Enhanced pigmentation effecting decreased protein secretion and pathogenicity in spontaneous melanin variants of Gaeumannomyces graminis var. tritici
by Tresa Len Quoss Goins

A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Microbiology
Montana State University
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Abstract:
Gaeumannomyces graminis var. tritici is the phytopathogenic agent of take-all, a severe root disease of wheat and barley. It is a member of the Gaeumannomyces-Phialophora complex of closely related homothallic, filamentous ascomycetes that are differentiated by pathogenicity and the ability to undergo meiosis. The population of Gaeumannomyces and Phialophora in native grasslands and take-all decline soils, where disease severity is absent or depressed, is characterized by darkly pigmented isolates. The parameters dictating this population dynamic have not been identified and there is no established relationship between fungal pigmentation and fungal virulence.

The spontaneous melanin variants of a virulent Gaeumannomyces graminis var. tritici isolate demonstrated Phialophora-like characteristics, loss of virulence and the progressive loss of the ability to undergo meiosis. G. graminis var. tritici effects penetration of intact host tissue by enzymatic means requiring the secretion of lytic enzymes for the extracellular degradation of macromolecules. The relationship between the secretion of fungal proteins [cellulases, pectinases, proteinases (plant-cell-wall degrading enzymes) and a polyphenol oxidase] and the melanin variant phenotype (enhanced pigmentation) was assessed in the wild-type, two spontaneous melanin variants, and two meiotically derived white-derivatives of the melanin variants. The effect of DHN-melanin biosynthesis inhibition on protein secretion was determined in melanin variants treated with tricyclazole.

The correlation between the melanin variant phenotype and pathogenicity was assessed in Triticum aestivum by shoot and root mass assay, root stelar lesion assay and root histopathology. A laccase-like polyphenol oxidase, not previously reported in G. graminis var. tritici, was assessed for plant phenolic detoxification (p-coumaric and caffeic acids) and lignin degradative (Poly B-411 decolorization) capacity and was identified as a putative fungal virulence factor. Results indicated 1) an inverse correlation between fungal pigmentation and protein secretion, 2) an inverse correlation between fungal pigmentation and pathogenicity and 3) an inferred direct correlation between protein secretion and pathogenicity.

The presentation of Phialophora-Yike characteristics by the spontaneous melanin variants is the first report of a sexual/asexual conversion in Gaeumannomyces. This observed phenotypic switching no doubt contributes to classification difficulties among members of the Gaeumannomyces-Phialophora complex.

ENHANCED PIGMENTATION EFFECTING DECREASED PROTEIN

SECRETION AND PATHOGENICITY IN SPONTANEOUS MELANIN

VARIANTS OF *GAEUMANNOMYCES GRAMINIS* VAR *TRITICI*

by

Tresa Len Quoss Goins

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LIST OF ABBREVIATIONS

CTAB	Cetyltrimethylammonium Bromide
DHN	Dihydroxynaphthalene
DMOP	2,6-Dimethoxyphenol
Ggt	*Gaeumannomyces graminis* var. *tritici*
MV	Melanin Variant
PAL	Phenylalanine Ammonia-Lyase
PDA	Potato Dextrose Agar
PGU	Polygalacturonase
PL	Pectin Lyase
PPO	Polyphenol Oxidase
PVP	Polyvinylpyrrolidone
RFLP	Restriction Fragment Length Polymorphism
TAD	Take-All Decline
TAL	Tyrosine Ammonia-Lyase

ABSTRACT

Gaeumannomyces graminis var. *tritici* is the phytopathogenic agent of take-all, a severe root disease of wheat and barley. It is a member of the *Gaeumannomyces-Phialophora* complex of closely related homothallic, filamentous ascomycetes that are differentiated by pathogenicity and the ability to undergo meiosis. The population of *Gaeumannomyces* and *Phialophora* in native grasslands and take-all decline soils, where disease severity is absent or depressed, is characterized by darkly pigmented isolates. The parameters dictating this population dynamic have not been identified and there is no established relationship between fungal pigmentation and fungal virulence.

The spontaneous melanin variants of a virulent *Gaeumannomyces graminis* var. *tritici* isolate demonstrated *Phialophora*-like characteristics, loss of virulence and the progressive loss of the ability to undergo meiosis. *G. graminis* var. *tritici* effects penetration of intact host tissue by enzymatic means requiring the secretion of lytic enzymes for the extracellular degradation of macromolecules. The relationship between the secretion of fungal proteins [cellulases, pectinases, proteinases (plant-cell-wall degrading enzymes) and a polyphenol oxidase] and the melanin variant phenotype (enhanced pigmentation) was assessed in the wild-type, two spontaneous melanin variants, and two meiotically derived white-derivatives of the melanin variants. The effect of DHN-melanin biosynthesis inhibition on protein secretion was determined in melanin variants treated with tricyclazole.

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The presentation of *Phialophora*-like characteristics by the spontaneous melanin variants is the first report of a sexual/asexual conversion in *Gaeumannomyces*. This observed phenotypic switching no doubt contributes to classification difficulties among members of the *Gaeumannomyces-Phialophora* complex.

INTRODUCTION

The *Gaeumannomyces-Phialophora* Complex

The *Gaeumannomyces-Phialophora* complex embraces phenotypically similar fungi isolated from the roots of grasses and cereals (145, 253). Members of the complex include non-pathogenic *Phialophora* (anamorph) and pathogenic and non-pathogenic *Gaeumannomyces* (teleomorph). It is postulated that the *Phialophora* members have teleomorph states in *Gaeumannomyces* that are undetected due to the inability to induce meiosis *in vitro*. In an exceptional case, a *Phialophora graminicola* var. *graminicola* isolate was induced to produce perithecia on laboratory grown wheat and barley (114) and the identification of the sexual state, *Gaeumannomyces cylindrosporus*, resulted in the discontinued use of the asexual varietal name which inaccurately depicted the isolate as imperfect. Even in this exceptional case, the holomorph (104) was not demonstrated; the ascospores germinated to form only the anamorph and the phialospores failed to germinate (116). As the *Phialophora* reproduce asexually via a uniform mechanism of conidiogenesis, phialospores produced in phialides supported on a phialophore, the identification of such phenotypically similar isolates is problematic; the name *Phialophora radicicola* was misapplied so frequently throughout the literature to a variety of isolates that its use was discontinued (239). It was therefore proposed

that the anamorphic members of the *Gaeumannomyces-Phialophora* complex be referred to as *Phialophora*-like without a specific or varietal name (239, 262).

Most *Phialophora* isolates readily produce phialospores *in vitro,* but phialospore production and germination is inconsistent (65, 131). Some isolates of *Gaeumannomyces* produce two types of phialospores; non-germinating lunate spores observed *in vivo* and germinating ovate to cylindrical spores observed only in culture (36, 66, 67, 239, 252, 262). The production of ascospores by *Gaeumannomyces* is inconsistent among isolates and within the same isolate (65) and contributes to classification uncertainty among members of the *Gaeumannomyces-Phialophora* complex.

