ABSTRACT

Vélez, Heriberto. *Alternaria alternata* mannitol metabolism in plant-pathogen interactions. (Under the direction of Dr. Margaret E. Daub)

Mannitol is purported to have role in fungi as a storage carbohydrate and has been shown to quench reactive oxygen species (ROS) both *in vitro* and *in vivo*. Mannitol metabolism in fungi is thought to occur through the mannitol cycle, which was proposed in the late 1970's from studies of cell free extracts of the fungus *Alternaria alternata.* In this cycle, mannitol 1-phosphate 5-dehydrogenase (MPDH; EC 1.1.1.17) reduces fructose 6-phosphate into mannitol 1-phosphate, which is dephosphorylated by a mannitol 1-phosphatase (EC 3.1.3.22) resulting in mannitol and inorganic phosphate. Mannitol also can be made through the enzyme mannitol dehydrogenase (MtDH; EC 1.1.1.138), which reduces fructose to mannitol. Here we report confirmation of these enzymes in the fungus *A. alternata*, the isolation of the genes, and the generation of strains mutated in *MPDH*, *MtDH*, or both genes. PCR confirmed gene replacement and enzyme assays using these mutants showed no activity for MtDH or MPDH. GC-MS analysis showed that strains deficient in both enzymes did not produce mannitol, while strains deficient in either MPDH or MtDH had reduced mannitol production. Mannitol, as a quencher of ROS, may also have a role in host-pathogen interactions, by allowing the fungus to suppress ROS-mediated plant defense responses. To assess the contribution of mannitol in plant-pathogen interactions, wild type, single and double mutants were used in pathogenicity assays on tobacco plants. Severity of lesions caused by the MtDH disruptant was not significantly different from that of the wild type. By contrast, the MPDH disruptant and the double mutant caused significantly less disease. Microscopy analysis and histochemical staining for H_2O_2 showed that both the wild type strain and the double mutant were able to germinate, produced appressoria, and elicited a defense response from the host. Quantitative PCR studies showed that genes for both enzymes were up-regulated in the presence of tobacco extracts, with *MPDH* having a stronger response. We conclude that mannitol biosynthesis is required for pathogenesis of *A. alternata* on tobacco, but is not required for normal spore germination either *in vitro* or *in planta* or for initial infection.

Alternaria alternata mannitol metabolism in plant-pathogen interactions

by

Heriberto Vélez

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Approved by:

Dr. Gary A. Payne	Dr. Marc A. Cubeta
Dr. Margaret E Daub, Chair	Dr. Greg G. Upchurch

DEDICATION

To Michael P. Meiners, for all the times we have shared, thank you for being you and for being there.

BIOGRAPHY

My name is Heriberto Vélez; however, I prefer my nick name "Hery" as my father would spell it or Eddie, as my English speaking friends call me. I was born in Puerto Rico and moved to Florida, with my siblings during the early 1980s. I attended Osceola High School, in Kissimmee, FL from which I graduated with honors. Prior to attending college, I decided to see the world and traveled to Boston, MA where I ended up working for Houghton Mifflin Co., a publishing company, for five years. However, having been interested in science since an early age, I decided to return to Florida where I began attending the University of Central Florida, while working for the Naval Air Warfare in Orlando. However, I transferred to North Carolina State University, where I got my degree in biochemistry. As an undergraduate working during a summer internship in the laboratory of Dr. Barry Goldfarb, I got interested in plants, so I decided to stay at NC State University and pursue a degree in Plant Pathology. And here I am…..

ACKNOWLEDGMENTS

I want to express my sincerest appreciation to Drs. Margo Daub, Gary Payne, Marc Cubeta, Greg Upchurch, and Scott Chilton….you are an inspiration to me. To Drs. Tanya Taylor, Sheri Denslow, and Sonia Herrero, for your encouragement…. and Beth Rueschhoff, is your turn now Beth! To Drs. Marilyn Ehrenshaft, John Williamson, and Mason Pharr… I hope that my findings are able to advance our knowledge…

TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER 3: Mannitol biosynthesis is required for pathogenicity of *Alternaria alternata*

LIST OF TABLES

Literature Review

Introduction

Mannitol is a six-carbon polyhydroxyalcohol (polyhydric alcohol; polyol; sugar alcohol) produced by many organisms including plants, algae, lichens, bacteria, and fungi, where it was first discovered by Henri Braconnot in 1811 (Cochrane 1958, Lewis and Smith 1967). In fungi, mannitol is the most common polyol, found in large quantities in spores, fruiting bodies, sclerotia, and mycelia (Lewis and Smith 1967). In *Agaricus bisporus*, for example, mannitol can contribute up to 20% of the mycelium dry weight and increases dramatically to 30-50% in differentiating sporophores (Stoop and Mooibroek 1998). In *Aspergillus niger* conidiophores, mannitol makes up to 10-15% of the dry weight (Ruijter et al. 2003). As much as 50% of the glucose consumed by some *Aspergillus* species may be excreted as mannitol in culture (Lewis and Smith 1967), presumably because such high concentrations cannot be retained in the cells (McCullough et al., 1986). It has been suggested that mannitol and other polyols are produced as carbohydrate reserves, act as translocatory compounds, function in osmoregulation, serve to regulate coenzymes and storage of reducing power and could also serve to regulate cytoplasmic pH by acting as sinks or sources for protons (Lewis and Smith 1967; Jennings 1984). More recently, mannitol has been shown to quench reactive oxygen species (ROS) both *in vitro* (Smirnoff and Cumbes 1989) and *in vivo* (Chaturvedi et al., 1997), and thus may have a role as an antioxidant in host-pathogen interactions (Jennings et al., 1998).

Mannitol metabolism in fungi

The mannitol cycle was proposed by Hult and Gatenbeck (1978) from studies of cell-free extracts of the fungus *A. alternata* (Fig. 1). Cell-free extracts from both an alternariol-producing and a non-producing strain were prepared from mycelia grown in Czapek-Dox medium. Specific activities of mannitol dehydrogenase, mannitol 1-phosphate 5-dehydrogenase, mannitol 1-phosphatase, hexokinase, glucose 6-phosphate dehydrogenase, glucose phosphate isomerase, phosphofructokinase and aldolase were measured to obtain information on the regulation of the cycle. In the proposed mannitol cycle, the enzyme mannitol 1-phosphate 5-dehydrogenase (MPDH; EC 1.1.1.17) would catalyze the reduction of fructose-6-phosphate to mannitol-1-phosphate using the cofactor NADH. Mannitol-1-phosphatase (M1Pse; EC 3.1.3.22), presumed to be specific for mannitol 1-phosphate (Yamada et al., 1961, Ramstedt et al., 1986), would hydrolyze mannitol 1-phosphate to mannitol and inorganic phosphate. Mannitol would then be oxidized to fructose by mannitol dehydrogenase (MtDH; EC 1.1.1.138) using the cofactor $NADP^+$. Finally, fructose would be phosphorylated to fructose 6-phosphate by a hexokinase (EC 2.7.1.1), completing the cycle. The cycle, as proposed, goes in one direction with the production of mannitol by MPDH and its utilization by MtDH. Thus, NADPH is produced at the expense of one molecule of ATP and NADH. Hult and Gatenbeck (1978) found no differences on the specific enzyme activities between the two strains of *A. alternata*, which could explain why the non-producing strain would synthesize more fat and oxidize more mannitol than the alternariol-producing strain. They further postulated that the cycle was regulated by the availability of the coenzymes NADH and $NADP^+$.

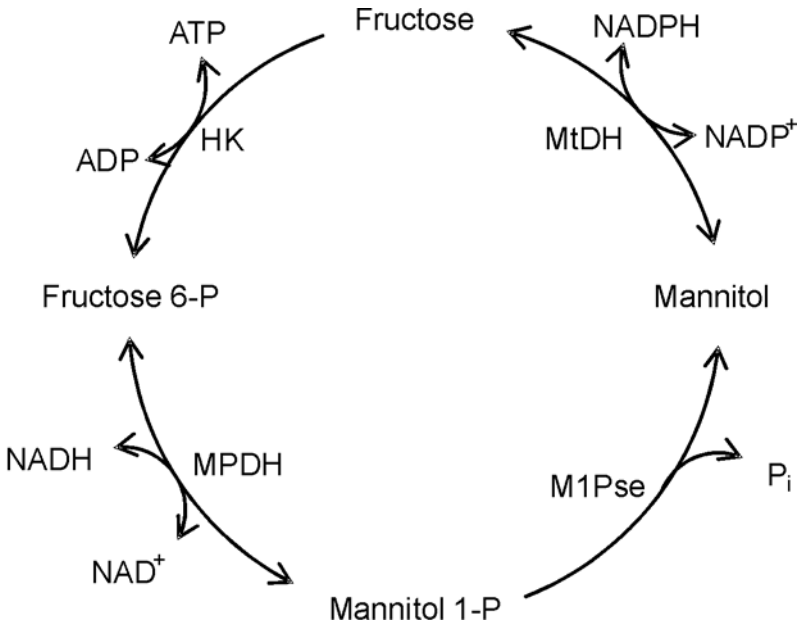

Figure 1. Mannitol cycle proposed for *A. alternata* (Hult and Gatenbeck, 1978)

Many researchers studied mannitol metabolism in fungi (Fig. 1, Table 1) during the 1960's through the 1980's and reported evidence for both MtDH and MPDH enzyme activities. Most researchers used cell-free extracts and some also purified and characterized the different enzymes in the cycle. Sometimes, MtDH activity was present but no MPDH activity would be found in spite of the presence of a mannitol 1-phosphatase as it was the case for *Lentinus edodes*, *Piloderma croceum*, *Siullus variegatus*, *Amanita muscaria*, *Marasmius scorodonius* and *Mycena metata* to name a few ((Ramstedt et al., 1987; Kulkarni 1990). In some cases, for example *Penicillium notatum*, MPDH activity would be found but no MtDH activity could be detected (Boonsaeng et al., 1976). Based on enzymatic assays, a different pathway for mannitol utilization was proposed by Ueng et al., (1976) for the zygomycete fungus *Absidia glauca*. Ueng, et al., (1976) found that extracts of *A. glauca* contained an NAD-dependent MtDH (EC 1.1.1.67) and a NAD-dependent MPDH

