium with trace copper than the other two strains.

A. JH4300



B. JH2033



C. JH4301

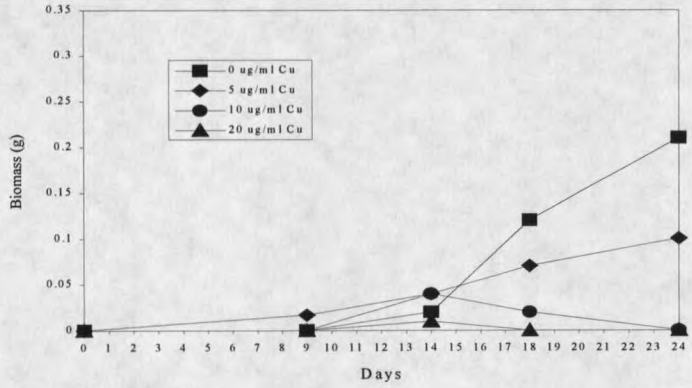


Figure 3.1. *G. graminis* var. *graminis* wild-type and melanin mutant growth in minimal medium containing varying concentrations of $CuSO_4$. Biomass is the total biomass in 200 ml culture. Each point is the average of 3 replicates.

Copper Binding to Hyphae

Copper accumulation with the dark mutant was greater than with the light mutant or wild-type for all copper concentrations, and JH4300 accumulated less copper than the two pigmented strains (Table 3.1) following 14 days growth in copper-containing minimal medium. The dark mutant JH4301 accumulated more copper than the other strains at all copper concentrations. In addition, fourteen day-old cultures of JH4301 grown in minimal medium without copper absorbed significantly more copper than light mutant JH4300 or the wild-type after 1 h exposure to 40 mM $CuSO_4$ (Table 3.2).

Silver Enhancement of Copper Sulfide

Silver staining demonstrated the presence of copper sulfide in hyphae of the wild-type and strain JH4301 following growth for 14 days with 40 μM $CuSO_4$ (Fig. 3.2). Silver deposition was observed within septa, which stained a dark brown, and within cell walls which imparted a yellowish-brown color to hyphae. No silver deposits were observed in the light strain JH4300 after growth in 40 μM $CuSO_4$, and this suggested that CuS did not accumulate in this strain.

Table 3.1. Cu accumulation of wild-type *G. graminis* var. *graminis* and two melanin mutant strains following 14 days growth in minimal medium with Cu. Concentrations are mg Cu/g fungal biomass and the average of 4 replicates. Means followed by different letters across a row are significantly different by Tukey's Studentized Range (HSD) at $P < 0.05$.

	Strain		
Medium Cu Concentration (μM)	Light Mutant JH4300	Wild-type JH2033	Dark Mutant JH4301
Trace (8 nM)	0.00625 a	0.00375 a	0.027 b
20	0.665 a	2.17 b	4.36 c
40	2.57 a	4.73 b	7.97 c
60	3.09 a	9.82 b	16.2 c
80	3.72 a	9.42 b	19.3 c

Table 3.2. Copper bound to 14 day-old cultures of wild-type *G. graminis* var. *graminis* and two melanin mutants following 1 h exposure of hyphae to 40 mM $CuSO_4$ after 14 days growth in minimal medium with trace Cu. Concentrations are mg Cu/g fungal biomass and the average of 2 replicates. Means followed across treatment rows with different letters are significantly different at $P < 0.05$ (Tukey's Studentized Range test, HSD).

	Strain		
Treatment	Light Mutant JH4300	Wild-type JH2033	Dark Mutant JH4301
No Cu	0* a**	0 a	0 a
Cu	22.85 a	32.15 b	67.30 c

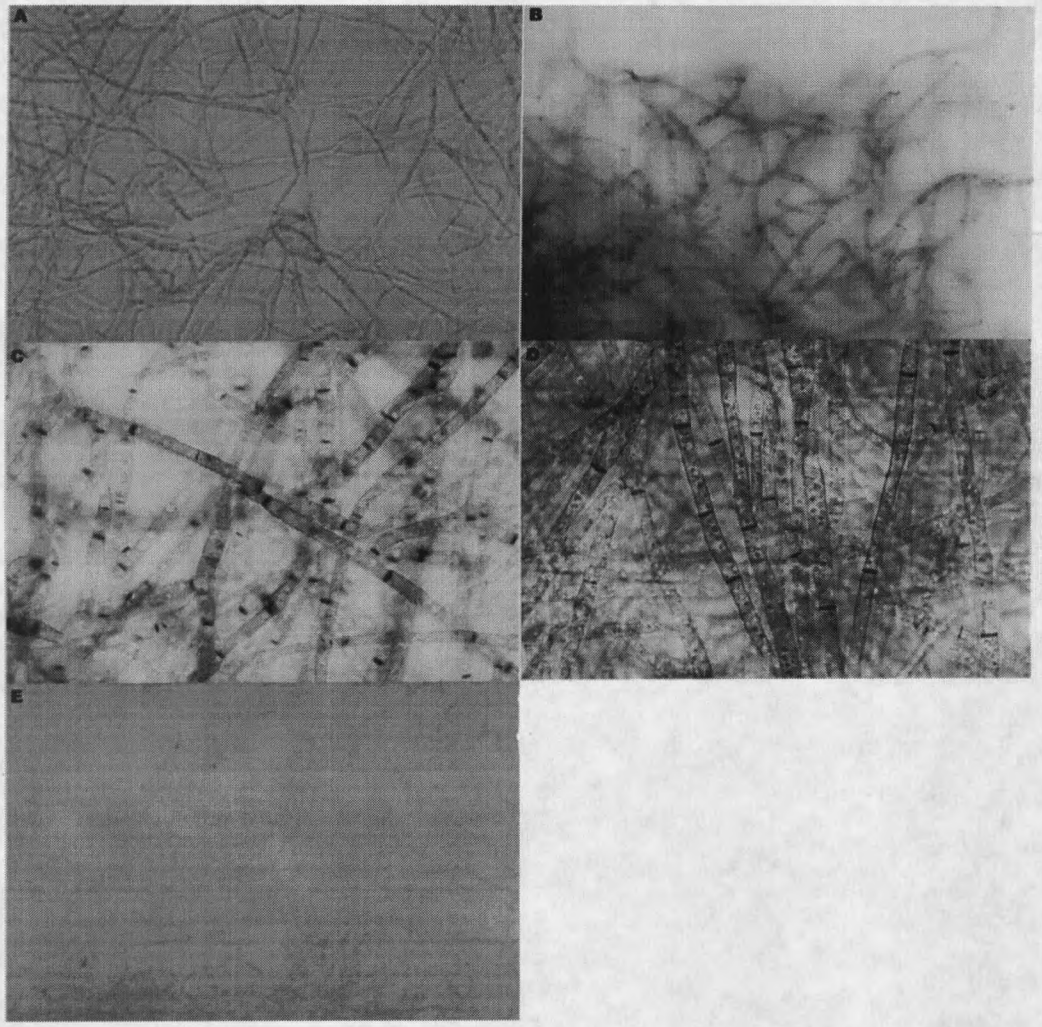


Figure 3.2. Silver-stained hyphae showing localization of CuS. Strains were grown for 14 days in minimal medium containing 40 μM $CuSO_4$. A. Light mutant JH4300 (500 X), B. Wild-type JH2033 (500 X), C. Wild-type JH2033 (1250 X), D. Dark mutant JH4301 (1250 X), E. Wild-type unstained (500 X).