The initial identification of members in the complex is based on the sometimes ambiguous phenotypic characteristics of growth rate, pathogenicity and the ability to produce perithecia; *Phialophora* are generally slower growing, are non-pathogenic and are unable to undergo meiosis (65, 218). Despite its limitations, classification of complex members by phenotypic differences has been confirmed by molecular characterization. Nucleic acid sequence analysis reveals some *Phialophora* species are more closely related to *Gaeumannomyces* than they are to other *Phialophora* (37, 244) and gives credence to the member grouping in the *Gaeumannomyces-Phialophora* complex and indicates that the *Phialophora*-like members are not simply a form-taxa of genetically unrelated fungi. *G. graminis* varieties include a relatively benign parasite of grasses and rice, *G. graminis* var. *graminis,* and two closely related varieties, *G. graminis* var. *tritici* (Walker) and *G. graminis* var. *avenae* (Turner) which are pathogenic to wheat, barley, rye and oats

(1, 111, 239). Classification of the *Gaeumannomyces* members of the complex based on host susceptibility and the morphology of specialized fungal structures has also been augmented by molecular characterization. Restriction fragment length polymorphisms in families of repeated DNA sequences have recently been used to confirm the variety pathotypes (176, 223). All three varieties produce hyphopodia, attachment and penetration structures, however, those of *G. graminis* vars. *tritici* and *avenae* are simple (unlobed) whereas those of *G. graminis* var. *graminis* are both simple and lobed (111). Sequence homology analysis of ribosomal RNA genes and the internal transcribed spacer regions (37) and random amplified polymorphic DNA (85) confirms relatedness based on hyphopodial structure; the pathogens *G. graminis* var. *tritici* and *G. graminis* var. *avenae* being more closely related than the parasitic *G. graminis* var. *graminis*. The *Phialophora*-like members of the complex produce both simple and lobed hyphopodia (239) and may be correlated to hyphopodial form of its proposed sexual state. The closely related varieties *tritici* and *avenae* can also be differentiated by ascospore length. While the ranges of spore length overlap in the two varieties, those of var. *tritici* are shorter overall (230, 239).

Gaeumannomyces graminis var. tritici

Gaeumannomyces graminis (Sacc.) von Arx & Olivier are plant parasitic ascomycetes that infect the roots, crowns and lower portions of stems and leaf sheaths of *Poaceae* and *Cyperaceae*. *G. graminis* (Sacc.) Arx & Olivier var. *tritici* (Walker) exhibits a wide host range in wild grasses in global temperate regions. It

causes a severe root disease, take-all, of wheat (*Triticum*) and barley (*Hordeum*) (237), and is the most damaging disease of cultivated wheat worldwide (120). The term take-all is a graphic description of the disease: a loss of crop yield due to decreased number of tillers per plant, decreased grain weight and fewer grains per tiller (10, 24). The intensive cultivation of wheat prompted early recognition of the disease in South Australia in the 1850's and identification of the fungal etiological agent in France in 1890 (193). Much of the early literature refers to *G. graminis* var. *tritici* as *Ophiobolus graminis*, a Loculoascomycete. The misapplication of the genus name *Ophiobolus* remained in use into the 1970's despite the designation of the new genus, *Gaeumannomyces*, by von Arx & Olivier in 1952 to reflect the organisms correct identification as a Pyrenomycete (113, 126).

G. graminis var. *tritici* is homothallic but capable of out-crossing among compatible anastomosis groups. Repeated hyphal fusion results in multinucleate conjugate cells (analogous to the female ascogonium) which give rise to numerous ascogenous hyphae (126). Repeated crozier formation and septal separation between nuclei pairs in the ascogenous hypha forms transient diploid cells which undergo meiosis immediately after karyogamy occurs. Four haploid daughter cells undergo mitosis forming four pairs of genetically identical unordered haploid ascospores within the ascus. The mature ostiolate perithecium contains scattered unitunicate asci that germinate to form a haploid vegetative mycelium with predominately uninucleate hyphal cells (45, 162).

Wild grasses, e.g. *Agropyron*, *Bromus*, and *Hordeum*, are natural hosts of *G. graminis* var. *tritici* and are a source of fungal inoculum in pastures cleared for

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cultivation and are responsible for "edge" or "border" outbreaks of take-all that occur along the perimeter of a cultivated plot (210, 211). In the perennial wild grass hosts, *Ggt* exists as a parasite year-round in the living rhizome (236, 211). In a monoculture, the life cycle of *G. graminis* var. *tritici* alternates between the parasitic phase when it acts as a pathogen and the saprophytic phase when it must survive between susceptible host crops. Plowing under infected plant debris that contains hyphal fragments provides inoculum for subsequent monoculture and the planting of sequential susceptible crops may result in an increase in take-all severity unless non-susceptible break-crops are planted in rotation (57, 203). The "spontaneous" outbreak of take-all in a field not previously cultivated in a susceptible crop is attributed to fungal dispersal by wind blown infected plant material (89, 161). Ascospores are produced in the field and may be disseminated by rainfall (98) but are very susceptible to environmental conditions where survival, as determined by germination *in vitro,* ranges from two days to two years (83, 116). Also, ascospore maturation rarely coincides with the optimum time to perpetuate infection in the subsequent crop (211) and ascospores fail to germinate in non-sterile soil (34, 89). The production of germinable phialospores has never been observed in the field.

The pathogen has a very low competitive saprophytic ability and demonstrates highly restricted colonization of sterile straw which is further diminished in the presence of antagonistic soil microflora (115, 210, 211). Antagonistic microflora alter the wheat rhizosphere chemistry by depleting specific plant exudates and producing inhibitory metabolites (208, 222). Up to 0.15% of total

plant assimilated carbon is lost to the soil as root exudates containing polysaccharides, mucopolysaccharides, proteins, amino acids, organic acids and sugars (43). *G. graminis* var. *tritici* responds chemotactically to root exudates as a nutrient source and responds to the chemical gradient to direct fungal growth toward its host target (94, 115, 191). Pseudomonads compete for nutrients with *G. graminis* var. *tritici* but also secrete antibiotics inhibitory to the pathogens growth (160, 188) and act as a biocontrol agent to suppress take-all (159, 160).