Table 1. MtDH and MPDH enzyme activities reported in fungi

Organism	MK[1]	FK[2]	M1Pse[3]	MtDH[4] Cofactor	MPDH[5] Cofactor	Sequence Reported[6]	Phyla[7]	Reference
Alternaria alternata		X	X	NADP	NAD	AAN28666 AAQ63948	A	Hult and Gatenbeck 1978; Hult et al., 1980 This work
Aspergillus candidus	N		X	NADP	NAD	N/N	A	Strandberg 1969
Aspergillus fumigatus					NAD	N	A	Boonsaeng et al., 1976
Aspergillus nidulans		X	X	NADP	NAD	N/N	A	Hankinson and Cove 1975; Singh et al., 1988
Aspergillus niger		X	X	NADP	NAD	AAL89587	A	Boonsaeng et al., 1976; Hult et al., 1980 Kiser and Niehaus 1981 Ruijter et al., 2003
Aspergillus oryzae					NAD	N	A	Boonsaeng et al., 1976
Aspergillus parasiticus			X	NADP	NAD	N/N	A	Foreman and Niehaus 1985; Niehaus Jr and Dilts 1982
Botrytis cinerea		X	X	NADP	NAD	N/N	A	Hult et al., 1980
Candida magnoliae				NADP		Y	A	Lee et al., 2003
Candida utilis		X	X	NADP	N	N	A	Hult et al., 1980
Cenococcum graniforme		X		NADP	NAD	N/N	A	Martin et al., 1985
Cephalosporium chrysogenum				NADP		N	A	Birken and Pisano 1976
Ceratocystis multiannulata		X	X	NADP	N	N	A	Hult et al., 1980
Chaetomium globosum		X		NAD		N	A	Adomako et al., 1972
Chaetomium thermophile var. dissitum					NAD	N	A	Boonsaeng et al., 1976
Cladosporium cladosporioides		X	X	NADP	NAD	N/N	A	Hult et al., 1980
Cladosporium fulvum				NADP		AF387300	A	Noeldner et al., 1994
Coccidioides immitis					NAD	N	A	Lones and Peacock 1964
Diplodia viticola				NADP		N	A	Strobel and Kosuge 1965
Geotrichum candidum				NADP		N	A	Chang and Li 1964
Gibberella zeae		X	X	NADP	N	AAP33281	A	Hult et al., 1980 Trail and Xu 2002
Humicola lanuginosa					NAD	N	A	Boonsaeng et al., 1976
Malbranchea pulchella var. sulfurea					NAD	N	A	Boonsaeng et al., 1976
Neurospora crassa		X	X	NADP	N	N	A	Hult et al., 1980
Penicillium chrysogenum				NADP	NAD	N/N	A	Boonsaeng et al., 1976 Boutelje et al., 1983
Penicillium cyclopium		X			NAD	N	A	Boonsaeng et al., 1976
Penicillium frequentans		X	X	NADP	NAD	N/N	A	Hult et al., 1980
Penicillium islandicum		X	X	NADP	NAD	N/N	A	Hult et al., 1980
Penicillium duponti		X			NAD	N	A	Boonsaeng et al., 1976
Penicillium notatum		X	X		NAD	N	A	Boonsaeng et al., 1976
Penicillium urticae					NAD	N	A	Boonsaeng et al., 1976
Pyrenochaeta terrestris			X		NAD	N	A	Wright and Le Tourneau 1966
Pyricularia oryzae		X	X	NADP	NAD	N/N	A	Yamada et al., 1961 Hult et al., 1980
Saccharomycete cerevisiae				NAD		U18795	A	Perfect et al., 1996

Table 1. MtDH and MPDH enzyme activities reported in fungi (continuation)

Organism	MK[1]	FK[2]	M1Pse[3]	MtDH[4] Cofactor	MPDH[5] Cofactor	Sequence Reported[6]	Phyla[7]	Reference
Sclerotinia sclerotiorum			X	NADP	NAD	N/N	A	Wang and Le Tourneau 1972
Sphaerosporella brunnea		X	X	NADP	NAD	N/N	A	Ramstedt et al., 1987
Stagonospora nodorum					NAD	AAT84078	A	Solomon et al., 2005
Thermomyces lanuginosus		X	X	NADP	NAD	N/N	A	Hult et al., 1980
Trichothecium roseum		X	X	NADP	NAD	N/N	A	Hult et al., 1980
Agaricus bisporus		X		NADP		AAC79985	B	Stoop and Mooibroek 1998; Morton et al., 1985;
Agaricus campestris				NADP		N	B	Edmundowicz and Wriston 1963
Amanita muscaria		X	X	NADP		N	B	Ramstedt et al., 1987
Armillariella mellea		X	N	N	N		B	Ramstedt et al., 1987
Chondrosterium purpureum		X	N	NAD	N	N	B	Ramstedt et al., 1987
Cryptococcus neoformans					NAD	AAG09209	B	Suvarna et al., 2000
Heterobasidion annosum		X	N	NAD	N	N	B	Ramstedt et al., 1987
Laccaria laccata		X	X	NADP	N	N	B	Ramstedt et al., 1987
Lentinus edodes		X	X	NADP	N	N	B	Kulkarni 1990
Marasmius scorodonius		X	X	NAD	N	N	B	Ramstedt et al., 1987
Melampsora lini				NADP		N	B	Clancy and Coffey 1980
Mycena metata		X	X	NAD	N	N	B	Ramstedt et al., 1987
Piloderma croceum		X	X	NADP	NAD	N/N	B	Ramstedt et al., 1986
Pleurotus ostreatus		X	X	NADP	NAD	N/Y	B	Chakraborty et al., 2004
Schizophyllum commune				NAD		N	B	Niederpruem et al., 1965
Suillus bovinus		X	N	NADP	N	N	B	Ramstedt et al., 1987
Suillus variegatus		X	X	NADP	N	N	B	Ramstedt et al., 1987
Uromyces viciae-fabae				NADP		O00058	B	Voegele et al., 2005
Absidia glauca	X	X	N	NAD	NAD	N/N	Z	Ueng et al., 1976
Mucor genevensis		X					Z	Boonsaeng et al., 1977 Boonsaeng et al., 1976
Mucor miehei		X					Z	Boonsaeng et al., 1977 Boonsaeng et al., 1976
Mucor pusillus		X					Z	Boonsaeng et al., 1977 Boonsaeng et al., 1976
Mucor rouxii		X			NAD	N	Z	Boonsaeng et al., 1977 Boonsaeng et al., 1976

[1]MK = Mannitol kinase; X = activity confirmed by enzyme assays; N = no activity by enzyme assays
[2]FK = Fructokinase; X = activity confirmed by enzyme assays; N = no activity by enzyme assays
[3]M1Pse = Mannitol 1-phosphatase; X = activity confirmed by enzyme assays; N = no activity by enzyme assays
[4]Notation of co-factor indicates presence of MtDH activity; N = no activity by enzyme assays
[5]Notation of co-factor indicates presence of MPDH activity; N = no activity by enzyme assays
[6]N= no gene or sequence reported; Y = gene recovery reported but no sequence provided; NCBI Accession numbers provided for
[7]A: Ascomycota, B: Basidiomycota, Z: Zygomycota

(EC 1.1.1.17); however, no mannitol 1-phosphatase was found. This led them to propose a different cycle for mannitol metabolism in this fungus (Fig. 2). In this cycle, mannitol could be converted to fructose using the NAD-dependent MtDH or vice versa. Fructose could then be converted to fructose 6-phosphate and enter the glycolytic pathway. A mannitol kinase could also add a phosphate group to make mannitol 1-phosphate, which could them be converted to fructose 6-phosphate through the NAD-dependent MPDH. They also showed that mannitol 1-phosphate would inhibit MtDH at high concentrations. No other studies have been carried out to corroborate this cycle or to determine if this cycle is limited to members of the Zygomycota. Although, Boonsaeng, et al., (1976) found enzyme activity for MPDH in another zygomycete *Mucor rouxii,* he found little or no activity in *Mucor miehei*, *Mucor pusillus*, or *Mucor genevensis*.

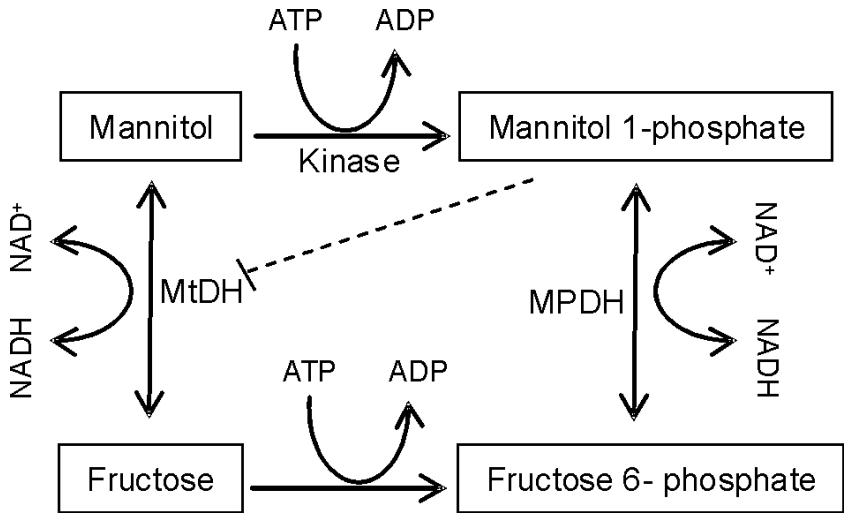

Figure 2. Mannitol cycle proposed for *Absidia glauca* (Ueng et al., 1976). Notice the absence of a mannitol 1-phosphatase, the presence of a mannitol kinase, and the feedback inhibition by mannitol 1-phosphate on MtDH.

MtDH genes have been characterized from a number of Ascomycetes as well as the Basidiomycetes *A. bisporus* (Stoop and Mooibroek 1998) and *U. fabae* (Voegele et al., 2005) (Table 1). The NADP-dependent MtDH from Brewer's yeast appears to be the first enzyme for mannitol metabolism studied by Müller in 1937 (Müller 1937). MPDH was thought to be limited to Zygomycetes and Ascomycetes, hence the mannitol cycle was thought to be absent in Basidiomycetes. Recently, however, MPDH activity was found in *Cryptococcus neoformans* and *Pleurotus ostreatus* (Suvarna et al., 2000, Chakraborty et al., 2004). MPDH was initially studied by Yamada et al., (1959) in *A. niger, Aspergillus oryzae*, and *Pyricularia oryzae* (*Magnaporthe grisea*).

The only characterized genes for MPDH are those from *Stagonospora nodorum* (Solomon et al., 2005) and *A. niger* (Ruijter et al., 2003). In 2003, Ruijter et al., disrupted the MPDH in the fungus *A. niger,* which resulted in a 70% reduction of mannitol in conidiospores and abolished mannitol synthesis in the mycelium. The genomic sequence reported for *A. niger* has putative binding sites for CREA, the broad domain regulator for carbon catabolite repression, as well as putative sites for binding by the development specific transcription factors BrlA and AbaA. Conidiospores from the mutants were sensitive to many stresses (e.g., high temperature, oxidative stress, freezing and lyophilization), but had normal viability. Solomon et al., (2005) disrupted the same enzyme in the fungus *S. nodorum*, and found no significant differences between wild type and mutant in terms of sporulation, spore viability, and growth rate. However, mutants were unable to sporulate *in planta*. Thus far, no disruption experiments have been performed for MtDH or mutants for both genes ever have been created to study the cycle.

Most fungi can use mannitol as a sole carbon source (Cochrane 1958; Lewis and Smith 1967). Interestingly, *Saccharomyces cerevisiae* does not make mannitol (Strandberg 1969; Adomako et al., 1972); however, some polyploid and haploid strains can use mannitol as a carbon source (Quain and Boulton 1987). Enzyme assays showed the presence of a NAD-dependent MtDH (EC 1.1.1.67), and there was no activity if the cofactor was changed to NADP or if the mannitol was replaced with mannitol 1-phosphate. Recently, it was shown that haploid strains of *S. cerevisiae* encode a cryptic MtDH (EC 1.1.1.67). When a putative regulatory protein encoded by *Mtl-1* from *C. neoformans* was expressed in *S. cerevisiae* (Sc41 YJO), a NAD-dependent MtDH was expressed in the fungus allowing it to grow on mannitol media (Perfect et al., 1996). In contrast, only the mycelial form of the dimorphic fungus *Coccidioides immitis* can grow on mannitol as a sole carbon source. However, extracts of both spherules and mycelial forms were shown to contain an NAD-dependent MPDH (EC 1.1.1.17) and no activity was present using mannitol as a substrate (Lones and Peacock 1964). Other enzymes such as ribitol 5-phosphate 2-dehydrogenase (EC 1.1.1.137), xylitol dehydrogenase (EC 1.1.1.9) and glycerol dehydrogenase (EC 1.1.1.72) were shown to utilize mannitol as a substrate during enzyme assays, but it is not clear what the product of the reaction was or if this also occurs *in vivo* (Jennings 1984; Sealy-Lewis and Fairhurst 1992).