Discussion

Previous studies show that L-DOPA melanin absorbs copper (22, 30, 31). In addition, tricyclazole-treated *G. graminis* var.

graminis cultures bind less copper than untreated cultures (7), a finding that suggests that DHN-melanin is involved in copper binding. Our heavily-melanized mutant strain bound significantly more copper than the wild-type strain or the light mutant when grown in Cu. These findings suggested that pigment overproduction was associated with copper binding. Alternatively, increased mucilage secretion by the dark mutant may have contributed to copper binding. Our light mutant did not bind as much copper as the wild-type strain when grown in Cu or when grown with trace copper and then exposed. The mucilage layer or other cellular component of the light mutant may have bound some copper.

Silver staining is caused by silver precipitation around CuS nucleation sites in which 10 or more molecules of CuS exist (9-11) and confirmed the presence of CuS in septa and cell walls of the wild-type and over-melanized mutant grown in $CuSO_4$. CuS was not apparent in hyphal cell walls or septa of the unmelanized mutant, a finding that suggested that cell wall melanin is required for copper sulfide accumulation. CuS staining may be more indicative of copper accumulation associated with melanin than ICP-AES analysis of whole cells, which may have bound copper in forms other than CuS.

Melanin serves as general protection and may provide a physical barrier against copper. While our heavily melanized mutant accumulated 1.6-2X as much copper as the wild-type and

3-6.6X as much copper as our light mutant (Table 3.1), growth was almost completely inhibited with this strain at higher copper concentrations. We observed a similar delayed growth of the melanized wild-type in the presence of 80 μM copper, but surprisingly, the unmelanized mutant was not inhibited by higher concentrations of Cu. Copper binding to melanin may actually inhibit growth if it reaches levels toxic to the fungus and, while the wild-type strain was able to overcome this toxicity, the slow growing dark mutant could not. We speculate that since melanized strains accumulated more Cu than the unmelanized mutant, melanin may be involved in sequestering metals, such as Cu or Fe that are necessary for growth. Rizzo et al. (1992) suggested that a coating of metal ions on fungal surfaces of *Amarillia* also act as protection from antagonistic microorganisms. Perhaps copper binding to *G. graminis* var. *graminis* provides a similar function in the environment.

Literature Cited

1. Ayers, R. U. 1992. Toxic heavy metals: Materials cycle optimization. Proc. Natl. Acad. Sci. USA **89**:815-820.

2. Bell, A. A. and M. H. Wheeler. 1986. Biosynthesis and functions of fungal melanins. Ann. Rev. Phytopathol. **24**:411-451.

3. Brady, D. and J. R. Duncan. 1994. Binding of heavy metals by the cell walls of *Saccharomyces cerevisiae*. Enyz. Microb. Technol. **16**:633-638.

4. Brady, D. and J. R. Duncan. 1994. Bioaccumulation of metal cations by *Saccharomyces cerevisiae*. Appl. Microbiol. Biotechnol. **41**:149-154.

5. Brady, D., A. D Stoll, L. Starke and J. R. Duncan. 1994. Chemical and enzymatic extraction of heavy metal binding polymers from isolated cell walls of *Saccharomyces cerevisiae*. Biotechnol. Bioeng. **44**:297-302.

6. Bruenger, F. W., B. J. Stover and D. R. Atherton. 1967. The incorporation of various metal ions in *in vivo*- and *in vitro*-produced melanin. Rad. Res. **32**:1-12.

7. Caesar-TonThat, T. C., F. van Ommen Kloeke, G. G. Geesey and J. M. Henson. 1995. Melanin production by a filamentous soil fungus in response to copper and localization of copper sulfide by silver-staining. Appl. Environ. Microbiol. **61**:1968-1975.

8. Cervantes, C. and F. Gutierrez-Corona. 1994. Copper resistance mechanisms in bacteria and fungi. FEMS Microbiol. Rev. **14**:121-138.

9. Danscher, **G.** 1981. Histochemical demonstration of heavy metals. Histochemistry **71**:1-16.

10. Danscher, G. 1984. Autometallography. A new technique for light and electron microscopic visualization of metals in biological tissues (gold, silver, metal sulfides and metal selenides). Histochemistry **81**:331-335.

11. Danscher, G. 1991. Detection of metals in tissues. Histochemical tracing of zinc, mercury, silver and gold. Prog. Histochem. Cytochem. **23**:273-285.

12. Foster, P. L. 1977. Copper exclusion as a mechanism of heavy metal tolerance in a green algae. Nature **269**:322-323.

13. Fourest, E. and J.-C. Roux. 1992. Heavy metal biosorption by fungal by-products: mechanisms and influence of pH. Appl. Micobiol. Biotechnol. **37**:399-403.

14. Fourest, E., C. Canal and J.-M. Roux. 1994. Improvement of heavy metal biosorption by mycelial dead biomasses (*Rhizopus arrhizus, Mucor miehei* and *Penicillium chyrsogenum*): pH control and cationic activation. FEMS Microbiol. Rev. **14**:325-332.

15. Fürst, P., S. Hu, R. Hackett and D. Hamer. 1988. Copper activates metallothionein gene transcription by altering the conformation of a specific DNA binding protein. Cell **55**:705-717.

16. Gadd, G. M. 1993. Interactions of fungi with toxic metals. New Phytol. **124**:25-60.

17. Gadd, G. M. and L. deRome. 1988. Biosorption of copper by fungal melanin. Appl. Microbiol. Biotechnol. **29**: 610-617.

18. Gadd, G. M. and A. J. Griffiths. 1978. Microorganisms and heavy metal toxicity. Microb. Ecol. **4**:303-317.

19. Gadd, G. M. And A. J. Griffiths. 1980. Influence of pH on toxicity and uptake of copper by *Aureobasidium pullulans*. Trans. Br. Mycol. Soc. **75**:91-6.

20. Gadd, G. M. and J. L. Mowll. 1985. Copper uptake by yeast-like cells, hyphae and chlamydospores of *Aureobasidium pullulans*. Exp. Mycol. **9**:230-240.

21. Hughes, M. N. and R. K. Poole. 1989. Metals and micro-organisms. Chapman and Hall, London, New York.

22. Larsson, B. and H. Tjälve. 1978. Studies on the melanin-affinity of metal ions. Acta Physiol. Scand. **104**:479-484.

23. Lepp, N. W. 1992. Uptake and regulation of metals in bacteria and fungi, p. 277-298 *In* D. C. Adriano (ed.), Biogeochemistry of trace metals. CRC Press, Boca Raton, FL.

24. Lin, C.-M., B. F. Crawford and D. J. Kosman. 1993. Distribution of ^{64}Cu in *Saccharomyces cerevisiae*: cellular locale and metabolism. J. Gen. Microbiol. **139**:1605-1615.