A spontaneous reduction in take-all severity and an increase in crop yield is a phenomenon known as take-all decline (96, 115). Take-all decline (TAD) soils have a greater population of antagonistic microflora than non-TAD soils. *G. graminis* var. *tritici* responds chemotactically to root exudates if the fungus is located within a rhizosphere response zone of 9-14 mm (94, 191, 261). The *G. graminis* var. *tritici* rhizosphere chemotactic response zone in TAD soils is reduced by nearly fifty-percent (12, 115, 191, 213, 261). Numerous *G. graminis* var. *tritici* isolates produce a self-inhibiting Q-factor (163) but its growth inhibiting activity is believed to function during the saprophytic rather than the pathogenic phase of the *G. graminis* var. *tritici* life cycle. Debate on the causes of take-all decline continues, and theories that have lost favor include the loss of fungal virulence (56, 58) and hypovirulence associated with the presence of a mycovirus (87, 146, 195). The take-all decline phenomenon observed in monoculture may merely be a reflection of the complex balance evolved between *G. graminis* var. *tritici* and its wild grass hosts. There are few reports of severe take-all in native or long term grasslands (238) and the severity of take-all symptoms in TAD soils diminish without a concomitant

decrease in the incidence of infected plants (210). The *Gaeumannomyces* and *Phialophora* isolates from monocultures displaying TAD and native grasses are typically darkly pigmented (51, 63, 65).

At the rhizoplane, *G. graminis* var. *tritici* demonstrates an initial extensive ectotrophic growth habit which is indistinguishable from that of non-pathogenic *Gaeumannomyces* and *Phialophora*-like species (114, 215). This shared manifestation of root surface colonization by pigmented runner-hyphae (macrohyphae) makes it impossible to assess risk from take-all by inspecting root systems (68, 215). The presence of *G. graminis* var. *tritici* should be verified by the development of perithecia on wheat culms, but diagnostic results are variable and require four to ten weeks (114, 253, 260). As the *G. graminis* var. *tritici* infection of wheat progresses, the pigmented runner hyphae originate hyaline infection hyphae (non-pigmented microhyphae) (126, 154) that penetrate the root cortex layers and endodermis and culminates in colonization of the root stele (215). It is the colonization of the root stele containing the phloem and xylem that cause plant wilting and death by interrupting root transport functions (53, 215). Non-pathogenic *Gaeumannomyces* and *Phialophora*-like species are unable to penetrate the stele and form darkly pigmented growth cessation structures against the endodermis or in any of the six cortex layers where penetration has been halted (65, 68, 109).

Factors of Host Resistance

There are no wheat cultivars that are highly resistant to *G. graminis* var. *tritici* (186). No host resistance genes have been identified and reported cases of resistance may result from an ill-defined interaction between environmental conditions and the cultivar genotype (112, 185). A good deal of non-specific plant disease resistance to fungal infection is imparted by fungitoxic plant phenolic compounds (121, 123, 232, 233). These low molecular weight phenolics are inhibitory metabolites that accumulate at wound or penetration sites to inhibit fungal growth by disrupting fungal plasma membrane function and fungal cell wall synthesis (216). Fungitoxic phenolic compounds include phytoalexins that are synthesized *de novo* in cells adjacent to those damaged by physical wounding, phytoanticipins that are present in plant tissue prior to infection (97, 182, 233, 243), and phenolic lignin precursors and intermediates that occur as normal plant constituents for the synthesis of lignin, a macromolecule that imparts rigidity and compression strength to all higher plant cell walls. No phytoalexin response has been detected in wheat infected with *G. graminis* var. *tritici* (231), however, resistant oat (*Avena sativa* L.) cultivars produce avenacin in response to infection with *G. graminis* var. *avenae* (32, 62, 179). Cultivars of oat that are susceptible to *G. graminis* var. *avenae* do not produce avenacin (178) and are also susceptible to infection with *G. graminis* var. *tritici* (180). Wheat tissue contains a relatively high concentration of the phenylpropanoid lignin precursor caffeic acid (60) that may act as a phytoanticipin. This fungitoxic monolignol precursor acts as a phytoalexin in

potato cultivars resistant to *Phytophthora infestans* where it occurs at high concentrations in damaged tissue (204).

The production of lignified zones in response to infection or wounding is a. common phenomenon (135, 257). Lignification in wheat is specific for filamentous fungal infections regardless if the infecting agent is saprophytic or pathogenic (149, 183, 231). In the *G. graminis* var. *tritici* infection of wheat, cortical lignitubers (82), or lignin impregnated papillae, are formed around the advancing hyphal tip as a physical barrier to fungal penetration (9, 82, 201). Induced lignification may be accompanied by the *de novo* synthesis of phenylalanine ammonia-lyase (PAL) and tyrosine ammonia-lyase (TAL), enzymes that catalyze the deamination of the respective amino acids to form *p*-coumaric acid (61, 148). PAL activity has been detected in all plant systems but lignification via TAL is unique to cereals and grasses (61). *p*-Coumaric acid is the substrate for the hydroxylated and methylated derivatives, caffeic , ferulic and sinapic acids. The reduction of *p*-coumaric, ferulic and sinapic acids yields the three lignin precursors, *p*-coumaryl, guaiacyl, and syringyl alcohol, respectively. The formation of ether bridges between the monolignols is catalyzed by plant peroxidase (149, 231, 257).

The proportion of *p*-coumaryl, guaiacyl, and syringyl alcohol units present in the mature lignin structure, and the prevalence of specific intermolecular bond types, is determined by phylogenetic origin (86, 170, 209). *Poaceae* lignin is composed of approximately equal numbers of all three monolignol derivatives but contains a comparatively high concentration of *p*-coumaric acid bound in ester

bridges to plant cell-wall arabinoxylans (144, 146) with a free hydroxyl group that is not covalently bound to lignin in an ether bridge.

Factors of Fungal Pathogenicity

G. graminis var. *tritici* effects penetration of intact host tissue and infection does not require the presence of a wound or lesion. *G. graminis* var. *tritici* secretes lytic enzymes for the extracellular degradation of cellulose in the plant primary wall and pectin and protein complexes in the middle lamella (20, 76, 156). Filamentous fungal protein secretion is believed to be restricted to nascent hyphal tips (184, 212, 247). The fungal cell wall is composed of a rigid microfibrillar network of chitin, a linear polymer of $\beta(1\rightarrow4)$-linked *N*-acetyl glucosamine, and glucan, polymers of $\beta(1\rightarrow3)$-linked and $\beta(1\rightarrow6)$-linked glucose residues (14), that become increasingly cross-linked as the cell wall matures to form a rigid structure that is impermeable to large proteins, demonstrated by molecular sieving (166, 227) and by immunogold labeling of specific proteins (267). Cell wall rigidity is further increased with the onset of secondary metabolism and the deposition of melanin in the fungal cell wall. Fungal melanins are synthesized in a variety of biosynthetic pathways shared across phyla; DOPA melanin formed by the oxidation of tyrosine (40) , DGHB melanin synthesized from γ-glutaminyl-3,4-dihydroxybenzene (194), catechol melanin formed by the oxidation of catechol (16), and 1,8-dihydroxynaphthalene (DHN) melanin synthesized from pentaketides (16, 254, 255). The synthesis of DHN-melanin is differentially expressed among members of the *Gaeumannomyces-*

Phialophora complex and in specialized structures within a single *Gaeumannomyces* strain (78).