When *Candida albicans* was grown in a mixture of glucose and mannitol, mannitol was not utilized until all the glucose was exhausted. An increase in the NAD-dependent MtDH (EC 1.1.1.67) activity was seen as the glucose was being depleted. When the protein synthesis inhibitor trichodermin was used, no activity for MtDH was seen. Mannitol uptake

was also inhibited in the presence of glucose, fructose, and to a lesser extent galactose, mannose and sucrose, suggesting that this MtDH enzyme is subject to carbon catabolite repression. Growth of *Aspergillus* species and *Sphaerosporella brunnea* on mannitol causes a decrease in both MtDH and mannitol 1-phosphatase activity, and an increase in MPDH activity (Lee 1967; Strandberg 1969; Ramstedt et al., 1987). This would suggest that MPDH could also degrade mannitol 1-phosphate, a role attributed by Hult and Gatenbeck (1978) to MtDH, as mannitol could be phosphorylated and converted to fructose 6-phosphate.

The accumulation of mannitol in the mycelium is a characteristic shared by many fungi (e.g., *Byssochlamys fulva, M. grisea*, *Pyrenochaeta terrestris Dendryphiella salina, A. alternata,* and *C. neoformans*) and can vary from 2-20% depending on the fungus and the substrate in which it is grown (Raistrick and Smith 1933; Yamada et al., 1961; Wright and Le Tourneau 1966; Holligan and Jennings 1972; McCullough et al., 1986, Witteveen and Visser 1995; Jennings et al., 1998; Suvarna et al., 2000). As much as 50% of the glucose consumed by some *Aspergillus* species may be excreted as mannitol in culture (Lewis and Smith 1967; Nelson et al., 1971), presumably because such high concentrations cannot be retained in the cells (McCullough et al., 1986). A possible explanation may be that, as in *S. cerevisiae*, a rapid increase in the glycolytic flux may lead to depletion of inorganic phosphate pool (Thevelein and Hohmann 1995) and an increase in the hexose-phosphates. These hexose-phosphates could be converted into mannitol 1-phosphate, then into mannitol and inorganic phosphate thereby providing, not only a more reduced carbohydrate that could be used later, but also restoring the inorganic phosphate pool.

Other polyols

There are many polyols found in fungi including: glycerol, D-erythritol, D-threitol, D-arabitol, L-arabitol, D-xylitol, D-ribitol, D-galactitol, D-sorbitol, D-mannitol, D-glycero-D-manno-heptitol, messo-glycero-ido-heptitol, and D-glycero-D-ido-heptitol (Lewis and Smith 1967; Fig 3). However, glycerol and mannitol are the most common and abundant (Griffin 1984; Jennings 1984). In *S. cerevisiae*, glycerol metabolism occurs via a NAD-dependent glycerol 3-phosphate dehydrogenase, which reduces dihydroxyacetone phosphate to glycerol 3-phosphate in the cytosol (Fig. 4.). Glycerol 3-phosphate is dephosphorylated to glycerol by the enzyme glycerol 3-phosphatase encoded by the *gpp2* gene. For glycerol catabolism, the enzyme glycerol kinase phosphorylates glycerol into glycerol 3-phosphate, which is then oxidized by a FAD-dependent glycerol 3-phosphate dehydrogenase located in the mitochondria where the newly formed dihydroxyacetone enters the glycolytic pathway (Nevoigt and Stahl 1997). Glycerol has a role in osmoregulation in many fungi and yeasts. In yeast, the NAD-dependent glycerol 3-phosphate dehydrogenase is encoded by *gpd1* and *gpd2*. The GPD1 isoform is induced by osmotic stress; hence it plays a role during osmoregulation. The GPD2 isoform is induced under anoxic conditions; hence glycerol is very important in maintaining the redox balance of the cell (Ansell et al., 1997). A different glycerol 3-phosphatase encoded by the *gpp1* gene is also expressed under anoxic conditions. During glucose fermentation ethanol and small amount of glycerol are produced, which is excreted by the yeast cell, through the Fps1p channel. This channel is also involved in osmoregulation as it closes and prevents glycerol from exiting the cell during osmotic shock (Tamás et al., 1999). Recently, a glycerol/H^+ symporter has been reported for *S. cerevisiae*

(Ferreira et al. 2005). Ferreira and coworkers have isolated the gene *STL1* (sugar transporter like) and showed that it encodes a symporter that can take up glycerol by proton symport. They also discussed the presence of many *STL1* homologues in *Debaromyces hansenii*, *C. albicans*, *Eremothecium gossypii*, *Aspergillus nidulans*, and *M. grisea*.

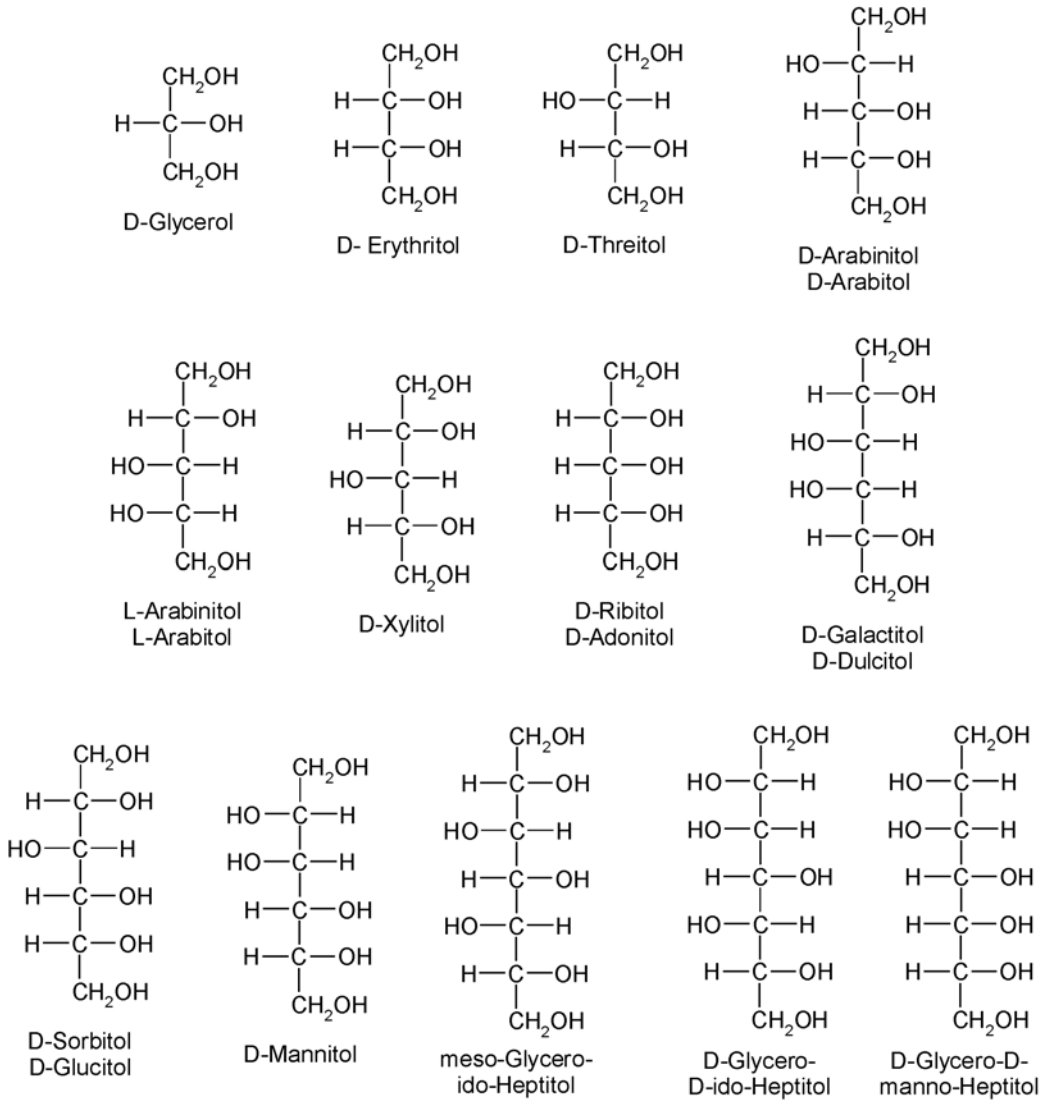

Figure 3. Polyols found in fungi. Mannitol and glycerol are the most common and abundant.

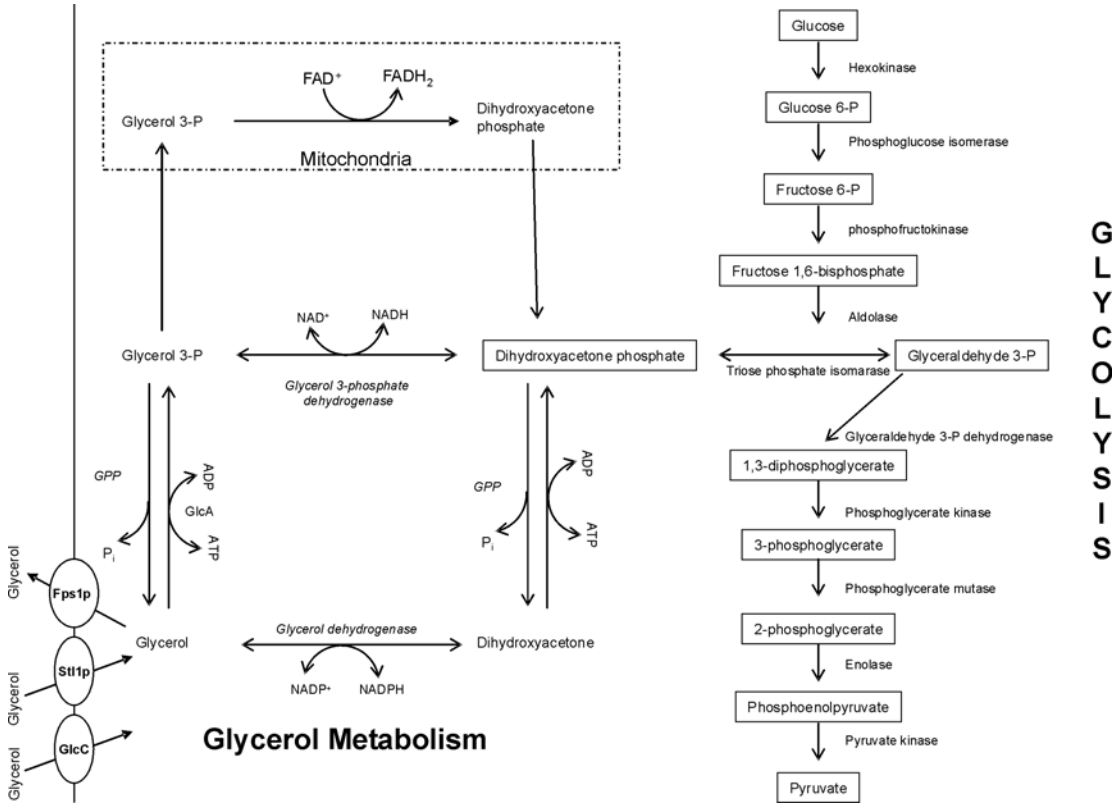

Figure 4. Glycerol metabolism in fungi

In *A. nidulans*, catabolism of glycerol begins by uptake of glycerol from the environment through a transporter (GlcC), addition of a phosphate group by glycerol kinase (GlcA), and conversion to dihydroxyacetone phosphate by a mitochondrially located FAD-dependent glycerol 3-phosphate dehydrogenase (GlcB, GlcD, and GlcG) (Fig. 4). As in *S. cerevisiae*, glycerol synthesis also occurs through a NAD-dependent glycerol 3-phosphate dehydrogenase converting dihydroxyacetone phosphate to glycerol 3-phosphate, which is dephosphorylated to glycerol by the enzyme glycerol 3-phosphatase (GPP). The same phosphatase can remove the phosphate group from dihydroxyacetone phosphate making dihydroxyacetone, which can then be converted to glycerol by a NADP-dependent glycerol

dehydrogenase (Fillinger et al., 2001). This NADP-dependent glycerol dehydrogenase is encoded by the *gldB* gene and has been shown by de Vries et al. (2003) to be important for glycerol biosynthesis of *A. nidulans* under osmotic stress. The appressoria of both *E. gossypii* and the rice blast fungus *M. grisea* accumulate large amounts of glycerol, which provide the osmotic force necessary for penetration of the leaf (De Jong et al., 1997). Aside from glycerol having a role in osmoregulation, glycerol precursors (i.e., glycerol 3-phosphate and dihydroxyacetone phosphate) are used to synthesize membrane phospholipids (Daum et al., 1998).