25. Rogers, H. J., H. R. Perkins and J. B. Ward. 1980. Microbial cell walls and membranes. Chapman-Hall, London, New York.

26. Rizzo, D. M., R. A. Blanchette, and M. A. Palmer. 1992. Biosorption of metal ions by *Armillaria* rhizomorphs. Can. J. Bot. **70**:1515-1520.

27. Rogers, H. J., H. R. Perkins and J. B. Ward. 1980. Microbial cell walls and membranes. Chapman-Hall, London, New York.

28. Saiz-Jimenez, C. and F. Shafizadeh. 1984. Iron and copper binding by fungal phenolic polymers: an electron spin resonance study. Curr. Microbiol. **10**:281-286.

29. Sarna, T., W. Fronicz and J. S. Hyde. 1980. Cu^{2+} probe of metal-binding sites in melanin using electron paramagnetic resonance spectroscopy. Arch. Biochem. Biophys. **202**:304-313.

30. Sarna, T., J. S. Hyde and H. M. Swartz. 1976. Ion exchange in melanin. An electron spin study with lantanide probes. Science **192**:1132-1134.

31. Senesi, N., G. Sposito and J. P. Martin. 1987. Copper (II) and iron (III) complexation by humic acid-like polymers (melanins) from soil fungi. Sci. Total Environ. **62**:241-252.

32. Thiele, D. J. 1988. *ACE1* regulates expression of the *Saccharomyces cerevisiae* metallothionien gene. Mol. Cell. Biol. **8**:2745-2752.

33. Whiteside, S. G. And D. J. Plocke. 1992. Selection and charaterization of a copper-resistant subpopulation of *Schizosaccharomyces pombe*. J. Gen. Microbiol. **138**:2417-242.

CHAPTER 4

CONCLUSIONS

1. Melanin protects fungi from UV light damage and lytic enzyme activity. Overmelanization results in decreased enzyme secretion. Melanin contributes to the competitive ability of fungi in mixed communities by enhancing protection against environmental stressors, but overexpression of melanin compromises enzyme secretion needed for nutrient acquisition and pathogenicity.

2. Melanized *G. graminis* var. *graminis* strains bind more Cu^{2+} than unmelanized strains, and the amount of copper binding is proportional to the melanin concentration.

3. Melanized *G. graminis* var. *graminis* strains bind more metals in mine tailings than the unmelanized strain, but with the exception of lead, the presence of the melanized strains do not affect metal uptake into plant tissue. Melanized *G.*

graminis var. *graminis* in the rhizosphere enhances lead uptake into plant tissue.

APPENDICES

APPENDIX A

RHIZOSPHERE COMPETITIVENESS OF *Gaeumannomyces graminis* var. *graminis* MELANIN MUTANTS

The rhizosphere is the zone surrounding plant roots and the site of intense microbial activity. Plants release nutrients into the rhizosphere that are utilized by soil microorganisms, and rhizosphere inhabitants in turn mineralize soil-bound nutrients for plant uptake (3). Many soil-borne fungal pathogens can exist saprophytically in the rhizosphere, where they are in competition with other rhizosphere microorganisms for substrates. In addition, competing microorganisms may possess inhibitory or antagonistic mechanisms that can affect other organisms in the same environment.

Fungal melanins are heterogeneous pigments that serve as a protection from environmental stressors (1). Some plant pathogens such as *Colletotrichum lagenarium* and *Magnaporthe grisea* require melanization for effective pathogenicity (4, 8). These fungi develop a melanized adhesion structure that allows the turgor pressure needed for leaf penetration (6, 7). The filamentous soil fungus *Gaeumannomyces graminis* var. *graminis* is also melanized, but there is no evidence to suggest a direct relationship between melanogenesis in this fungus and pathogenicity (5). However, melanin may enhance survivability in a competitive environment and thus contribute to pathogenicity indirectly. In

this experiment, we attempted to determine the effect of *G. graminis* var. *graminis* melanization on rhizosphere competitiveness, using mutants affected in the melanin pathway. We also tried to characterize the rhizosphere fungal community using PCR amplification of the ITS region (Figure A.1). Eukaryotic ribosomal genes are arranged in a tandem repeat with the 5.8S coding region flanked by internal transcribed spacers (ITS). Insertions and/or substitutions occur within the sequence of the multiple ITS copies (2). We expected to tentatively detect the survivability of *G. graminis* var. *graminis* in the presence of competing fungi by amplification of the ITS region consistent in size with *G. graminis* var. *graminis*.

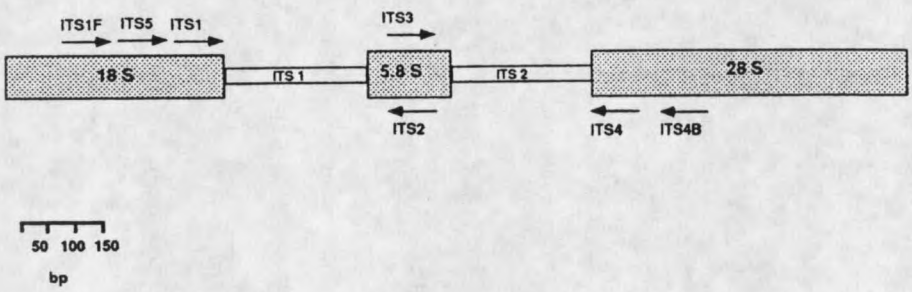


Figure A.1. Internal transcribed spacer (ITS) region of ribosomal DNA (2). Shadowed boxes represent ribosomal subunits, ans arrows indicate position and direction of PCR primers.

Materials and Methods

Competition Assay

Rhizosphere inoculum was prepared by weighing 50 g of vermiculite into each of 4 pots, adding 20 g of garden soil, and mixing well. Twenty non-sterile rice seeds (*O. sativa* Lemont) were added to each pot, the pots transferred to a greenhouse and watered with 100 ml deionized water and 50 ml ¼ strength Hoagland's solution. Pots were watered on alternate days with 50 ml ¼ strength Hoagland's or water and plants allowed to grow for 28 days. At harvest, plants were removed and contents of the four pots were turned into a sterile glass pan. The inoculated vermiculite was thoroughly mixed with a flame-sterilized spatula and used for the rhizosphere inoculum.

Sterile rice seedlings were prepared by removing hulls from seeds, soaking the seeds overnight in 100 ml sterile deionized water, then adding 25 ml Clorox® bleach and 250 µl Tween 80 (Sigma). After swirling to mix, seeds were left in the Tween 80/ bleach solution for 20 minutes, with occasional swirling. Contents of flasks were poured into sterile petri dishes and aseptically transferred to petri dishes containing sterile deionized water.

Seeds were rinsed again with sterile deionized water, then transferred to petri plates containing ¼ strength Hoagland's solution with 0.75% Bacto agar. Five day-old seedlings were used for the competition assay.