The deposition of melanin, resulting in a pigmented phenotype, is frequently observed during fruit body formation in the sexual (perithecia) and asexual (conidia) phases of reproduction (54, 272) and imparts structural integrity to fungal elements requiring rigidity (226, 246, 248, 264). In the phytopathogenic fungi *Colletotrichum lagenarium* (137, 138) and *Magnaporthe grisea* (52, 118), melanization of appressoria is required for virulence. The appressoria are specialized attachment and penetration structures with an internal high turgor pressure that effects the mechanical penetration of intact host plants. Melanin-deficient mutants of these fungal cells are not sufficiently rigid to attain the turgor pressure required to mechanically breach the plant cell wall (52). However, no melanized penetrative structure has been identified in *G. graminis* var. *tritici* and transmission electron microscopy of *Gaeumannomyces* cell walls reveals DHN-melanin deposited as an amorphous granular layer (41) that is indicative of a protective rather than a penetrative function where the melanin is deposited as a smooth homogeneous layer (136). Melanin may enhance fungal survival and competitive ability by protecting melanized (pigmented) fungal structures from dehydration (16), solar irradiation (16) microbial lysis (23, 39, 190, 192, 229), lytic activity of soil constituents (152) and insect predation (33).

Melanin synthesis is associated with fungal morphological changes during growth and development (40, 138) and melanization of *G. graminis* var. *tritici* ectotrophic runner hyphae may impart protection from adverse environmental

conditions in the rhizosphere during the pathogen's initial colonization of the root surface and in the soil where hyphal fragments persist in plant tissue debris and function as an important inoculum source (16, 207, 256). However, during the parasitic phase, repressed melanin deposition in the infection hyphae may be required to facilitate lytic enzyme secretion for the penetration and colonization of root tissue.

To establish an infection, the successful *G. graminis* var. *tritici* pathogen must counter the effect of fungitoxic plant phenolic compounds and breach the intact host tissue and lignituber barriers. The fungal polyphenol oxidases (157, 158), laccase (*para*-diphenol: oxygen oxidoreductase, EC 1.10.3.2) (197) and tyrosinase (inclusive of cresolase, EC 1.14.18.1, and catecholase, EC 1.10.3.2, with monophenolase and diphenolase activities respectively) (202) are copper containing metalloenzymes that catalyze the detoxification of plant phenolic compounds. The phytoalexins (pisatin and cucurbitacin) produced by the pea and the cucumber in response to infection with *Fusarium solani* and *Botrytis cinerea*, are detoxified by laccase via enzymatic demethylation (232, 233) and the formation of phenoxy radicals which undergo spontaneous transformation or condensation reactions (5, 29, 221, 228, 245). The onion (*Allium cepa*) phytoalexin, catechol, is a precursor of fungal catechol melanin and is detoxified by *Colletotrichum circinans* tyrosinase (182). Fungitoxic phytoalexins and phytoanticipins are susceptible to oxidation by fungal polyphenol oxidases due to their inherent cyclic conjugated structures (5, 29, 31, 107, 129, 130, 228, 271). Even in the absence of an identified phytoalexin/fungal detoxification interaction between wheat and *G. graminis* var. *tritici,* the

importance of plant phenolic detoxification in the establishment of a successful infection can not be ruled out. Virulent *G. graminis* var. *avenae* strains produce avenacinase, a secretory protein that detoxifies oat avenacin. Virulent *G. graminis* var. *avenae* mutants that lose avenacinase activity also lose their pathogenicity on oats (178).

Laccase catalytic activity has also been associated with the depolymerization of the high molecular weight lignin macromolecule. Lignin degradation has been most extensively studied in the white-rot basidiomycetes that are defined on the basis of their ability to degrade all components of the plant cell wall leaving a white residue indicative of lignin mineralization. Fungal enzymes participating in lignin mineralization include two heme peroxidases, lignin peroxidase and manganese dependent lignin peroxidase, and laccase. Ascomycetes are soft-rot fungi (21) that were once considered capable of only limited chemical modification of lignin due to the absence of one or more of the lignin degradative enzymes. However, the elucidation of variable and highly complex lignin degradation pathways have demonstrated the important contribution of soft-rot fungi to lignin mineralization (44).

Degradation assays using ^{14}C-labeled dehydrogenation polymers (128, 132, 174) and processed natural lignins (124) have demonstrated that the phenylpropanoid is susceptible to Cα-oxidation, demethoxylation, aryl-Cα cleavage and Cα-Cβ cleavage in reactions initiated by laccase oxidation (31, 107, 122, 133, 147, 271,). The enzymatic abstraction of one electron from phenol hydroxyl groups by laccase (107, 130, 271) yields spontaneously reactive radicals that cause the

demethylation, side chain cleavage and ring opening in residues of the lignin polymer (107, 130, 147). *In vivo* evidence of the role of laccase in lignin degradation is provided by a laccase-minus mutant strain of *Sporotrichum pulverulentum* (4). The mutant was unable to degrade a natural processed lignin substrate unless supplemented with exogenous laccase and a laccase-plus revertant regained lignolytic ability. Previous characterization of the extracellular enzymes produced by *G. graminis* var. *tritici* failed to detect enzymes that may be involved in lignin degradation (250, 251).

Fungal polyphenol oxidase activity is also associated with cellular differentiation (157, 158) and the synthesis of melanin. Rhizomorph formation, aggregations of hyphae covered with a rind of thick-walled pigmented cells, by *Armillaria* species is proportional to laccase activity (264). Melanization of the zoopathogenic fungi *Wangiella dermatitidis* (72, 73, 74,) and *Cryptococcus neoformans* (141, 142, 199, 242) is associated with polyphenol oxidase activity and fungal dimorphism required for virulence. Laccase is required for DHN-melanin biosynthesis in *Aspergillus nidulans* (42, 54, 106, 140) and strongly implicated in DHN-melanin biosynthesis in *Cochliobolus heterostrophus* (224).

Fungal Phenotype and Pathogenicity

The classification of members of the *Gaeumannomyces-Phialophora* complex remains somewhat dependent on the assessment of phenotypic characteristics because the genetics of these Ascomycetes remains ill-defined. The non-pathogenic

Phialophora generally grow more slowly in culture than *Gaeumannomyces* (65), however, growth rate in culture rarely reflects growth rates observed on root tissue (11) and can not be used to rationalize pathogenicity differences among fungal isolates (19). The assessment of pathogenicity is also complicated by the sometimes rapid and significant loss of virulence in culture. Predominantly virulent field isolates can exhibit an 80% loss of virulence after serial transfer (47, 56, 168, 169) and a 50% loss of virulence if ascosporogenesis occurs in culture or on dead versus living host tissue (47).