In fungi, tetritols (i.e., erythritol and threitol) and pentitols (i.e., arabitol, ribitol, and xylitol) are made from sugars metabolized in the pentose phosphate pathway (Fig. 5 and Fig. 6). Isenberg and Niederpruem (1967) studied a NAD-dependent erythritol dehydrogenase in the fungus *Schizophyllum commune.* Enzyme activity could be detected when the fungus was grown in either glycerol or erythritol, but not glucose. Interestingly, Braun and Niederpruem (1969) studied erythritol metabolism in wild-type *S. commune* and a mutant unable to grow on erythrose or erythritol. They showed that erythritol metabolism involved an NADPH-dependent reduction of erythrose to produce erythritol, followed by induction of an NAD-coupled erythritol dehydrogenase to form erythrulose. The mutant strain failed to express an erythritol dehydrogenase and therefore could not grow on erythrose or erythritol. In *Saccharomyces rouxii*, *Saccharomyces millis* and *D. hansenii*, ribulose 5-phosphate made in the pentose phosphate pathway (Fig. 6), is dephosphorylated and then converted to arabitol by a NADP-dependant pentitol dehydrogenase. In *D. salina*, xylulose 5-phosphate is dephosphorylated and then converted to D-arabitol by a NAD-dependant arabitol

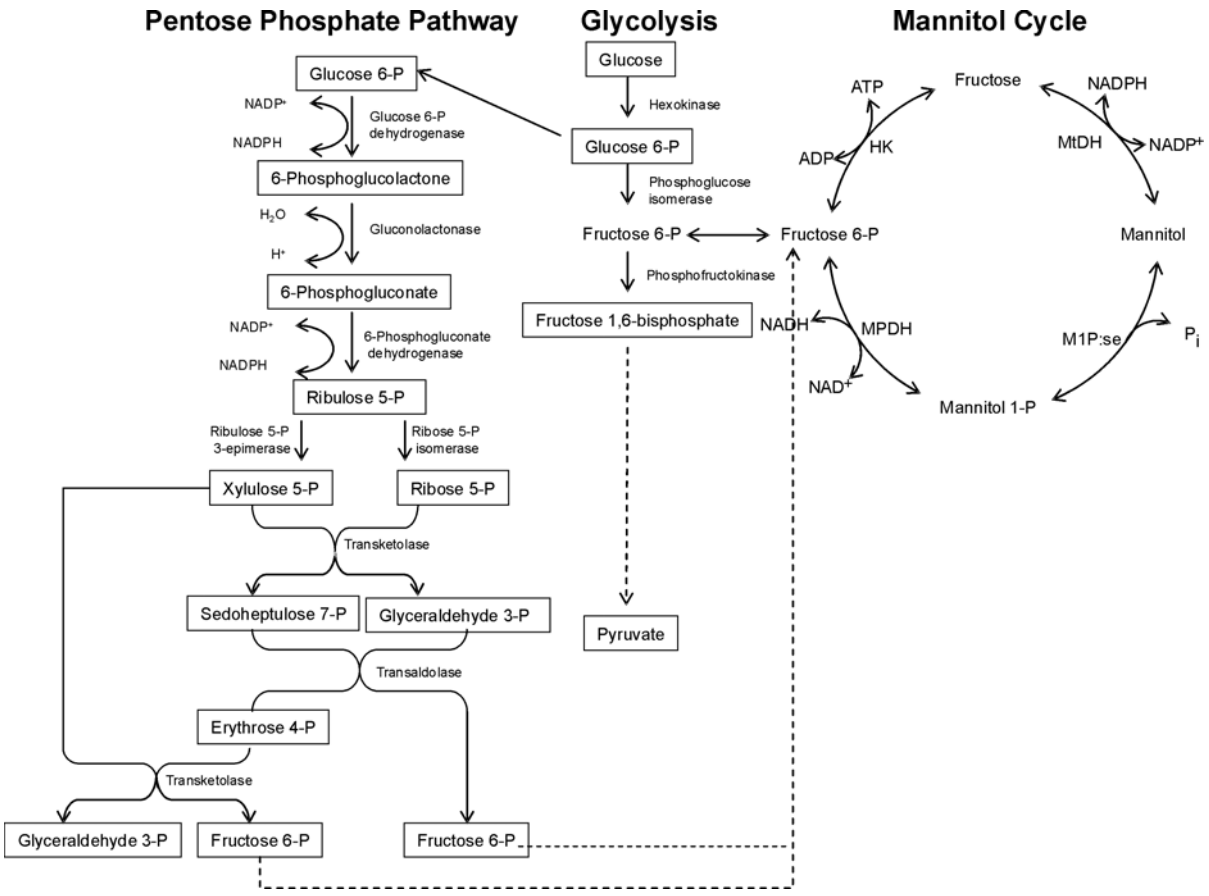

Figure 5. The pentose phosphate pathway and the mannitol cycle in relationship to the glycolysis cycle.

dehydrogenase (EC 1.1.1.12). *C. albicans*, and *Candida tropicalis* appear to share the same metabolic pathway in which ribulose 5-phosphate is dephosphorylated and then converted to arabitol by a NAD-dependent arabitol dehydrogenase (EC 1.1.1.250; Wong et al., 1993; Quong, et al., 1993). However, Wong et al., (1995) later showed that a *C. albicans* mutant lacking a NAD-dependent arabitol dehydrogenase could not grow on minimal medium with arabitol and arabinose, but could still make arabitol. Ruijter et al., (2004) also showed that a NAD-dependent D-arabitol dehydrogenase was involved in arabitol utilization, but was not required for arabitol biosynthesis in the fungus *A. oryzae*. *S. cerevisiae* has a xylitol

dehydrogenase whose activity cannot be detected when the yeast is grown in glucose. Only when the *S. cerevisiae* begins to use ethanol as a carbon source is the NADP-dependent xylitol dehydrogenase expressed (Richard et al. 1999). Chiang and Knight (1960) proposed a pathway for the catabolism of D-xylose and L-arabinose in the fungus *Penicillium chrysogenum* (Fig. 6). A similar catabolic pathway has been proposed for *A. niger* (de Groot et al., 2005), which can utilize the polysaccharide arabinan found in plant cell wall. *A. niger* is able to degrade arabinan to L-arabinose which is further metabolized via the non-oxidative pentose phosphate pathway (Fig. 6; de Groot et al., 2003). The metabolic pathways for tetritols and pentitol in fungi have only been elucidated in a few cases. More research is needed to understand how they are made and utilized by the fungus.

Roles for mannitol and other polyols

Osmoregulation

Polyol production is common among fungi; however, relatively little is known about their function. Recent studies are beginning to shed some light into some of their ascribed roles in fungi. In most filamentous fungi and especially in yeasts, glycerol is the favored osmoprotectant. In the yeast *S. cerevisiae*, glycerol has a role in regulating the osmotic potential of the cell. A decrease in glycerol production and increased sensitivity to osmotic stress were seen in *S. cerevisiae* when the *pgd1* gene (Fig. 4) was disrupted (Albertyn et al., 1994). Transformation of an *S. cerevisiae* mutant in glycerol synthesis with genes for

bacterial mannitol 1-phohsphate 5-dehydrogenase and plant sorbitol 6-phosphate dehydrogenase resulted in mannitol and sorbitol production. However, mannitol/sorbitol

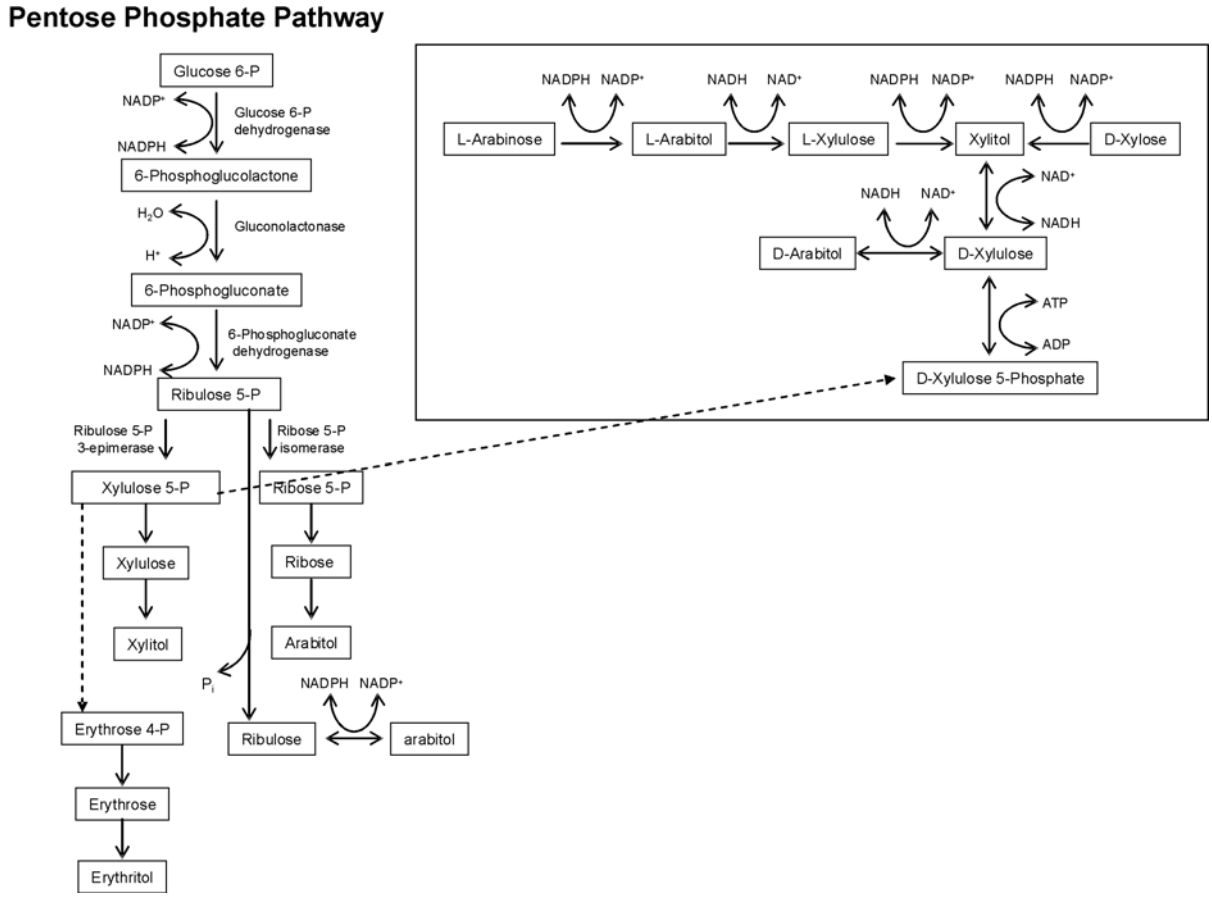

Figure 6. Tetritols and pentitols are made from carbohydrate precursors from the pentose phosphate pathway. The proposed pathway for the metabolism of D-xylose and L-arabinose is shown in the square.

transformants were more sensitive to salt stress than mutants transformed with the *gpd1* gene (Shen et al., 1999). They suggested that glycerol had specific functions that could not be replaced with mannitol and sorbitol. They concluded that polyol accumulation has two functions, facilitating osmotic adjustment and supporting redox control.