The uninoculated (no rhizosphere inoculum) treatment was prepared by weighing 50 g of vermiculite into 20 cm^2 plastic pots with Whatman #1 filter paper in the bottom of the pots to cover drain holes. Pots were covered with aluminum foil and sterilized by autoclaving. Twenty seedlings were transferred to each pot, along with 12 X 3 mm LB agar plugs containing wild-type, JH4300 or JH4301 or no inoculum spaced between seedlings. The inoculated (with rhizosphere inoculum) treatment consisted of plastic pots with 40 g sterile vermiculite with 20 g rhizosphere inoculum added to the top of the pot after autoclaving. Seedling and fungal inoculation were the same as for the no rhizosphere treatment. All treatments were prepared in triplicate.

Plants were grown for 28 days in a greenhouse with watering every other day, alternating with 50 ml sterile 1/4 strength Hoagland's solution or sterile deionized water. At harvest, plants were removed from pots, vermiculite adhering to roots was collected aseptically into sterile tubes, and frozen at -20 °C until

further analysis. Plant roots were washed under running water to remove any additional vermiculite, then separated from shoots, and both roots and shoots freeze-dried overnight to obtain dry weights.

Rhizosphere DNA Extraction and PCR Amplification

Rhizosphere DNA was extracted as described by Tsai and Olson (1991) with the following modifications: 2 g of vermiculite were extracted; following the lysis step, 1 ml of a solution containing 1% CTAB and 0.1 M NaCl was added, mixed and incubated at 60 °C for 1 hour with gentle shaking; and finally, the extracted DNA was brought up to a 1 ml volume and purified by passing through a Sephadex G200 column.

Purified DNA was PCR-amplified using primers ITS4 (TCCTCCGCTTATTGATATGC) and ITS5 (GGAAGTAAAGTCGTAA CAAGG). The temperature program was 94 °C, 2.5 minutes for 1 cycle, then 94 °C (15 s), 53 °C (30 s), 72 °C (1.5 minutes) for 40 cycles, followed by 72 °C for 10 minutes. PCR products were electrophoretically separated on a 3.5% agarose gel. Each treatment triplicate was amplified individually.

Results

Competitiveness

In the competition assay, the interaction between *G. graminis* var. *graminis* strain inoculation and rhizosphere inoculum was significant for both shoot ($P < 0.018$) and root ($P < 0.036$) biomass. Rhizosphere inoculation significantly decreased root and shoot biomass in control and JH4301-treated plants (Fig. A.2), as well as root biomass of JH4300-inoculated plants. Our wild-type strain JH2033 significantly decreased both root and shoot biomass in the absence of rhizosphere competition. However, in the competition, plant biomass was not significantly reduced compared to plants inoculated with JH2033 alone.

PCR

PCR successfully amplified a PCR product consistent in size with the ITS region of JH2033 for some treatments, but not others (Fig. A.3). More PCR products were generated from DNA extracted from samples without rhizosphere inoculum. In addition, *G. graminis* var. *graminis* was amplified in most non-rhizosphere treatments inoculated with *G. graminis* var. *graminis*. However, without sequence analysis of the PCR products, we cannot

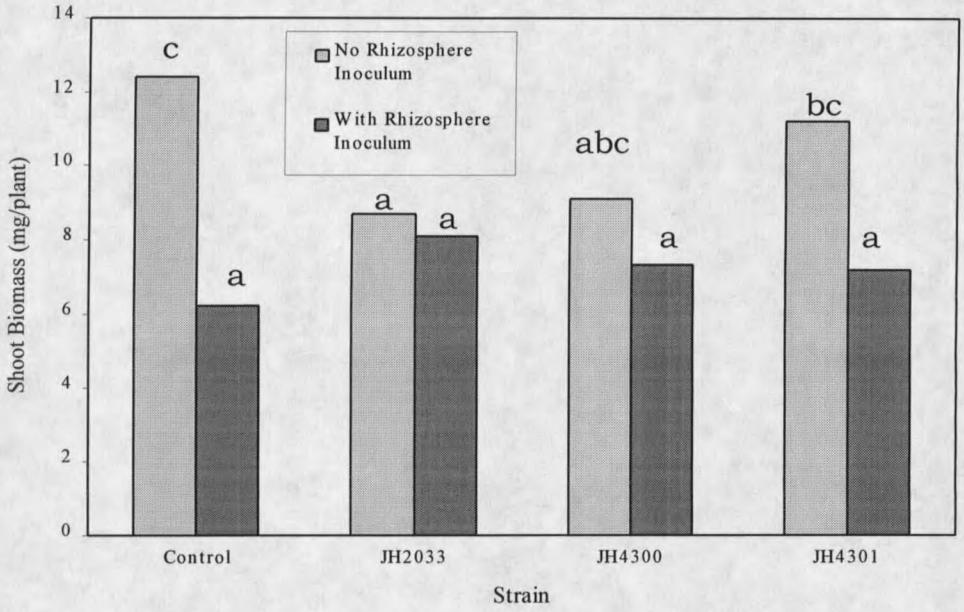
definitively conclude that the 600 bp band represents *G. graminis* var. *graminis.* To determine the ability of ITS primers to successfully amplify *G. graminis* var. *graminis* DNA in soil extracts, JH2033 DNA was purified through a Sephadex G200 column in the same manner as soil DNA, then PCR-amplified with or without soil DNA from the rhizosphere inoculum. The PCR products from JH2033 DNA were less concentrated than those found in the soil inoculum, although it did not appear that amplification of JH2033 was inhibited in the spiked rhizosphere inoculum (Fig.A.4).

Discussion

Melanized *G. graminis* var. *graminis* strains are not more pathogenic than unmelanized strains in pathogenicity tests with *G. graminis* var. *graminis* as the sole inoculum (see Chapter 2). However, because melanin provides protection from environmental factors such as UV light and lytic enzymes, pigmented strains may be better competitors in mixed communities; thus melanin may indirectly enhance pathogenicity. We addressed this issue by devising a competition experiment comparing pathogenic effects of the three strains on rice in the presence and absence of additional rhizosphere microorganisms. At harvest, we extracted rhizosphere

DNA in an attempt to characterize the rhizosphere fungal communities of the different treatments, using PCR amplification of the ITS regions (Figure A.1). Because the ITS regions are sites of insertions and deletions, we hoped to determine differences in fungal communities based on the size differences of the PCR prodicts. While we successfully amplified a 600 bp PCR product from rhizosphere DNA (Figure A.3, JH2033), it should be emphasized that sequence analysis of the PCR product would be required for unequivocal identification. It is evident from PCR amplification of DNA extracts that even treatments without rhizosphere inoculum had acquired fungal rhizosphere inhabitants in addition to *G. graminis* var. *graminis*; contamination probably originated from the greenhouse. However, our two melanin mutant strains were unable to sufficiently compete with these rhizosphere inhabitants to cause significant plant biomass reduction. The rhizosphere inoculum apparently contained pathogens that were more virulent than *G. graminis* var. *graminis*. PCR results also suggest a shift in rhizosphere communities when the initial rhizosphere inoculum ITS "profile" is compared to profiles at harvest (Figure A.3b).