All complex members establish an initial extensive ectotrophic colonization of the root rhizoplane with melanized runner hyphae and root surface colonization can not be used to assess risk from disease. Differences in the non-pathogen/pathogen infection processes become evident only after the fungal isolate initiates an endotrophic infection. Pathogenic *Gaeumannomyces* penetrate the stele causing intense vascular discoloration whereas non-pathogenic members of the complex present darkly-pigmented growth cessation structures within the cortex (65, 68). The absence of darkly-pigmented growth cessation structures in the *G. graminis* var. *tritici* infection may be indicative of required repression of melanin deposition in infection hyphae to facilitate lytic enzyme secretion for effective penetration of root tissue and colonization of the stele. With age, *G. graminis* var. *tritici* hyaline microhyphae differentiate into melanized macrohyphae (126,153) and suggest the chemical composition of the non-melanized and melanized fungal cell walls are identical prior to the onset of secondary metabolism and the deposition of DHN-melanin (40). The absence of melanin in the infection hyphae (microhyphae)

would facilitate increased lytic enzyme secretion and invasive capability during the pathogenic phase while the presence of melanin in the runner hyphae (macrohyphae) would offer protection to pigmented fungal structures during colonization of the root surface.

A relationship between *G. graminis* var. *tritici* pigmentation and pathogenicity has been assessed but has not been demonstrated. Virulence analysis in the out-cross progeny of light and dark field isolates indicated that pigmentation and pathogenicity were independently inherited (12, 19, 259). However, ascosporogenesis was induced in culture and cold incubation for an extended period of time was sometimes required for development of pigmentation differences. In a survey of 115 *G. graminis* var. *tritici* field isolates, it was reported that "dark mycelial color was not essential for pathogenicity" and therefore "correlation between the two characteristics was poor" (267). The survey results also described two highly virulent isolates which were not pigmented and an isolate with more pigment that was less pathogenic. The possible significance of this observed inverse correlation between pigmentation and pathogenicity may have been ignored due to accepted bias of the basic tenet that fungal melanization enhances pathogenicity.

Statement of Thesis

This study was undertaken to establish a correlation between *G. graminis* var. *tritici* pigmentation and virulence consistent with the *G. graminis* var. *tritici* infection process and the observed predominance of darkly-pigmented isolates from sources displaying little or no disease symptoms (51, 63, 65). Filamentous fungal

protein secretion is limited to non-melanized hyphal elements (184, 212, 247) and the deposition of melanin in the *G. graminis* var. *tritici* cell wall would result in decreased protein secretion efficiency with a concomitant decrease in fungal virulence.

To minimize the influence of variable genetic backgrounds on phenotype presentation, a single virulent *G. graminis* var. *tritici* isolate and its isogenic melanin variant strains were assessed *in vitro* for pigment phenotype and protein secretion capacity. The fungal strains were then assessed *in vivo* for pathogenicity on wheat to test the hypothesis that protein secretion efficiency is directly related to virulence and pigment phenotype.

The occurrence of the enhanced pigment phenotype and the presentation of *Phialophora*-like characteristics is noted and discussed.

MATERIALS AND METHODS

Fungal Strain Selection

Four strains of non-melanized *Gaeumannomyces graminis* (Sacc.) Arx & Olivier var. *tritici* (Walker) from discontinuous geographical sources were screened for enhanced pigmentation following protoplast formation and regeneration. The strains, from Denmark (H. Shultz, 1868), England (D. Hornby, 1805), France (P. Lucas, 1819) and the United States (D. Mathre, DM528), were all field isolates from wheat (*Triticum aestivum* L.) that had been purified by single ascospore isolation. Protoplasts were prepared as described (220), with the exception of a shortened cell-wall-digest incubation period (45 minutes), and regenerated on protoplast regeneration medium (103). Enhanced pigmentation in the melanin variant strains became evident within 7-14 days at room temperature (24-26 °C). The rates of occurrence of the enhanced pigment phenotype were determined in the four strains based on total number of regenerated protoplasts for that individual strain.

The wild-type strain selected for further study (D. Mathre, DM528) was isolated from Montana wheat and maintained in culture for more than eight years without apparent loss of pathogenicity. Wild-type morphology on maintenance LB agar [LB medium plus 1% (w/v) agar; 165] was characterized by non-pigmented hyphae lacking aerial growth. When grown on potato dextrose agar (PDA, Difco), which enhances pigmentation of *G. graminis* var. *tritici* (*Ggt*) strains, the culture

color remained ecru through four days but darkened slightly upon prolonged incubation (10-14 days) or refrigeration (4 °C). This wild-type *Ggt* strain undergoes meiosis to produce mature perithecia and fertile ascospores but phialospore production has never been observed.

Enhanced pigmentation was presented as melanin variant colonies with abundant aerial hyphae; the melanin variant strains (designated MV01, MV02, etc.) were isolated by transfer of the aerial hyphae to LB agar. The isolation plates were incubated for three days and the cultures transferred to LB maintenance plates by hyphal tipping to ensure the selected variant strains are homokaryotic (162).

Mitotic stability of the melanin variants was assessed by sequential serial transfer every 3-4 days on PDA. To assess meiotic stability, perithecia were produced on autoclaved wheat leaves on the surface of sterile Whatman no.1 filter papers on 1% (w/v) water-agar plates, and incubated for more than thirty days. Ascospores were harvested from individual perithecia and spread plated onto LB agar for germination. After 1-2 days incubation, the germinated ascospores were transferred to PDA to assess pigment phenotype. Post-meiotic, non-pigmented derivatives of the melanin variant strains (designated MV01-W, MV02-W, etc.) were purified by hyphal tipping and assessed for mitotic stability as described above.

All incubations, regardless of media type, were carried out at room temperature (24-26 °C). Maintenance plates were stored at 4 °C for up to two weeks. The source plates for all assays done in the characterization of the wild-type and variant strains were 3-4 day old plates that were not previously refrigerated.

Fungal Strain Characterization

Restriction Fragment Length Polymorphism Analysis

To verify *Ggt* as the source of the phenotypically dissimilar melanin variant strains, restriction fragment length polymorphism (RFLP) analysis was done with the restriction endonucleases *Sau*3A, *Dpn*II, *Msp*I, *Hpa*II, *Xho*I, *Xba*I, and *Bgl*II (all from New England Biolabs). Agarose gels (0.6%; Type I-A: Low EEO, Sigma) were run at 60 volts and stained with ethidium bromide for detection of the DNA bands.

Linear Growth Rates

To compare the linear growth rates of the five strains selected, 80 ml of LB medium in a 300-ml Erlenmeyer flask were inoculated with twelve plugs of fungal hyphae transferred from the margin of a four-day old LB agar maintenance plate using a sterile Pasteur pipette. Flasks were incubated at room temperature on a rotary shaker and mycelia were harvested by vacuum filtration through Whatman no. 1 filter paper supported in a Buchner funnel. Mycelial dry weights of lyophilized cultures were determined after 3-6 days incubation in triplicate trials.