In *Aspergillus nidulans*, glycerol and erythritol are the major polyols involved in osmoregulation. When *A. nidulans* was grown in medium osmotically amended with NaCl, KCl, or glucose, the glycerol and erythritol levels were elevated. The same resulted after growth in medium amended with increasing concentrations of NaCl (Beever and Laracy 1986). Although mannitol was present in the mycelium, it did not change in response to the changes in osmotic potential, and only decreased, with a concomitant increase in glucose, when *A. nidulans* was exposed to hypoosmotic shock.

Some fungi use D-arabitol for osmoregulation. Dixon et al., (1999) showed that *M. grisea* accumulated arabitol and small quantities of glycerol during hyperosmotic stress adaptation. In another study, the fungus *Cladosporium fulvum* was grown axenically under hyper-osmotic stress. The levels of glycerol, arabinitol, and mannitol were also followed in *C. fulvum*-infected tomato plants under normal and restrictive watering schedules for a period of eight days (Clark et al., 2003). Mannitol was the most abundant polyol detected at low osmotic pressure, while arabinitol levels accumulated at higher osmotic pressure, with glycerol having a transitory accumulation. Furthermore, the levels of mannitol increased during infection of the tomato plants, but not in response to water stress. Arabinitol increased during infection and exceeded that of mannitol in water-stressed plants eight days post inoculation. Clark, et al., (2003), suggested that, since well-watered infected plants contained mannitol and arabinitol accumulated in response to water stress, the fungus was using arabinitol to osmoregulate during *in planta* growth.

Research with the mycorrhizal fungi *Suillus tomentosus* and *Hebeloma crustuliniforme* showed mannitol accumulation in the presence of increasing concentrations

of NaCl (Bois et al., 2005). These fungi were subjected to increasing concentrations of NaCl at 0, 50, 100, 200 mM. *S. tomentosus* accumulated mannitol and proline, while *H. crustuliniforme* accumulated mannitol, which the authors suggested was used to counteract osmotic stress. In the fungus *A. bisporus*, mannitol also accumulates in response to salt stress (Stoop and Mooibroek 1998).

Storage Carbohydrate

Mannitol is purported to have a role in fungi as a storage carbohydrate (Lewis and Smith 1967). Ballio et al., 1964 isolated glycerol, erythritol, arabitol, mannitol, and trehalose from conidia of *Penicillium chrysogenum*. Mannitol is also accumulated by sclerotia of *Sclerotinia sclerotiorum, Claviceps purpurea, Claviceps nigricans, and Sclerotinia curreyana* (Cooke 1969; Cooke and Mitchell 1969) Mannitol has also been found in spores of *Aspergillus oryzae*, *Myrothecium verrucaria*, *Neurospora sitophila*, *Neurospora crassa*, *A. bisporus*, *Sterostratum corticoides*, *Puccinia coronata*, *Puccinia graminis* f sp. *tritici*, *Erysiphe graminis* f sp. *hordei,* and *Aspergillus clavatus* (Cochrane et al., 1963; Lewis and Smith 1967). According to Horikoshi et al., (1965), mannitol found in the conidia of *A. oryzae* is metabolized at a very early stage during germination. They concluded mannitol was being used as the carbon source for endogenous respiration during the first steps of germination, which was later sustained by glucose. For *Fusarium solani* f sp. *phaseoli*, this did not seem to be the case. According to Cochrane et al., (1963), lipids are initially used; while the major source of energy comes from exogenous carbohydrates. Also, in the initial stages of germination of *Neurospora tetrasperma*, lipids are utilized slowly with the major

source of carbohydrate being endogenous trehalose (Lingappa and Sussman 1959). The role of mannitol as carbon storage in conidia of *A. niger* is not clear. During the first hours of conidia germination the levels of trehalose and mannitol, which exceeded that of glycerol by five to six fold, quickly dropped, while the levels of glycerol increased (Witteveen and Visser 1995). However, Ruijter et al., (2003), disrupted the MPDH in *A. niger* and found some interesting results. MPDH-mutants contained approximately 70% less mannitol than wild-type, however, glycerol accumulation in germinating conidia from MPDH-mutants was not different than wild type conidia, suggesting that mannitol was not required for glycerol production.

Moreover, studies with *A. nidulans* using ^{13}C-labeled carbon showed that mannitol, arabitol, erythritol, and glycerol were present and upon starvation, the first carbon sources to be depleted were glycerol followed by the rest of the polyols, strengthening their role as storage compounds (Dijkema et al., 1985). Voegele et al., (2005) found that sucrose, fructose, and glucose were below the detection limit of 10μM in spores, but mannitol was found in large quantities, which rapidly disappeared during germination. For some fungi, there seems to be an interrelationship between trehalose, glycogen, and the polyols in that an increase in any given carbohydrate during germination may be accompanied by a reduction in another. Whether or not mannitol or other polyols are involved as storage carbohydrates in spores, the evidence supports a role during germination (Lewis and Smith 1967).

In mycorrhizal fungi, mannitol serves as sink for the translocation and storage of carbohydrate, which presumably is not available to the host plant (Lewis and Smith 1967; Martin et al., 1985; Koide et al. 2000). This concept has also been applied to interaction

between the fungus *C. fulvum* and tomato (*Lycopersicon esculentum* L). A wall-bound invertase of fungal origin hydrolyses apoplastic sucrose into glucose and fructose. These sugars can then be converted by the fungus into mannitol, which cannot be metabolized by the plant, allowing the retention of carbon solely for use by the fungus (Joosten and de Wit, 1999). Wedding and Harley (1976) suggested that the accumulation of mannitol could inhibit some glycolytic enzymes in beech (*Fagus sylvatica*) and hence increase the accessibility of glucose to the fungus. However, Jirjis et al., (1986) later reported that mannitol did not inhibit glucose 6-phosphate dehydrogenase, hexokinase, or phosphoglucose isomerase from *Pinus sylvestris* or *Fagus orientalis* as initially proposed.

Martin et al., (1998) used labeled [1-^{13}C]glucose and nuclear magnetic resonance spectroscopy to study carbohydrate metabolism in free and associated *Pisolithus tinctorius* and its host *Eucalyptus globulus*. Martin and coworkers showed that in free living *P. tinctorius* most of the labeled carbon was incorporated into mannitol, trehalose, glutamine and alanine. Arabitol, erythritol and glutamate were labeled weakly. However, in the ectomycorrhizal state, most of the labeled carbon was incorporated into arabitol and erythritol. An interesting observation by Ramstedt et al., (1987) was that most of the non-mycorrhizal basidiomycetes tested contained an NAD-dependent MtDH versus a NADP-dependent MtDH for mycorrhizal basidiomycetes. The significance of this is not understood.

Regulating cofactors

Strobel and Kosuge (1965) studied the MtDH and glucose 6-phosphate dehydrogenase from the fungus *Diplodia viticola* for approximately 16 days. They noticed

that formation and loss of mannitol was reflected in the activity of the enzyme glucose 6-phosphate dehydrogenase and not of MtDH. It was suggested that mannitol was being synthesized for the recurrent regeneration of NADP in order for hexose oxidation to occur. Hult and Gatenbeck (1978) suggested a similar concept in that the mannitol cycle could be used to regulate the coenzymes NADH and NADP+, that is, a way to generate NADPH at the expense of NADH and ATP with each turn of the cycle. However, studies with the fungus *A. nidulans*, provided no support for the operation of the mannitol cycle or for NADPH generation in this fungus (Singh et al., 1988). According to Singh, et al., *A. nidulans* grown on NO_3 as a nitrogen source would increase the demand for NADPH, and thus produce an increase in the maximal specific activities of the enzymes in the mannitol cycle. However, this was not the case. Only enzymes in the pentose phosphate pathway (glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase) (Fig. 3) showed an increased in the maximal specific activity. This has also been supported by Ruijter et al., (2003) and Solomon et al., (2005) on *A. niger* and *S. nodorum*, respectively. Disruption of the mannitol cycle by deletion of MPDH from *A. niger* or *S. nodorum* did not seem to have any effect in these fungi. The idea of transhydrogenation from NADH to NADPH was also studied by Lagunas and Gancedo (1973) in *S. cerevisiae*. They found that the pentose phosphate pathway supplied most, if not all, the required NADPH and that re-oxidation of NADH was coupled to organic compounds like acetaldehyde or dihydroxyacetone phosphate and their reduction by enzymatic reactions. Furthermore, Juhnke et al., (1996) isolated a mutant that was sensitive to H_2O_2 and found that the mutation occurred in the gene encoding ribulose 5-phosphate epimerase, an enzyme of the pentose phosphate pathway. They also created

mutants for other enzymes in the pentose phosphate pathway (i.e., gluconate 6-phosphate dehydrogenase, transketolase, and transaldolase) and found that all the mutants were sensitive to oxidative stress. They showed the importance of the pentose phosphate pathway in generating NADPH, which is needed during oxidative stress. The pentose phosphate pathway, the NADP-dependent isocitrate dehydrogenase (in the cytosol and mitrochondria), and the NADP-dependent acetaldehyde dehydrogenase enzymes are believed to be the primary sources for NADPH required for many biosynthetic reactions in the cell.

Regulate pH

Jennings (1984) proposed that polyols could also be used by fungi to regulate their pH. Polyols could accomplish this by serving as sink or sources for protons, as the polyols are made (reduced) or oxidized into other carbohydrates. Using [^{3}H/^{14}C]glucose, Corina and Munday (1971) devised an experiment to show if mannitol could be involved in hydrogen-acceptor mechanisms. Based on the changes in ^{3}H/^{14}C ratios with respect to glucose, ribitol was found to exhibit stronger labeling patterns than mannitol. Ribitol accumulation occurred after mannitol depletion and during fatty-acid degradation when a hydrogen-acceptor may be required.