a. Shoot dry weights



b. Root dry weights

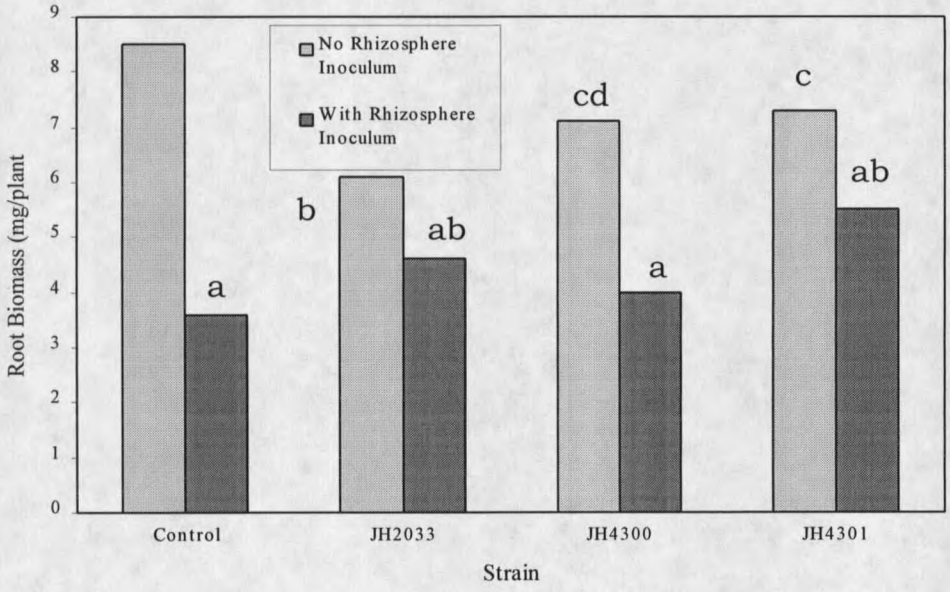
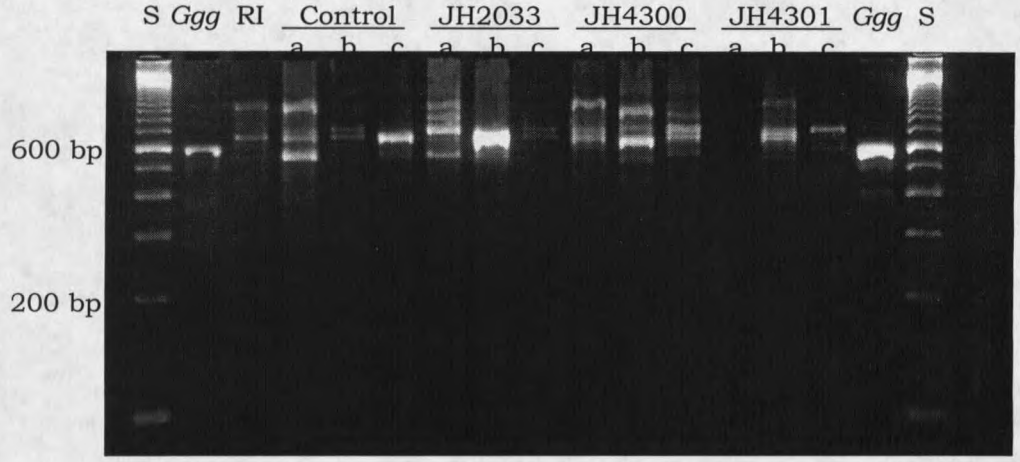


Figure A.2. Significant interactive effect between strain inoculation and rhizosphere inoculum on root and shoot dry weight. Different letters above bars indicate significant differences at $P < 0.05$ (Tukey's Studentized Range test).

a. Without Rhizosphere Inoculum



b. With Rhizosphere Inoculum

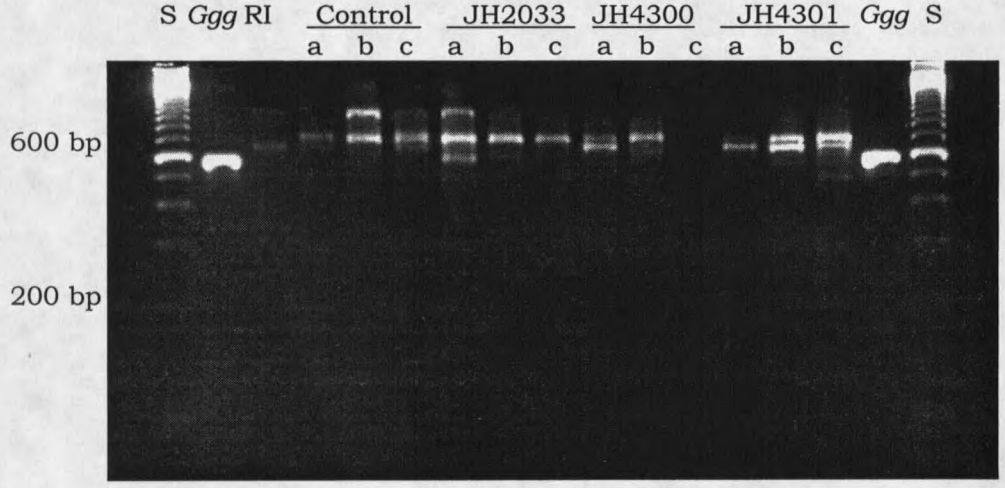


Figure A.3. PCR-amplified ITS regions of DNA extracted from rhizosphere material with or without rhizosphere inoculum. Triplicate pots = a, b and c. S = 100 bp standard; *Ggg* = PCR-amplified JH2033; RI = rhizosphere inoculum. Negative controls (no template) showed no amplification (not shown).

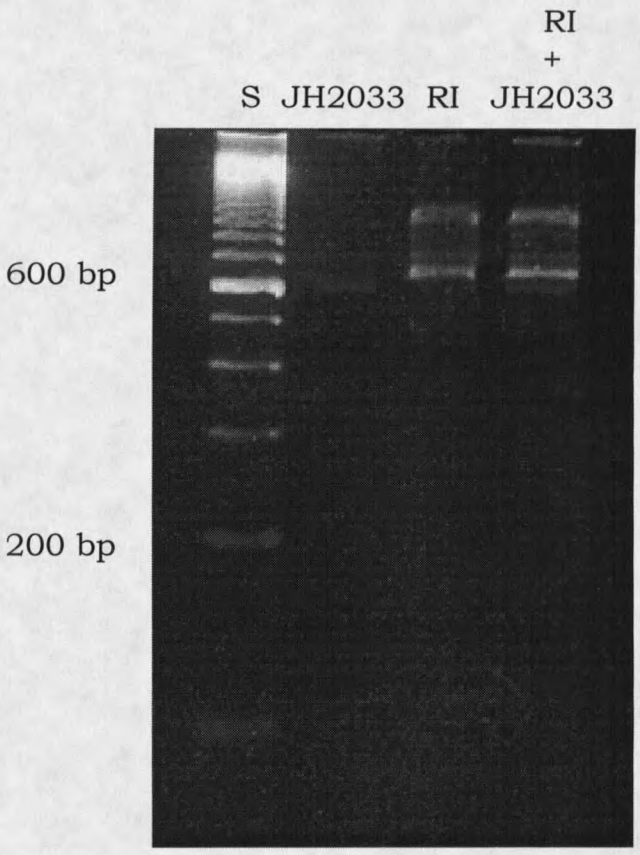


Figure A.4. PCR-amplified ITS regions of JH2033, rhizosphere inoculum extract and rhizosphere inoculum spiked with JH2033. S = 100 bp standard ladder; RI = rhizosphere inoculum.