Methylation Determination

To determine if the *Ggt* "melanin" gene(s) were silenced via DNA methylation, the strains were treated with 5-azacytidine (Sigma) to inhibit DNA methylation (75, 125). Protoplasts of *Ggt*, MV01 and MV01-W are suspended in protoplast buffer containing 150, 300, 500 and 800 μM 5-azacytidine and incubated

overnight on a rotary shaker at room temperature. Aliquots of the treated protoplasts were plated onto regeneration medium and incubated at room temperature for 7-8 days. Control plates consisted of untreated protoplasts and dilution plates were done to determine protoplast recovery for the control treatment and at all 5-azacytidine concentrations. In an alternate treatment, protoplasts of *Ggt* and MV01 were recovered on regeneration medium containing 500 μM 5-azacytidine. Individual regenerated colonies were transferred to PDA and incubated for 7-10 days to assess the pigment phenotype.

Phialospore Formation

Agar plugs from the colony margin of 4-day old LB maintenance plates were transferred to 1% (w/v) water-agar plates with or without surface-sterilized pre-germinated wheat seeds. The wheat (*Triticum aestivum* L.) seeds were surface-sterilized with 1% AgNO$_3$ for three minutes, rinsed once with 2.5 M NaCl and three times with sterile distilled H$_2$O, and pre-germinated for 48 hours on moistened sterile Whatman no. 1 filter paper. The water agar plates were incubated in natural light at room temperature for five days. The inoculation plugs were removed and placed in a drop of sterile distilled H$_2$O on a slide and cover slipped. Gentle pressure applied to the cover slip released phialospores into suspension for transfer to water-agar plates to assess germination. Germinated phialospores were transferred to PDA and incubated at room temperature for 5 days to assess pigment phenotype.

Protein Secretion and Enhanced Pigmentation

Relative Total Protein Secretion

Relative total protein secretion was assessed for the five strains grown in 80-ml LB as described. Aliquots of the culture filtrates were removed at 24-hour intervals and frozen at -20 °C until assayed for relative total protein content by SDS-PAGE (3-10% gradient) (143) and silver staining (22). The cultures were harvested on day 6 and the fungal mass was determined.

The effect of enhanced pigmentation on relative total protein secretion was assessed in the wild-type and the melanin variant strains MV01 and MV02. Duplicate cultures were grown in 80-ml LB as described and tricyclazole was added at 8 μg/ml on day two and day three to one set of LB culture flasks. On day four, the fungal mycelia were harvested and lyophilized to determine dry weights and the culture filtrate samples were analyzed by SDS-PAGE and silver stained to determine relative total proteins secreted. The volume of the culture filtrate sample assayed for each individual strain was adjusted to compensate for fungal mass differences.

Plant-Cell-Wall Degrading Enzyme Secretion

The plant-cell-wall degrading enzymes secreted by *Ggt* are well characterized and the effect of enhanced pigmentation on their secretion was assessed. Base medium to assess pectinase secretion contained 0.1% yeast extract, 0.125% each

biotin and thiamin, and 2% agar. To detect pectin lyase (PL, EC 4.2.2.2), the base medium was supplemented with 1% apple pectin (Sigma) and prepared with 10-mM citrate-phosphate and 10-mM glycine buffers to maintain the pH required, pH 5.0 (250), and pH 9.0 (156), respectively. Secretion of PL was assessed by positive hydrolysis indicated by a clear halo surrounding the colony after flooding the plates with 1% cetyltrimethylammonium bromide (CTAB; Sigma). To assess polygalacturonase (PGU, EC 3.2.1.15) secretion, the base medium contained 1% sodium-polypectate (Sigma) at pH 6.5 in 10-mM sodium-phosphate buffer and positive hydrolysis was detected as a cleared zone after flooding the plates with 5 N hydrochloric acid (250).

Base medium for the detection of cellulases contained 2.5% Czapek Dox Broth (Difco), .125% each biotin and thiamin, and 2% agar. The endo-β-gluconase substrate was provided as 1% carboxymethyl cellulose (Sigma) and hydrolysis was detected by flooding plates with 0.5% Congo red, rinsing with 1M NaCl and measuring the cleared area (2). The cellobiohydrolase substrate was supplied as 1% α-cellulose and hydrolysis was detected as a cleared yellow zone after developing the plates with Lugol's solution (Sigma, St. Louis, MO) (269).

Media used to detect the enzymatic hydrolysis of skim milk protein (234) and gelatin (100) were prepared and developed as described in the references cited.

To assess the effect of DHN-melanin biosynthesis inhibition on the secretion of plant-cell-wall degrading enzymes, the above plating media were prepared with tricyclazole (16). Tricyclazole, 5-methyl-1,2,4-triazolo-(3,4-6)-benzothiazole (Eli Lilly Research Laboratories, Greenfield, IN.) was dissolved in 100% ethanol (2.5 mg/ml

stock), filter-sterilized and added to cooled, autoclaved medium to a final concentration of 16 μg/ml. Hydrolysis of the test substrate was assessed as described above and positive reaction area was determined and compared to that observed on identical media without added tricyclazole.

Phenolic Detoxifying Enzymes

The growth of *Ggt* within the host tissue may require the secretion of phenolic detoxifying enzymes. Therefore, the effect of enhanced pigmentation on fungal growth rates was assessed on PDA supplemented with the fungitoxic phenolic compounds *p*-coumaric acid and caffeic acid (Sigma). Stock phenolics were prepared as 0.25 M solutions in 95% ethanol, filter sterilized and added to autoclaved media after cooling to final concentrations of 1.0 mM. The plates were centrally inoculated with a 0.5-cm plug from the margin of a four-day old PDA culture and incubated in the dark at room temperature for eight days. The mycelium was harvested by liquefying the agar in 100 ml water in a microwave and collecting the mycelia by filtration through a Whatman no. 1 filter. Dry fungal weight was determined after lyophilization as an indication of fungal growth rate. The control plates consisted of PDA without added phenolic compounds.

Polyphenol Oxidase

Spot Test Reagents. A polyphenol oxidase (PPO) has not been previously reported in *Ggt* and several potential substrates were used as spot test reagents to

screen four-day old cultures on LB plates. Syringaldazine, (N,N'-bis-3,5-dimethoxy - 4-hydroxybenzylidene hydrazine, Sigma) (100), prepared as a 0.1% solution in 95% ethanol, α-Naphthol (1-hydroxy naphthalene) (155, 258), L-tyrosine and *p*-cresol (64) (both from Sigma) prepared as 1.44%, 0.5% and 1% aqueous solutions, respectively, and 2,6-dimethoxyphenol (DMOP, Fluka Chemika, Switzerland) prepared as a 1.25 mM solution in 10 mM citrate phosphate buffer, pH 5.0, were dropped onto the agar surface after the fungal mass was removed and color development determined over a range of time periods specific to each spot test reagent.