Quencher of reactive oxygen species

When both deoxyribose and mannitol are oxidized by hydroxyl radicals (OH)• generated by gamma radiolysis, in the presence of thiobarbituric acid, thiobarbituric-reactive substances are produced. However, when the hydroxyl radicals are produced by the iron-

driven Fenton reaction, mannitol was able to protect the deoxyribose from degradation presumably by chelating the iron (Franzini et al. 1994). Smirnoff and Cumbes (1989) showed that 20mM mannitol, sorbitol, and proline could inhibit hydroxylation of salicylate by hydroxyl radicals and protect enzymes from inactivation. The products of the reaction between mannitol and hydroxyl radicals remain unidentified. According to Shen et al., (1997), hydroxyl radical reactions can be classified in three types: hydrogen abstraction, addition, and electron transfer. Hence, hydrogen abstraction by hydroxyl radicals would form water, with mannitol having an unpaired electron on the C1 or C6 (Fig. 3). This mannitol radical could react with oxygen and form mannose, or may undergo bimolecular disproportionation to form ketones or dimerize. The mannitol radical could form deoxyketones or deoxyketo-dimers if it undergoes elimination of water prior to the disproportionation and dimerization reactions.

Mannitol and probably other sugar alcohols may be used by fungi to protect themselves against reactive oxygen species. Mannitol has been shown to quench reactive oxygen species (ROS) both *in vivo* and *in vitro* (Smirnoff and Cumbes 1989; Chaturvedi et al., 1997). ROS play a major role in pathogen defense for both plants and animals. In animals, ROS are generated by phagocytic leukocytes (macrophages/neutrophils) (Rotrosen and Gallin 1987), while in plants, ROS are produced by a NADPH oxidase localized in the plasma membrane (Grant and Loake 2000). In plants, ROS have a regulatory role in triggering plant defenses (e.g., lignin production, phytoalexin productin, lipid peroxidation and the hypersensitive response) as well as having antimicrobial activity (Baker and Orlandi 1995).

The hypothesis for a role for mannitol in host-pathogen interactions has come from direct and indirect observations. While some have reported mannitol accumulation in liver tissue and blood of rats suffering from aspergillosis (Wong, et al., 1989), others have shown that mutants of *C. neoformans* that produced less mannitol were found to be less virulent than wild type (Chaturvedi et al., 1996[a]). *C. neoformans* produces large amounts of the mannitol in culture and infected animals. A UV generated mutant of *C. neoformans* that produced less mannitol was more susceptible to stresses such as heat and high NaCl concentrations than wild type. In addition, mice that were inoculated with the mutant survived, while the ones inoculated with the wild type at the same inoculum concentration died. The same mutant and wild type were used in an assay with polymorphonuclear neutrophils. Polymorphonuclear neutrophils killed more mutants than wild type *C. neoformans* after 2 and 4 hrs of exposure ($p <$ than 0.05). While the usage of catalase in the assay did not prevent the death of the two strains, using superoxide dismutase, mannitol, and DMSO prevented both strains from being killed. It was suggested that *C. neoformans* produced and secreted mannitol to protect itself against oxidative killing mechanisms of phagocytic cells (Chaturvedi et al., 1996[b]). The yeast *C. albicans* produces arabinitol in cultured, as well as in animals and humans suffering from candidiasis (Kiehn, et al., 1979). Link et al., 2005 recently showed that arabinitol, which is also made by the fungus *U. fabae*, can also quench ROS. However, similar studies as those with *C. neoformans* have not been done with *C. albicans* and arabinitol.

Another study expressed a bacterial mannitol 1-phohsphate dehydrogenase in a strain of *S. cerevisiae* unable to produce glycerol resulting in mannitol production. Mannitol-producing mutants and wild type *S. cerevisiae* were less sensitive to hydroxyl radicals (HO·)

and other reactive oxygen intermediates than the glycerol-deficient mutants suggesting that mannitol could protect *S. cerevisiae* from oxidative damage by scavenging toxic oxygen intermediates (Chaturvedi et al. 1997).

More evidence that fungi use polyols, in particular mannitol, to prevent oxidative damage can be found in *A. alternata,* a fungal pathogen of tobacco (*Nicotiana tabacum* L). When *A. alternata* was grown in culture medium and amended with tobacco extracts, an increase in mannitol levels and secretion was observed (Jennings et al. 1998). Furthermore, Jennings et al., (1998) discovered that tobacco, which does not synthesize mannitol, has an endogenous mannitol dehydrogenase (MTD; EC 1.1.1.255), a 1-oxidoreductase which converts mannitol to mannose, that was induced by fungal colonization, salicylic acid, and 2,6-dichloroisonicotinic acid. They concluded that upon infection, the fungus produced mannitol to quench ROS, while the plant's MTD would convert the fungal mannitol into mannose and thus allow for ROS-mediated plant defenses to be effective against the fungus. To test this hypothesis, Jennings et al., (2002) inoculated transgenic tobacco plants expressing a MTD from celery with *A. alternata* in a detached leaf assay. The transgenic plants expressing the MTD enzyme had enhanced resistance to *A. alternata*.

Voegele et al., (2005) have also identified a MtDH in the rust fungus *Uromyces fabae.* The MtDH was localized to the lumen of haustoria and in the lumen of uredospores. Mannitol was detected at concentrations from 0-10.5 μmol/ g fresh weight in cell extracts to 5.5 mM in apoplastic fluids. An *in vitro* assay for ROS quenching by mannitol showed that, at 5 mM, mannitol could suppress ROS to approximately one-half the level found in the absence of mannitol. They found evidence that mannitol is not only a mobile storage

compound, but is also released into the apoplast where it could have a role in ROS quenching.

Mannitol, which is also made by the tomato pathogen *C. fulvum,* was found in intercellular fluids of tomato leaves infected with virulent races of *C. fulvum.* However, no mannitol was found if avirulent races were used (Joosten et al. 1990). Glucose and fructose from plant origin were converted to mannitol by the fungus and stored. The mannitol could then be used to provide energy during sporulation or could be translocated to the spores directly (Joosten et al., 1990). Since mannitol is exported or leached passively into the apoplast, it could also have a role in ROS quenching. However more studies would be necessary to determine if this were the case.

Other ways fungi defend themselves from ROS

Most of what we know about redox regulation and other defenses against oxidative damage in fungi come from studies of *S. cerevisiae*. Since ROS can also be generated by the fungus as part of normal cellular metabolism (e.g., mitochondria respiration, peroxisome, etc.), *S. cerevisiae* has many defenses to deal with oxidative stress. As reviewed by Jamieson (1998), antioxidant defenses in *S. cerevisiae* can be divided into enzymatic and non-enzymatic responses. Examples of enzymatic defenses are catalases, superoxide dismutases, glutathione peroxidases, glutathione reductase, pentose phosphate pathway enzymes, thioredoxin peroxidase and thioredoxin reductase, methionine sulphoxide reductase, and apurinic endonuclease /3'-diesterase. Catalases catalyze the breakdown of H_2O_2 to O_2 and H_2O. There are two catalases in *S. cerevisiae*, catalase A, located in the peroxisome and

catalase T, located in the cytosol. Superoxide dismutases convert the superoxide anion to H_2O_2 to O_2. There are two superoxide dismutases, MnSod located in the mitochondria and Cu/ZnSod located in the cytoplasm. Enzymes in the pentose phosphate pathway, glucose 6-phosphate dehydrogenase and 6-phosphoguconate dehydrogenase, are involved in the regeneration of NADPH, which will be required by both glutathione reductase and thioredoxin reductase. Glutathione reductase reduces oxidized glutathione and maintains the ratio of reduced/oxidized glutathione in the cell. Glutathione peroxidase uses glutathione to reduce hydroperoxides. Thioredoxin peroxidase and thioredoxin reductase reduce H_2O_2 and alkyl hydroperoxides in combination with thioredoxin and NADPH. Although untested in *S. cerevisiae,* methionine residues on proteins could serve as antioxidants thereby protecting the active site of the protein. The enzyme methionine sulphoxide reductase would reduce the oxidized methionine, restoring the protein. *S. cerevisiae* also has a system for repairing oxidative damage to DNA. The apurinic endonuclease /3'-diesterase enzyme is in charge of this process (Jamieson 1998).

Examples of non-enzymatic defenses include glutathione, phytochelatins, polyamines, trehalose, metallothioneins, metal ion homeostasis, flavohaemoglobin, thioredoxin, and glutaredoxin. Glutathione contains a redox-active sulphydryl group which reacts with oxidants producing reduced glutathione and acting as a radical scavenger. Although, only isolated in *Schizosaccharomyces pombe*, phytochelatins have a similar role as glutathione. The amino acid-derived polyamines spermine and spermidine also have a role in oxidative stress, as null mutants were hypersensitive to oxygen. Since H_2O_2 exposure did not induce genes involve in trehalose synthesis, there are doubts about trehalose being involved

as an antioxidant. However, some studies have shown that trehalose protects yeast cells from oxidative damage after trehalose accumulation due to heat shock treatment (Benaroudj, et al., 2001). Metallothioneins are small cysteine-rich proteins that can bind metal ions. Metal ions have been linked to oxidant resistance/sensitivity, and proteins encoded by the genes *bsd1*, *bsd2*, and *atx1* are involved in the metal ion homeostasis in yeast cells. Null mutants of *atx1* were hypersensitive to both H_2O_2 and superoxide anion generators. Flavohaemoglobin is another metallo-protein involved in oxidative stress protection as expression of the encoding gene was induced by oxygen, and mutants were slightly sensitive to oxidants. *S. cerevisiae* has two genes encoding thioredoxin, which is another sulphydryl-rich protein used as a reductant by thioredoxin peroxidase and for ribonucleotide reductase. *S. cerevisiae* also has two genes encoding glutaredoxins, which are small proteins whose active site contains two redox sensitive cysteines. Although both proteins are very similar, one protects the cell against H_2O_2, while the other protects against superoxide anions. Interestingly, lipid composition is also important in resisting oxidative damage. *S. cerevisiae* cell membranes that have a higher level of saturated fatty acids are more resistant to oxidative stress than those that have higher levels of polyunsaturated fatty acids (Jamieson 1998).

Recent studies have shown that catalases are secreted by fungi during host-pathogen interactions. *Aspergillus fumigatus*, a fungal pathogen of immuno-compromised humans, has two catalases named F and S for fast and slow based on their electrophoretic mobility in non-denaturing polyacrylamide gel electrophoresis (Calera et al., 1997). The S catalase, a secreted catalase used as a diagnostic antigen for aspergillosis, is encoded by the *cat1* gene. Disruption of the *cat1* resulted in mutants that did not produce the slow catalase; however,

pathogenicity assays in mice showed no differences between the mutant and wild type (Calera et al., 1997). *Claviceps purpurea* also secretes catalases both in axenic and parasitic culture (Garre et al., 1998). Out of four catalase isoforms (i.e., CATA, CATB, CATC and CATD) produced by *C. purpurea*, CATC and CATD were found in both mycelium and culture fluids. CATC was the major catalase found in axenic culture, while CATD was the major catalase secreted during infection (Garre et al., 1998). Deletion of the gene *cpcat1*, which encodes both CATC and CATD, resulted in mutants for both catalases, however, pathogenicity assays on rye showed no reduction in virulence of the fungus. The fungal pathogen *Botrytis cinerea* also secretes a catalase encoded by the *Bccat2* gene. mRNA transcript levels for the catalase were detected both in cultures treated with H_2O_2 and during initial stages of infection of tomato plants. Disruption of the gene resulted in mutants that were more sensitive to H_2O_2 *in vitro*, however, *in planta* assays on bean and tomato leaves did not show a consistent reduction in virulence (Schouten et al., 2002). Keissar et al., (2002) showed that *Exserohilum turcicum* expresses seven catalases over a period of 72 hrs encompassing spore germination, germ tube elongation, and hyphal formation *in vitro*. Spores of *E. turcicum* can germinate and survive in high concentrations of H_2O_2 (up to 15mM). Isoform #3 was found to be expressed during the compatible interaction between *E. turcicum* and maize. Robbertse et al., (2003) created triple mutants for CAT1, CAT2 and CAT3, the catalases produced by *Cochliobolus heterostrophus*. Mutants deficient in CAT3, which has a secretory signal sequence, were more sensitive to H_2O_2 than wild type or the other mutants during *in vitro* assays. However, triple and single mutants had normal virulence on maize. Zhang et al., (2004) have published evidence that *Blumeria graminis* f.

sp. *hordei* also secretes a catalase during host infection. Using *A. fumigatus* polyclonal antibody anti-CATB they showed that during infection of barley by *B. graminis* f. sp. *hordei*, the fungus secretes a protein from the germ tube tips and seeps down on to the host. A catalase also has been reported for *A. alternata* (Caridis et al. 1991), however, it is not know whether catalases are secreted during plant-*Alternaria* interactions.