While this experiment provides some evidence to support the indirect effect of melanin on pathogenicity, more controlled experiments are needed to address the uncertainties associated with the results. From the PCR amplification, it is evident that replicate treatments probably had different rhizosphere microorganisms, and perhaps a defined rhizosphere inoculum that

is not itself pathogenic as the competition factor would ameliorate replication. In addition, steps to eliminate contamination in the greenhouse would enhance the quality of data collected. From Figure A.4, it is also apparent that the PCR reaction is not optimized for amplification of the *G. graminis* var. *graminis* ITS region.

Literature Cited

1. Bell, A. A. and M. H. Wheeler. 1986. Biosynthesis and functions of fungal melanins. Ann. Rev. Phytopathol. **24**:411-451.

2. Boysen, M., M. Borja, C. del Moral, O. Salazar and V. Rubio. 1996. Identification at strain level of *Rhizoctonia solani* AG4 isolates by direct sequence of asymmetric PCR products of the ITS region. Curr. Genet. **29**:174-181.

3. Coleman, D. C., C. P. P. Reid and C. V. Cole. 1983. Biological strategies of nutrient cycling in soil systems. Adv. Ecol. Res. **13**:1-55.

4. Chumley, F. G. and B. Valent. 1990. Genetic analysis of melanin-deficient, non-pathogenic mutants of *Magnaporthe grisea*. Mol. Plant Microbe interact. **3**:189-198.

5. Elliott, M. L. 1995. Effect of melanion biosynthesis inhibiting compounds on *Gaeumannomyces* species. Mycologia **87**:453-459.

6. Howard, R. J. and M. A. Ferrari. 1989. Role of melanin in appressorium function. Exp. Mycol. **13**:403-418.

7. Howard, R. J., M. A. Ferrari, D. H. Roach, and N. P. Money. 1991. Penetration of hard substrates by a fungus employing enormous turgor pressure. Proc. Natl. Acad. Sci. USA **88**:11281-11284.

8. Takano, Y., Y. Kubo, K. Shimizu, K. Mise, T. Okuno and I Furusawa. 1995. Structural analysis of *PKS1*, a polyketide synthase gene involved in melanin biosynthesis in *Colletotrichum lagenarium*. Mol. Gen. Genet. **249**:162-167.

9. Tsai, T.-L. and B. H. Olson. 1991. Rapid method for direct extraction of DNA from soil and sediments. Appl. Environ. Microbiol. **58**:1070-1074.

APPENDIX B

PHYTOREMEDIATION APPLICATION OF *Gaeumannomyces graminis* var. *graminis* MELANIN MUTANTS

The Anaconda Copper Company extensively mined the Butte, Montana area for approximately 80 years, and smelted the ore at the Anaconda smelter, 30 miles west of Butte. The resulting tailings from the smelting process were deposited into tailings ponds covering several square miles. The tailings ponds are part of the largest U.S. E.P.A. Superfund site in the United States, and strategies are currently being developed to remediate and/or reclaim the tailings ponds.

One such strategy is phytoremediation of the contaminated site. Phytoremediation is defined as the use of green plants to remove pollutants such as metals from the environment or to render them harmless (8). The major factor in availability of metals to plants in soil is the solubility and thermodynamic activity of the uncomplexed ion. Only a soluble species adjacent to the root is likely to be translocated into the root (1). Stable metal-organocomplexes such as those used in fertilization may be highly soluble (6) and readily taken up by roots. Plants may secrete metal-chelating proteins (10) or phytochelatins (9) that act as phytosiderophores and solubilize "soil-bound" metals. Plant uptake of non-essential heavy metals may also be enhanced by rhizosphere microorganisms (8, 12). Rhizosphere fungi have been

shown to acidify soil surrounding roots (2), which would increase metal availability for plant uptake. Organic fungal components such as melanin may also bind metals and influence metal solubility. In this study, we investigated the interactions between melanized and unmelanized *G. graminis* var. *graminis* strains and tailings metal uptake by range grasses.

Materials and Methods

Metal Sorption of Tailings Material to Biomass

Tailings material was collected from three sites in the "New River Pond" near Anaconda, Montana in June 1996, by the Montana State University Reclamation Research Department. The top 5cm of material was removed and tailings were collected to a depth of 20 cm. Material was sieved through a 2 mm screen, and stored at 4 °C in covered containers until use.

Shake flasks containing 200 ml minimal medium supplemented with 1% Anaconda tailings pond material were inoculated and incubated as described for copper binding studies. At harvest, fungal biomass and associated tailings material were collected by filtration through a Whatman #1 filter, rinsed 2X to

remove residual tailings, then dried overnight. Samples were digested as previously described in Chapter 3, and analyzed by ICP-AES for iron, zinc, copper and lead.

Metal Uptake by Plant Biomass

The experiment was designed as a 2 X 4 X 5 factorial, with the following treatment factors: 1) tailings pond addition, 2) wild-type or melanin mutant strains of *G. graminis* var. *graminis*, and 3) plant species.

Pots were prepared by rinsing 500 g (dry weight) sand (Pakmix, Inc., Toledo, OH) 2X with tap water, then transferring into 10 cm pots with Whatman #1 filter paper covering the drain holes. Air bubbles were removed by gently shaking the pots and excess surface water was poured off.

Outer seed hulls of *Agropyron dasystachyum, A. elongatum, A. trachycaulum, Elymus cinereus,* or *E. juneus* were removed and seeds were placed in 250 ml Erlynmeyer flasks containing 25 ml Clorox® bleach and 1% Tween 80. After shaking for 20 minutes, contents of flasks were transferred to sterile petri dishes. Seeds were washed in sterile water, then aseptically transferred to 25 X 150 mm slip-cap tubes containing 20 ml of ½ strength nutrient

agar (Difco). Five to six day-old seedlings were transferred to pots along with 4 - 1cm diameter plugs of LB agar containing no inoculum (control), JH2033, JH4300 or JH4301. Plugs were placed 0.5 cm below the sand surface at a distance of 1.5 cm from the plant. Pots were transferred to a greenhouse, and watered on alternate days with either 50ml deionized H_2O or ¼ strength Hoagland's solution. After 14 days, 50 g of sand surrounding the plant were removed and mixed with 50 g of Anaconda tailings pond material. The mixture was replaced in the pot and the plants grown for an additional 14 days. At harvest, plants were removed and material adhering to roots was collected into sterile tubes and stored at -20 °C. Plants were then rinsed with tap water, roots and shoots separated, lyophilized overnight, then weighed. Shoots were cut into 1cm pieces and digested for metal analysis by EPA method 3050A, with a final sample volume of 50 ml. Growth material tightly adhered to roots even following extensive washing, making any assessment of metal content within root tissue suspect.