Quantitative Reagent. PPO activity was quantitated with the DMOP colorimetric assay where the colorless monohydroxyphenol substrate is oxidized to an orange product, 3,5,3',5'-tetramethoxydiphenoquinone (25, 26). Reaction duration and color intensity limits were determined at a range of PPO concentrations so that the colorimetric reaction accurately reflected the concentration of PPO present. The sample volume containing the range of PPO concentrations was kept constant at 0.5 ml by premixing the culture filtrate with an appropriate volume of LB. All cuvettes containing the DMOP substrate were blanked to zero absorbance at 468 nm prior to the addition of the enzyme solution and all assays at each enzyme concentration are done in triplicate. One unit of enzyme activity was defined as that amount that catalyzed a change in absorbance of 1.0 at 468 nm over a one minute interval.

Ggt culture filtrates were prepared in 300-ml Erlenmeyer flasks containing 80-ml LB medium inoculated and incubated as described for determination of fungal growth rates. After four days, the culture filtrate was harvested by filtration through a Whatman no. 1 filter. The assay mixture consists of 2.5 ml of 1.25 mM DMOP in 10 mM citric phosphate buffer, pH 5.0, plus 0.5 ml culture filtrate.

Effect of Redox Agents on Polyphenol Oxidase Activity. Due to the lack of PPO substrate specificity, the effect of redox agents on activity was determined to facilitate PPO identification. The DMOP assay was used to determine the effects of pretreatment of the culture filtrate with oxidation inhibitors specific for laccase (sodium azide and cetyltrimethylammonium bromide), tyrosinase (polyvinyl-pyrrolidone) and peroxidase (catalase) (all from Sigma). NaN_3 inactivates laccase by forming an azide bridge between copper sites 2 and 3 (157) and cetyltrimethylammonium bromide (CTAB) inhibits oxidation by laccase (13, 241), possibly by altering the tertiary structure to lessen its catalytic activity (240) or anionic inactivation of the Type 2 Cu^{2+} (7). Polyvinylpyrrolidone (PVP) acts as a competitive inhibitor of tyrosinase activity (240). Catalase was added to the culture filtrate to remove endogenous H_2O_2, a cofactor required by peroxidase for catalytic activity. Exogenous H_2O_2 was added to culture filtrates to stimulate peroxidase catalytic activity (101, 102). Controls consisted of untreated culture filtrate and all assays are done in triplicate.

Dependence of Polyphenol Oxidase Secretion on Culture Age. The temporal pattern of PPO secretion by the selected fungal strains was determined in triplicate

LB cultures. A 2-ml aliquot of culture filtrate was removed from each flask on days three through six and frozen at -20 °C until processed. The collected culture filtrates were warmed to 4 °C and PPO activity was determined using DMOP as substrate.

Effect of Tricyclazole on Polyphenol Oxidase Secretion. The effect of enhanced pigmentation on the secretion of PPO by MV01 was assessed using tricyclazole to inhibit DHN-melanin biosynthesis. Wild-type *Ggt* and MV01 were grown in 80 ml LB as described and tricyclazole was added at 8 μg/ml on day two and day three to one set of triplicate LB cultures. The culture filtrate and the fungal mycelia were harvested on day four to determine PPO activities, using DMOP as substrate, and to determine dry fungal weights, respectively.

Polyphenol Oxidase Characterization

Induction Medium for Polyphenol Oxidase Production.

For the production of PPO, *Ggt* was grown in a defined medium containing per liter: KH_2PO_4 (1.0g), $Na_2HPO_4 \cdot 2H_2O$ (0.1g), L-asparagine (0.5g), D-phenylalanine (0.1g), L-phenylalanine (0.1g), adenine hemisulfate (.04g), sucrose (20g) $FeSO_4 \cdot 7H_2O$ (200μg), thiamin-HCl (100μg), $MgSO_4 \cdot 7H_2O$ (0.5g), $MnSO_4 \cdot H_2O$ (100μg), $ZnSO_4 \cdot 7H_2O$ (100 μg), biotin (800μg), and $CuSO_4 \cdot 5H_2O$ (.01g) (modified from 80, 81). The salts and vitamin supplements were autoclaved separately and added after cooling to prevent precipitation. Eighty ml of medium in a 300-ml Erlenmeyer flask was inoculated and incubated as described for linear growth

determination. Two days after inoculation, additional $CuSO_4 \cdot 5H_2O$ was added to a final concentration of 400μM. The cultures were induced to secrete PPO by adding 2,5-xylidine (2,5-dimethylaniline, Aldrich Chemical Co., Milwakee, WI) to a final concentration of 200μM on day four and adding half that amount on day six (80). Culture filtrates were harvested on day eight by filtration through Whatman no. 1 filter paper and either processed immediately or frozen at -20° C until processed.

The concentration of copper sulfate to be added for maximal PPO production without inhibiting fungal growth was determined by supplementing the media with variable copper sulfate concentrations at day two. PPO secretion was determined in both static (195, 225) and shaken cultures (27, 263) over a nine day period using the optimal copper concentration.

<u>Crude Polyphenol Oxidase Preparation</u>.

Defined medium culture filtrate harvested at day-8 was cooled to 4 °C and 361 g/l ammonium sulfate (Sigma) was added (60% saturation) and the solution was equilibrated for 30 minutes on ice with gentle stirring. The preparation was centrifuged at 15,000 rpm for 20 minutes to remove precipitated protein. The supernatant was collected and an additional 279 g/l ammonium sulfate (100% saturated) was added with gentle stirring for 30 minutes and equilibrated over night at 4 °C. Centrifugation was repeated to collect the protein fraction containing the PPO. No pH adjustment was required during the salting out procedure. The precipitates from the 60% and 100% saturation steps, 60% and 100% fractions respectively, were resuspended in a minimal volume of 10 mM citrate phosphate

buffer, pH 5.0, and dialyzed extensively at 4 °C against the same buffer using a Spectra/Por 6 Membrane with a 25,000 molecular-weight-cut-off rating.

SDS-PAGE Electrophoresis.

Polyacrylamide gel electrophoresis was done according to Laemmli on 3-10% gradient SDS-PAGE gels. To accommodate the different polymerization rates of the 3% and 10% bis-acrylamide solutions (Acrylamide/bis-Acrylamide 40% Stock, 37:1 Ratio, Sigma), ammonium persulfate (Bio Rad) was added at .06% and .03% (w/v), respectively, prior to mixing the gradient. The protein standard used was SDS-6H (Sigma) with a molecular weight range of 29-205 kDa. The gels were run at 60V for 18 hours at 4°C and the protein bands were localized with either the silver stain as described (22) or activity stained by immersing the gel in a 1.25 mM solution of DMOP in 10 mM citrate phosphate buffer, pH 5.0, for 15 to 20 minutes. Samples included both native and denatured (heated to 100 °C for 90 seconds) PPO preparations as indicated in the results.