Plant defense responses

ROS in plants are produced by a NADPH oxidases, cell-wall-bound peroxidases, xanthine oxidases, and amine oxidases in the apoplast (Allan and R 1997, Grant and Loake 2000, Neill et al. 2002, Kawano 2003). The NADPH oxidase is thought to be similar to the mammalian neutrophils, which is composed of a b-type cytochrome with two subunits, $p22^{phox}$ and $gp91^{phox}$. These proteins will also interact with $p67^{phox}$ and phosphorylated $p47^{phox}$ to complete the holoenzyme (Grant and Loake 2000). *Arabidopsis,* tomato, potato, and rice homologues to $gp91^{phox}$ have been found, but not for *p47* or *p67* (Simon-Plas et al., 2002; Torres et al., 2002).. Recently, (Torres et al., 2002), isolated two mutants for the *gp91* homologues in *Arabidopsis, atrbohD* and *atrbohF*. Mutations in these two genes largely eliminate ROS accumulation during disease-resistance reactions between *Arabidopsis* to avirulent *Pseudomonas syringae* and *Peronospora parasitica*. Simon-Plas et al., (2002) also isolated a pg91 homologue in tobacco, *NtrbohD,* and studied transgenic cells knocked-out for *NtrbohD* expression by antisense technology. They showed that the NADPH oxidase localized to the plasma membrane. No hydrogen peroxide could be detected in the cell line gp3, while some of the cell lines (i.e., gp1, gp2, gp4) accumulated only 2–6% of ROS,

suggesting that NtrbohD was implicated in ROS production. In plants, ROS can inhibit pathogen ingress by strengthening cell walls through oxidative cross-linking of cell wall structural proteins, by triggering the hypersensitive response (HR), and by their antimicrobial activity (Levine et al., 1994; Baker and Orlandi 1995).

Alternaria alternata – Brown spot

The fungus *Alternaria alternata* (Fr. Ex Fr.) Keissler, belongs in the class Dothideomycetes, order Pleosporales, and family Pleosporaceae. Fungal colonies are usually black to gray. Conidiophores arise singly or in small groups. They can be simple or branched, straight or flexuous, and sometimes geniculate. The color appears pale to mid olivaceous or golden brown. They are smooth and can be 50µm long and 3-6µm thick with one or several conidial scars. Conidia are long, branched, obclavate, and obpyriform, ovoid or ellipsoidal. When ellipsoidal, they are beakless; otherwise they often have a short conical or cylindrical beak. Conidia are pale to mid golden brown, smooth or verruculose with up to eight transverse longitudinal or oblique septa. Their overall length is 20-63(37)µm and 9-18(13)µm thick in the broadest part. The beak can be 2-5µm thick and up to one-third the length of the conidium (Ellis 1971). Conidial size, shape and septation differ making it hard to identify species. The genus was first established in 1817, with the type isolate, *Alternaria tenuis* (now *Alternaria alternata*) (Rotem 1998).

A. alternata is responsible for causing Brown Spot, a major foliar disease of tobacco (*Nicotiana tabacum* L.). Initially, conidia land on the leaves closest to the ground and

germinate producing one or more germ tubes. Penetration is through stomata, basal cells of trichomes, leaf margins, wounds, or directly by appressorium formation. After penetration, the fungus develops a microsclerotium of aggregated cells, which later become branched, thick-walled, angular, and darkly pigmented. The hyphae grow intercellularly and breakdown of the middle lamellae occurs. The epidermal cells collapse first, then the spongy parenchyma, and finally the palisade cells. The fungus grows profusely on dead tissue and a necrotic lesion develops as the cells between the infected surface and lower surface collapse. Conidiophores develop in concentric rings or zones several days after penetration on either side of the leaf and the cycle repeats. The fungus overwinters in lignified plant debris (Lucas 1975).

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CHAPTER 2: **Mannitol metabolism in the phytopathogenic fungus *Alternaria alternata***

Heriberto Vélez[a], Norman J. Glassbrook[a], and Margaret E. Daub[b*]

[a] Department of Plant Pathology, NC State University, Raleigh, NC 27695 USA

[b] Department of Botany, NC State University, Raleigh, NC 27695 USA

[*]Corresponding author

Corresponding Author: Margaret E. Daub Department of Botany, Campus Box 7612, 2214 Gardner Hall, NC State University, Raleigh, NC 27695 USA

Telephone: 1-919-513-3807, Fax: 1-919-515-3436, Email: margaret_daub@ncsu.edu

Abstract

Mannitol metabolism in fungi is thought to occur through a mannitol cycle first described in 1978. In this cycle, mannitol 1-phosphate 5-dehydrogenase (EC 1.1.1.17) was proposed to reduce fructose 6-phosphate into mannitol 1-phosphate, followed by dephosphorylation by a mannitol 1-phosphatase (EC 3.1.3.22) resulting in inorganic phosphate and mannitol. Mannitol would be converted back to fructose by the enzyme mannitol dehydrogenase (EC 1.1.1.138). Although mannitol 1-phosphate 5-dehydrogenase was proposed as the major biosynthetic enzyme and mannitol dehydrogenase as a degradative enzyme, both enzymes catalyze their respective reverse reactions. To date the cycle has not been confirmed through genetic analysis. We conducted enzyme assays that confirmed the presence of these enzymes in a tobacco isolate of *Alternaria alternata*. Using a degenerate primer strategy, we isolated the genes encoding the enzymes and used targeted gene replacement to create mutants deficient in mannitol 1-phosphate 5-dehydrogenase, mannitol dehydrogenase, or both. PCR analysis confirmed gene disruption in the mutants, and enzyme assays demonstrated a lack of enzymatic activity for each enzyme. GC-MS experiments showed that a mutant deficient in both enzymes did not produce mannitol. Mutants deficient in mannitol 1-phosphate 5-dehydrogenase or mannitol dehydrogenase alone produced approximately 30 % and 70 %, respectively, of wild type levels. All mutants grew on mannitol as a sole carbon source, however, the double mutant and mutant deficient in mannitol 1-phosphate 5-dehydrogenase grew poorly. Our data demonstrate that mannitol 1-phosphate 5-dehydrogenase and mannitol dehydrogenase are essential enzymes in mannitol metabolism in *A. alternata*, but do not support mannitol metabolism operating as a cycle.

Keywords: Mannitol cycle, mannitol dehydrogenase, mannitol 1-phosphate 5-dehydrogenase, polyols, fungi

Introduction

Polyhydroxy alcohols or sugar alcohols are produced by many organisms including bacteria, plants, and fungi (21, 26). In fungi, mannitol is the most common polyol, found in large quantities in spores, fruiting bodies, sclerotia, and mycelia (26). In *Agaricus bisporus*, mannitol can contribute up to 20% of the mycelium dry weight and increases dramatically to 30-50% in differentiating sporophores, while in *Aspergillus niger* conidiophores, mannitol makes up to 10-15% of the dry weight (40, 46).

Mannitol is purported to have different roles in fungi, including osmoregulation, serving as a storage or translocated carbohydrate, serving as a source of reducing power, regulating coenzymes, and regulating cytoplasmic pH by acting as a sink or source for protons (21, 26). More recently, mannitol has been shown to quench reactive oxygen species (ROS) both *in vitro* and *in vivo* (9, 43, 52). *Cryptococcus neoformans* has been reported to produce and secrete mannitol to protect itself against oxidative killing mechanisms of phagocytic cells (10). A role for mannitol in antioxidant defense has also been supported by experiments with *Saccharomyces cerevisiae* (9), *Alternaria alternata* (19, 20), and the rust fungus *Uromyces fabae* (52).

The mannitol cycle was proposed by Hult and Gatenbeck in 1978 (17) from studies of cell-free extracts of the fungus *A. alternata* (Fig. 1). Cell-free extracts from both an alternariol- producing and a non-producing strain were prepared from mycelia grown in Czapek-Dox medium. Specific activities of mannitol dehydrogenase, mannitol 1-phosphate 5-dehydrogenase, mannitol 1-phosphatase, hexokinase, glucose 6-phosphate dehydrogenase, glucose phosphate isomerase, phosphofructokinase and aldolase were measured to obtain information on the regulation of the cycle. In the proposed mannitol cycle, the enzyme

mannitol 1-phosphate 5-dehydrogenase (MPDH; EC 1.1.1.17) would catalyze the reduction of fructose-6-phosphate to mannitol-1-phosphate using the cofactor NADH. Mannitol-1-phosphatase (M1Pse; EC 3.1.3.22), presumed to be specific for mannitol 1-phosphate (38, 56), would hydrolyze mannitol 1-phosphate to mannitol and inorganic phosphate. Mannitol would then be oxidized to fructose by mannitol dehydrogenase (MtDH; EC 1.1.1.138) using the cofactor $NADP^+$. Finally, fructose would be phosphorylated to fructose 6-phosphate by a hexokinase (EC 2.7.1.1). The cycle as proposed goes in one direction with the production of mannitol by MPDH and its utilization by MtDH. Thus, NADPH is produced at the expense of one molecule of ATP and NADH. Hult and Gatenbeck (17) found no differences in the specific enzyme activities between the two strains of *A. alternata*, that could explain why the non-producing strain would synthesize more fat and oxidize more mannitol than the alternariol-producing strain. They further postulated that the cycle was regulated by the availability of the coenzymes NADH and $NADP^+$.

Evidence for MtDH and MPDH enzyme activities have been reported in many fungi, but only very few gene sequences have been reported (Table 1). MtDH genes have been characterized from a number of Ascomycetes as well as the basidiomycetes *A. bisporus* (46) and *U. fabae* (52). The only characterized genes for MPDH are those from *Stagonospora nodorum* (44) and *Aspergillus niger* (40). MPDH was thought to be limited to Zygomycetes and Ascomycetes, hence the cycle was thought to be absent in Basidiomycetes. Recently, however, MPDH activity was found in *Pleurotus ostreatus* and *Cryptococcus neoformans* (7, 49).

In spite of the extensive reports on enzyme activities in fungi, questions concerning the importance of the mannitol cycle and whether or not it acts as a cycle have never been

definitively answered (31, 44). Since the initial report of the cycle in 1978, the genes encoding the *A. alternata* MtDH and MPDH enzymes have not been reported. Further, only *MPDH* has been disrupted in fungi (40, 44). The goals of this research were to confirm the proposed *Alternaria* mannitol cycle by genetic analysis. Here we confirm the enzymatic activity of both MtDH and MPDH in a tobacco isolate of *A. alternata*, and report the isolation of the genes, their disruption, and characterization of the mannitol cycle in disruption mutants.