Rhizosphere Hyphal Lengths

Fungal hyphal lengths were determined by thin agar film preparation (5) followed by microscopic examination of hyphal segment intersects on an ocular grid (7). A 0.5 g subsample of rhizosphere material was weighed into a 50 ml screw-capped tube containing 4.5 ml sterile deionized water. The suspension was vortexed vigorously for 30 s, then allowed to settle for 30 s. One ml of supernate was pipetted into a screw-capped tube containing 4 ml of molten 2% agar. The tube contents were mixed well, and then poured onto hematocytometers. A slide was placed over the center of the hematocytometer and the agar was allowed to solidify. Excess agar was cut away from around the center section of the hematocytometer and the agar film was floated onto a microscope slide in a shallow dish of water. The films were air-dried and observed with phase contrast microscopy at 100X magnification. Four films were prepared for each sample, and 10 random fields per film were counted.

Results

Tailings Sorption to Biomass

More iron and copper in the tailings pond material were bound to the dark mutant JH4301 than the light mutant, while the wild-type sorbed an intermediate concentration not significantly different from either mutant (Table B.1). This result is in contrast to experiments with copper accumulation of cultures grown with $CuSO_4$ (Table 4.1) and suggest that while the ionic copper species binds to melanized fungal biomass, other copper species may bind to *G. graminis* var. *graminis* biomass regardless of melanin status. Lead and zinc concentrations were similar for the three strains.

Tailings Metal Uptake by Plants Inoculated with *G. graminis* var. *graminis*

A summary of significant treatment effects is given in Table B.2. *G. graminis* var. *graminis* inoculation significantly increased hyphal lengths in the rhizosphere. Control treatments had less hyphae (2.39 m/g) than JH4300, JH2033 or JH4301 (3.84, 3.49 and 3.21 m/g, respectively). Inoculated treatments were not significantly different.

Table B.1. Metal concentrations of tailings pond material and metals sorbed to wild-type (JH2033) and melanin mutant strains of *G. graminis* var. *graminis*.

		Biomass Sorption		
Metal Species	Tailings Pond	JH4300	JH2033	JH4301
Fe (mg/g)	20.00 (0.54)*	17.66 a**	19.34 ab	26.10 b
Zn (μg/g)	96.68 (15.28)	121.40 a	116.56 a	95.39 a
Cu (μg/g)	157.78 (4.52)	139.07 a	141.20 ab	185.21 b
Pb (μg/g)	397.80 (9.99)	383.50 a	394.04 a	472.27 a

* standard error

** means followed by different letters across a row are significantly different at $P < 0.05$ (Tukey's Studentized Range test, HSD).

Plant biomass was not affected by either tailings pond addition or by inoculation, suggesting that the applied tailings concentration was not toxic, and that *G. graminis* var. *graminis* was not pathogenic to any of the plant species tested. Not surprisingly, both root and shoot biomass differed according to plant species (Table B.3). *A. elongatum* plant biomass was greatest of the five species tested. *A. dasystachyum* produced the least aboveground biomass, while *E. juneus* produced the least belowground.

Inoculation had no effect on iron uptake and transport to shoot tissue (Table B.2). However, a significant interaction between plant species and tailings addition suggested that these five plant species responded differently in iron uptake from tailings material. All species grown without tailings had similar iron

concentrations in shoot tissue (Figure B.1). Only one species (*A. elongatum*) had an increased iron concentration when grown with the tailings addition, suggesting that this species was perhaps either unable to regulate iron uptake, or had an enhanced iron uptake mechanism. In contrast, the only significant treatment effect on zinc concentration was tailings addition (Table B.2). Zinc shoot concentration of unamended treatments was 42.42 μg/g biomass and amended treatments had a zinc concentration of 94.29 μg/g biomass.

Table B.2. Significant treatment effects of plant metal uptake experiment. P = plant species, I = Fungal strain inoculum, T = tailings addition. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

	Treatment Effect						
Parameter	P	I	T	P x I	P x T	I x T	P x I x T
Hyphal Lengths	-	***	-	-	-	-	-
Shoot Biomass	***	-	-	-	-	-	-
Root Biomass	**	-	-	-	-	-	-
Shoot Iron	**	-	**	-	***	-	-
Shoot Zinc	-	-	***	-	-	-	-
Shoot Copper	-	-	-	-	-	-	-
Shoot Lead	-	-	-	-	-	*	-

The only significant effect of *G. graminis* inoculation was an interaction between inoculum and tailings addition on lead concentration in shoot biomas (Figure B.2). Control and JH4300-inoculated treatments gave similar responses; lead concentration

was significantly higher in shoots of unamended compared to tailings-amended treatments. Both the wild-type JH2033 and JH4301 strains had less shoot lead in the no tailings treatment than the uninoculated (control) and JH4300-inoculated plants. Interestingly, the heavily melanized mutant JH4301 significantly increased lead content in shoots with added tailings.

Table B.3. Significant main effect of plant species on root and shoot biomass. Means in column followed by different letters are significantly different at $P < 0.05$ (Tukey's Studentized Range test, HSD).

	Plant Biomass (mg dry weight)	
Plant Species	Root	Shoot
A. dasystachyum	64.99 a	136.11 ab
A. elongatum	134.39 b	260.00 c
A. trachycaulum	107.61 a	170.75 ab
E. cinereus	118.13 ab	189.34 b
E. juneus	74.44 a	112.20 a

Discussion

We found a positive correlation between melanin and metal binding of tailings material, indicating that melanized fungal biomass are capable of sorbing mixed metal species, particularly iron and copper. Because our ICP-AES analysis did not provide information concerning the type of metal complexes in the tailings material, comparisons between $CuSO_4$ binding experiments and tailings sorption should be viewed with caution. Most likely many

different metal complexes exist within the tailings material that may react with fungal components other than melanin.

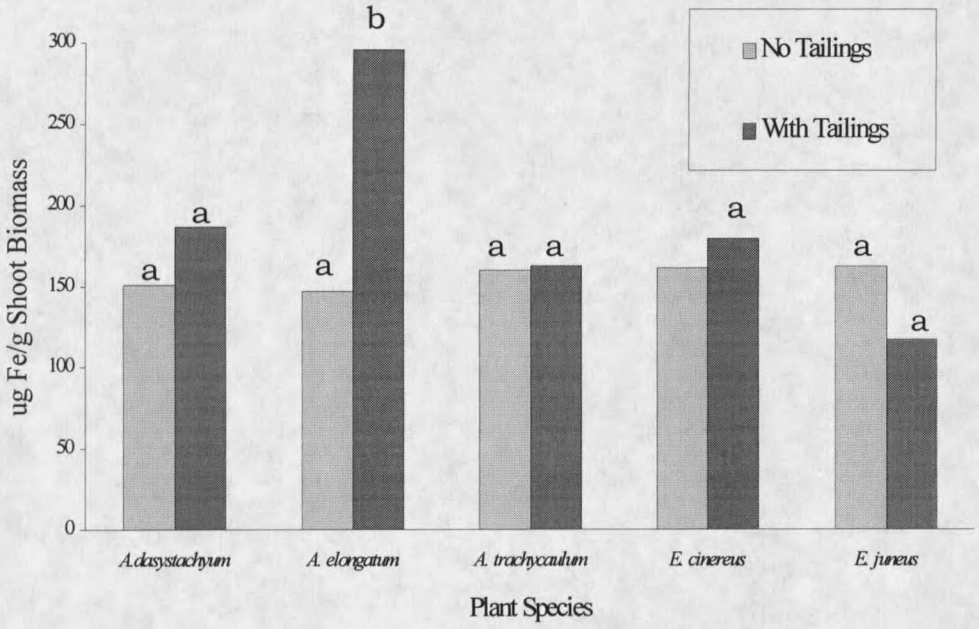


Figure B.1. Interactive treatment effect of plant species and tailings addition on iron uptake into shoot tissue. Means followed by different letters are significantly different at $P < 0.05$ (Tukey's Studentized Range test, HSD).