Preparative SDS-PAGE for Polyphenol Oxidase Characterization

The 100% fraction (defined in Crude Polyphenol Oxidase Preparation) was subjected to SDS-PAGE (3-10% gradient) and a portion of the gel was immersed in DMOP to locate PPO activity. A strip from the gel corresponding to the enzyme's location was cut from the untreated portion of the gel and the PPO was eluted into 10 mM citrate phosphate buffer, pH 5.0, by placing the gel strip in dialysis tubing (Spectra/Por 6 Membrane, 25,000 MWCO) in distilled H_2O at 4 °C for 72 hours.

This enzyme preparation was concentrated by centrifugation in an Ultrafree-4, Biomax 30 (Millipore), for 5 minutes at 2,000 g.

Polyphenol Oxidase Deglycosylation

The proteins present in the 60% fraction, the 100% fraction, and the PAGE-eluent were deglycosylated using PNGase F (New England Biolabs) per manufacturer's instructions. The protein bands were localized with silver staining.

pH Range of Polyphenol Oxidase Activity

The pH optimum for PPO activity was determined using 1.25 mM DMOP in 10 mM citric acid (pH 2.6 - pH 6.0), citrate phosphate (pH 3.8 - pH 6.8), and phosphate (pH 6.2 - pH 8.2) buffers.

Determination of Polyphenol Oxidase Isoelectric Point

The isoelectric point of the *Ggt* PPO was determined using a Rotofor Prep IEF CELL (Bio Rad) per manufacturers' instructions. Briefly, ampholytes pH 3-10 (Sigma) were prefocused for one hour at constant power, 12 W, using 0.1 M H_3PO_4 anode electrolyte and 0.1 M NaOH cathode electrolyte. The minimal induction medium culture filtrate (4 ml) was added at port number seven and separation was run at 12 W for three hours. The fractions were collected and PPO activity detected using syringaldazine as substrate.

Temperature Range of Polyphenol Oxidase Activity

To determine if PPO activity was lost during sample storage at -20 °C, activity was measured using DMOP as substrate at various time points after freezing. Temperature lability of the PPO was also determined after preincubation at various elevated temperatures.

Determination of Polyphenol Oxidase Km

The Km of the PPO was determined by Lineweaver-Burke plot using 0.1 - 3.0 mM DMOP, 10 mM citrate phosphate buffer, pH 5.0, as substrate. All colorimetric assays were done in duplicate as described within the range .02 - .20 units of PPO activity per reaction.

Lignin Degradation

The secretion of lignin degradative enzymes by *Ggt* was assessed by the Poly B-411 [Sigma (Poly B-411 is a trademark of Dynapol)] decolorization assay (49, 59, 95). There exists a good correlation between Poly B-411 decolorization and lignin degradative capability (49, 59). The decolorization of Poly B-411 is indicated by a decrease in absorbance at 593 nm (blue) with a concomitant increase in absorbance at 483 nm (brown). Decolorization is expressed as the ratio A_{593}/A_{483}.

Poly B-411 (a generous gift from L.J. Cookson; 0.2% w/v in water) was added to a final concentration of 0.01% to triplicate aliquots of LB culture filtrate (harvested after four days) and incubated at room temperature. The control

consisted of culture filtrate treated with 1 mM sodium azide prior to the addition of Poly B-411 at time point zero. At predetermined time intervals, 0.5 ml of the culture filtrate was removed and diluted 4-fold in 1 mM aqueous sodium azide and the absorbance ratio A_{593}/A_{483} was determined using a Gilford Instrument 2600.

Pathogenicity and Enhanced Pigmentation

G. graminis var. *tritici* Host

Wheat (*Triticum aestivum* L.) seeds were surface-sterilized with 1% AgNO3 for three minutes, rinsed once with 2.5 M NaCl and three times with sterile distilled water. The seeds were placed on moistened sterile Whatman no. 1 filter paper and pre-germinated for 48 hours at room temperature in natural light.

Plant Mass Assay

As an indirect measure of infection (10, 84), wheat shoot mass assays were replicated five times in 10-cm pots filled in layers from the bottom of the pot as follows: 50 mm vermiculite, nine 4-mm agar disks taken from the colony margin of a four day old LB culture, 15 ml vermiculite, 20 pre-germinated wheat seeds and 15 mm vermiculite. Sterile agar disks were used as the control treatment. Plants were watered to saturation with one-quarter strength Hoagland's solution (108) once a week, harvested after 23 days and lyophilized to determine the dry shoot and dry root weights.

Root Lesion Assay

As a direct measure of infection, the total length of root-stelar lesions in infected wheat plants was determined for nine replicates in a tube assay as described (198, 217). Conical 15-ml centrifuge tubes were filled to the 9 ml mark with vermiculite and an agar plug from the edge of a four-day old LB culture was added and covered with 4 ml vermiculite. Finally, a pre-germinated wheat seed was added and covered with 1 ml vermiculite. Plants were watered to saturation with sterile water two times a week and harvested after 14 days. All plants were grown at room temperature (22-25°C) with a 12- hour photo-period using a GE Grow-Lite providing 3000 Lux.

Histopathology

To assess infection patterns and histological changes in infected roots, cryostat sections were prepared (2800 Frigocut N, Reichert-Jung) of FAA preserved tissue sections embedded in O.C.T. Compound 4583 (Tissue Tek, Miles Inc. Elkhart, IN). To ensure adequate embedding, the FAA solution was replaced with O.C.T. Compound and allowed to sit at room temperature for 24 hours prior to embedding with fresh O.C.T. Compound. The root area in closest proximity to fungal inoculation depth was selected for embedding. Sections (12-μm) were stained with Toluidine Blue-O (.05% w/v in 0.1 mM phosphate buffer, pH 6.8) (175) and examined microscopically.

RESULTS

Fungal Strain Selection

Enhanced pigmentation resulting in the melanin variant (MV) phenotype was not unique to a single *G. graminis* var. *tritici* isolate but occurred in three of the four *Ggt* strains tested at a mean rate of 2 per 10^7 regenerated protoplasts. No melanin variants were detected in the wheat isolate from Denmark (H. Shultz, 1868). The rate of occurrence of the melanin variant phenotype in the Montana wheat isolate (D. Mathre, DM528) selected for further study was not significantly different at 4.5 per 10^7 regenerated protoplasts. Melanin variants were evident as heavily-melanized colonies producing abundant aerial hyphae in an ecru background lacking aerial growth (Fig. 1a).

The isolated melanin variants are not phenotypically identical (Fig. 1b) but the respective pigment phenotype remained stable for twelve isolates tested through fifteen serial transfers over a 55 day period. Meiotic stability of the enhanced pigment phenotype was assessed in self-crosses with 460 ascospores harvested from 19 perithecia. The ascospores yielded 254 (55%) melanin variant and 206 (45%) non-melanized colony types. Phenotypic mitotic stability was confirmed for thirty-nine of the post-meiotic melanin variant isolates through twelve serial transfers over a 42 day period. In contrast, many non-melanized derivatives displayed poor growth rates from serial transfer four and 2% died by transfer twelve.