Fungal melanins constitute a large portion of the soil humus and therefore affect the translocation and availability of metal ions to plants (11). Humic acids form water-soluble and water-insoluble complexes with metal ions. Stability constants of metal-humic acid complexes suggest that amino acids and hydroxy carboxylic acids secreted by plant roots will compete favorably with humic acids for metal ions and promote their uptake by plant roots (13). However, Cu and Pb accumulation in plants grown in sludge-

amended soils show only small increases compared to those grown on uncontaminated soils (3). We found no correlation between melanized fungal inoculum and copper accumulation in shoot tissue with tailings amendment. Our results suggest that fungal melanin does not significantly enhance copper availability by increasing solubility, or decrease plant copper uptake by sequestration. We were unable to accurately determine root tissue concentrations because of material tightly adhered to the root surface, and thus cannot reach any conclusions about metal concentrations of whole plants. Presumably higher metal concentrations in roots would lead to greater shoot translocation as well, particularly when metal concentration of roots is in excess. While copper concentrations did not vary with treatment, we did find increased lead accumulation in plants from amended soils inoculated with the heavily-melanized mutant, indicating that melanin may have increased solubility of lead in tailings material. Soil-applied chelating agents such as EDTA have been shown to greatly increase the translocation of heavy metals, including lead from soil into shoots (4). Perhaps fungal melanin acts in a similar manner as a chelator.

The results of these metal binding experiments with our melanin mutants provide evidence to support the relationship between fungal melanin and metal binding. Sorption of metals to DHN-melanin varies with metal species and different metal complexes, and melanin may act as a chelator in the soil

environment to increase metal solubility (particularly lead) and subsequent plant uptake on metal-contaminated sites. Further experiments with defined metal complexes are required to determine the exact relationship between fungal melanogenesis in the rhizosphere and metal uptake into plant tissue.

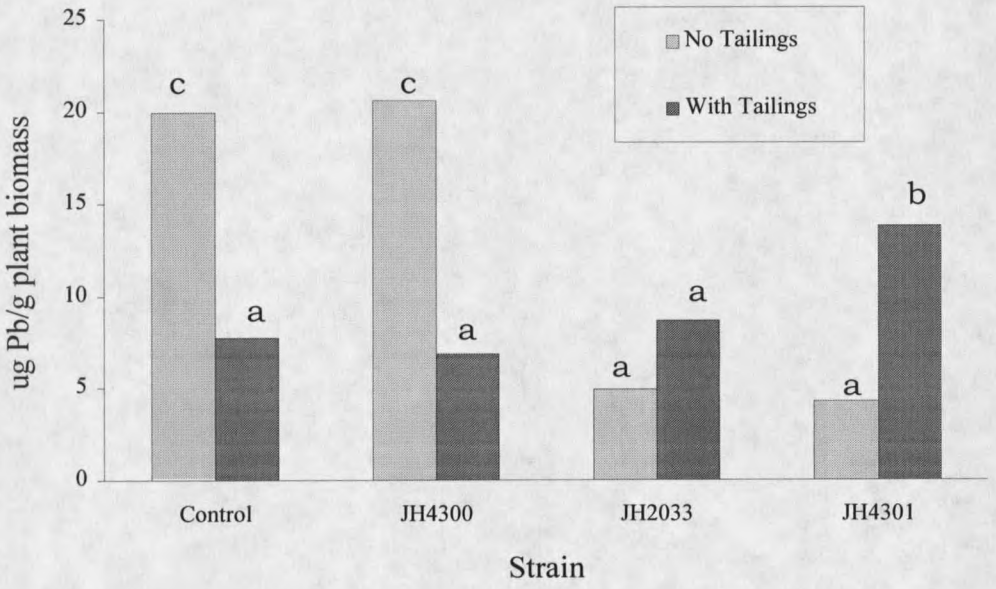


Figure B.2. Significant interaction between strain inoculum and tailings addition on lead concentration in shoot biomass. Means followed by different letters are significantly different at $P < 0.05$ (Tukey's Studentized Range test, HSD).

Literature Cited

1. Cataldo, D. A. and R. E Wildung. 1978. Soil and plant factors influencing the accumulation of heavy metals by plants. Environ. Health Perspec. **27**:149-159.

2. Devêvre, J. Garbaye, and B. Bottom. 1996. Release of complexing organic acids by rhizosphere fungi as a factor in Norway spruce yellowing in acidic soils. Mycol. Res. **100**:1367-1374.

3. Hooda, P. S., D. McNulty, B. J. Alloway, and M. N. Aitken. 1997. Plant availability of heavy metals in soils previously amended with heavy applications of sewage sludge. J. Sci. Food Agric. **73**:446-454.

4. Huang, J. W., S. D. Cunningham. 1996. Lead phytoextraction: species variation in lead uptake and translocation. New Phytol. **134**:75-84.

5. Jones, P. C. T. And J. E. Mollison. 1948. A Technique for the quantitative estimation of soil microorganisms. J. Gen. Microbiol. **2**:54-69.

6. Norvell, W. A. and W. L. Lindsay. 1969. Reactions of EDTA complexes of Fe, Zn, Mn, and Cu with soils. Soil Sci. Soc. Am. Proc. **33**:86.

7. Olson, F. C. W. 1950. Quantitative estimates of filamentous algae. Trans. Am. Micro. Soc. **69**:272-279.

8. Raskin, I., R. D. Smith and D. E. Salt. 1997. Phytoremediation of metals: using plants to remove pollutants from the environment. Curr. Opin. Biotechnol. **8**:221-226.

9. Rauser, W. E. 1995. Phytochelatins and related peptides. Plant Physiol. **109**:1411-1419.

10. Robinson, H. J., A. M. Tommey, C. Kuske and P. J. Jackson. 1993. Plant metallothioneins. Biochemistry **295**:1-10.

11. Saiz-Jimenez, C. and F. Shafizadeh. 1984. Iron and copper binding by phenolic polymers: an electron spin resonance study. Curr. Microbiol. **10**:281-286.

12. Salt, D. E., M. Blaylock, N. P. B. A. Kumar, V. Dushenkov, B. D. Ensley, I. Chet, and I. Raskin. 1995. Phytoremediation: a novel strategy for the removal of toxic metals from the environment using plants. Biotechnology **13**:468-474.

13. Stevensen, F. J. 1976. Stability constants of Cu^{2+}, Pb^{2+} and Cd^{2+} complexes with humic acids. Soil Sci. Soc. Am. J. **40**:665-672.

MONTANA STATE UNIVERSITY LIBRARIES
3 1762 10298046 1