Materials and Methods

Strain and media

All studies utilized *A. alternata* strain A5, isolated from brown spot-infected tobacco in Oxford, NC (provided by H. Spurr Jr, Oxford, NC; (45)). For enzyme assays, the fungus was grown in malt extract medium (15 g malt extract, 3 g peptone, 30 g glucose per L). For protoplast isolation, the fungus was grown on GYB medium (l0 g glucose, 5 g yeast extract per liter). Liquid regeneration medium (RM; 0.5 M sucrose, 0.1 % yeast extract, 0.1 % casein amino acids, and 0.1 % mannitol) was used for protoplast regeneration. Solid RM (1 M sucrose, 0.1 % yeast extract, 0.1 % casein amino acids, 0.1 % mannitol and 15 % agar) with either 150 µg/mL hygromycin B (Roche, Indianapolis, IN) or 10 µg/mL phleomycin (Research Products International Corp., Mt. Prospect, IL) was used for selection of transformants. V-8 medium (300mL V-8 juice (Campbell Soup Co., Camden, NJ), 4.5 g $CaCO_3$, and 15 g agar per liter) was used to grow transformants. Modified Richard's minimal medium (10 g KNO_3, 5 g KH_2PO_4, 2.5 g $MgSO_4$, 20 g sucrose, 1 g yeast extract, 15 g agar per liter) was used for sporulation. Minimal medium (10 g KNO_3, 5 g KH_2PO_4, 2.5 g

$MgSO_4$, and 20 g glucose, per liter) was used for i*n vitro* growth characteristics of wild type and mutants. Glassbrook minimal medium was used to grow the *A. alternata* wild type and mutants to determine the levels of mannitol. This medium contained (per liter) 7.5 mg NaCl, 300 mg KCl, 150 mg $CaCl_2 \cdot 2H_2O$, 200 mg $MgSO_4 \cdot 7H_2O$, 200 mg Na_2HPO_4, 35 mg KH_2PO_4, 150 mg $CH_3CO_2NH_4$, 2 g glucose, 3.5 mg EDTA-Na_2, 1 mg $FeSO_4 \cdot 7H_2O$, 1 mg $ZnSO_4 \cdot 7H_2O$, 250 µg $CuSO_4 \cdot 5H_2O$, 125 µg $MnSO_4 \cdot H_2O$, 75 µg $CoC1_2 \cdot 6H_2O$), 100 µg H_3BO_3, 200 µg $Na_2MoO_4 \cdot 2H_2O$, 100 µg KI). For DNA isolation, potato dextrose broth (Difco) was used to grow the fungus.

Genomic DNA isolation

Fungal genomic DNA was extracted as described (54). Lyophilized fungal tissue was ground to a powder in a mortar and pestle cooled with liquid nitrogen. One gram of tissue was suspended in fungal lysis buffer (100 mM LiCl, 10 mM EDTA, 10 mM Tris pH 7.5, 0.5 % SDS) for 10 min at 65°C. The suspension was extracted once with buffer-saturated phenol (Invitrogen), treated with RNase (20 mg/mL), and incubated at 37°C for 30 min. The suspension was extracted twice with phenol:chlorophorm:isoamyl alcohol (25:24:1; Fisher Scientific, Hampton, NH) and the DNA was ethanol-precipitated and re-dissolved in sterile distilled water. The DNA concentration was determined with a Beckman DU Series 650 spectrophotometer (Beckman Instruments, Fullerton CA).

Enzyme assays

Extraction procedures for cell-free extracts were carried out at 4°C. Five-day old cultures of the wild type strain grown in GYB medium were harvested, washed with sterile

distilled water, submerged in liquid nitrogen for five minutes and stored at -80°C until needed. Frozen tissue was ground with a mortar and pestle, using a 1:4 tissue-to-buffer ratio. The extraction buffer contained 50 mM morpholine propanesulfonic acid (MOPS; pH 7.5), 5 mM dithiothreitol (DTT), and 1 mM EDTA. The samples were centrifuged for 5 minutes at 16,000 × g, and the supernatant was desalted by centrifugal filtration on a Sephadex G25-50 column equilibrated with 50 mM MOPS (pH 7.5) containing 1 mM DTT or by PD-10 columns (Amersham Pharmacia Biotech, Piscataway, NJ) following the manufacturer's protocol.

MtDH and MPDH enzymatic activities were measured by monitoring the increase or decrease in the concentration of the cofactors NADPH or NADH spectrophotometrically at A_{340} using a Beckman DU Series 650 spectrophotometer (Beckman Instruments) at 25°C. For MtDH activity, the reduction of fructose to mannitol was assayed in a reaction mixture containing 100 mM MOPS-Na pH 7.5, 0.20 mM NADPH, and 10 µL cell-free extract in a 1 mL cuvette. After establishing a baseline of two minutes, the reaction was started by the addition of fructose to a final concentration of 800 mM. The oxidation of mannitol to fructose was conducted in the same manner using 100 mM Bis-Tris Propane pH 9.0, 0.20 mM $NADP^+$, and 10 µL cell-free extract. The reaction was started with the addition of 200 mM mannitol after establishing a baseline of two minutes.

For MPDH activity, the reduction of fructose 6-phosphate was assayed in a reaction mixture containing 100 mM MOPS-Na pH 7.5, 0.01 mM NADH, and 50 µL cell-free extract in a 1 mL cuvette. The reaction was started with the addition of 4 mM fructose 6-phosphate, after establishing a baseline of two minutes. For the reverse reaction, mannitol 1-phosphate barium salt (Sigma-Aldrich, St. Louis, MO) was converted to a potassium salt by the addition

of K_2SO_4. The solution was spun at 16000 × g and the supernatant (mannitol 1-phosphate potassium salt) was placed in a new tube, evaporated to dryness, and re-dissolved at 0.5 M. The oxidation of mannitol 1-phosphate was conducted in the same manner using 100 mM Bis-Tris Propane pH 9.0, 0.01 mM NAD^+, and 50 μL cell free extract. The reaction was started with the addition of 25 mM mannitol 1-phosphate after establishing a baseline of two minutes.

For both enzymes, specific activities were defined as the micromoles of NADP(H) or NAD(H) reduced or oxidized per minute per milligram of protein. Protein concentrations were determined spectrophotometrically using a Beckman DU Series 650 spectrophotometer (Beckman Instruments) by the Bradford method (6) with bovine serum albumin as a standard.

Isolation of *MtDH* and *MPDH* genes

For *MtDH*, a consensus amino acid sequence derived from sequences from *Botrytis cinerea* (Accession # AL110741, AL116577, AL115121, AL114933, AL113379, AL116869), *Cladosporium fulvum* (AAK67169), and *Aspergillus nidulans* (EAA62170) was used to generate degenerate primers (Table 2, MtDH degenerate) using CODEHOP software (http://blocks.fhcrc.org/blocks/codehop.html; (39). All primers were obtained from Sigma-Genosys (The Woodlands, TX). Amplification was performed using Amplitaq Gold (Applied Biosystems, Foster City, CA) with the following parameters: 94°C for one min, followed by 94°C for 30 s, 60°C for 30 s, 32 cycles at 68°C for 1min, and 72°C for 5 min. The PCR resulted in a single 234bp fragment which was gel purified, cloned in pGEM-T-Easy (Promega, Madison, WI) using Subcloning Efficiency DH5α Competent Cells

(Invitrogen), and sequenced. Inverted PCR was used to isolate the 5' and 3' regions of the gene to recover the full-length gene. Southern hybridization analysis was used to determine which restriction enzymes digested the *A. alternaria* genome into fragments suitable for inverse PCR amplification. *BamHI*-digested DNA was self-ligated at dilute concentrations and used as a template for an inverse PCR amplification. A 2091bp fragment was amplified using ELONGASE (Invitrogen) and primers (Table 2, MtDH inverted) using the following parameters: 94°C for 30 s, 60°C for 30 s, 32 cycles at 68°C for 2min 50s, and 72°C for 5 min. The fragment was cloned into pGEM-T-Easy as described above and sent for sequencing. Vector NTI (Informax of Invitrogen, Carlsbad, CA) was used to assemble the contigs and analyze the sequence. Analysis of the 2091bp fragment showed that the entire ORF plus upstream promoter and downstream sequences were present. Primers (Table 2, MtDH with *Pfu*) were designed to amplify the 2091bp fragment in the correct orientation from *A. alternata* genomic DNA using the high-fidelity DNA *Pfu* polymerase (Stratagene, La Jolla, CA). The amplified product was cloned into pGEM-T-Easy after the addition of dATP residues and sent for sequencing. Sequencing was performed by the Integrated Biotechnology Laboratories at the University of Georgia or by MWG-BIOTECH (High Point, NC).

In the same manner, a consensus amino acid sequence from *A. niger* (Accession # AAL89587), *Paracoccidioides brasiliensis* (AAO47089), and *Neurospora crassa* (EAA33240), was used to generate degenerate primers (Table 2) for MPDH. Amplification was performed with the following parameters: 94°C for one min, followed by 94°C for 30 s, 61°C for 30 s, 32 cycles at 68°C for 1 min, and 72°C for 5 min. The PCR resulted in a single 504bp fragment, which was gel purified, cloned, sequenced, and the sequences analyzed as

described for MtDH above. Inverted PCR was used to isolate the 5' and 3' regions of the gene as described for MtDH, using the amplification parameters of 94°C for 30 s, 60°C for 30 s, 32 cycles at 68°C for 4 min, and 72°C for 5 min. The resulting 2999bp fragment was cloned into pCR4-TOPO (Invitrogen) and sent for sequencing. As with MtDH, sequence analysis of the 2999bp fragment showed that the entire ORF plus upstream promoter and downstream sequences were present, and primers (Table 2, MPDH with *Pfu*) were designed to amplify the 2999 fragment in the correct orientation from *A. alternata* genomic DNA. The amplified product was cloned into pGEM-T-Easy after the addition of dATP residues and sent out for sequencing.

Gene disruption

For *MtDH,* restriction enzymes *EcoO109I* (New England Biolabs, Ipswich, MA) and *Acc65I* (Promega) were used to digest a 364bp fragment within the ORF of the gene cloned in pGEM-T-Easy (Fig.2). The same enzymes were used to digest plasmid pUCATPH (28) to recover the gene encoding hygromycin resistance (*hph*) flanked by a fungal promoter (*A. nidulans trpC* promoter) and terminator (*A. nidulans trpC* terminator). The *hph* fragment was ligated into the *MtDH* gene, replacing the 364bp fragment and providing a selectable marker flanked by 958bp and 735bp of *MtDH* DNA sequence on the 5' and 3' end, respectively (Fig. 2). The reconstituted pGEM-T Easy plasmid was cloned into DH5α competent cells and named pGHyg.

For *MPDH*, the enzyme *EheI* (Fermentas International Inc., Burlington Canada) was used to digest a 1324bp fragment from the *MPDH* gene. High-fidelity DNA *Pfu* polymerase and PCR primers (Table 2) were used to amplify the *ble* gene (encoding phleomycin

resistance) flanked by a fungal promoter (*A nidulans gpdA* promoter) and terminator (*S. cerevisiae* CYC1 terminator) from the plasmid pBC-Phleo (30). Amplification conditions were 94°C for one min, followed by 94°C for 30 s, 51°C for 30 s, 32 cycles at 68°C for 3 min, and 72°C for 5 min. The resulting 3060bp band was gel purified and ligated into the *MPDH* gene, replacing the 1234bp fragment and providing a selectable marker flanked by 684bp and 1000bp of *MPDH* DNA sequence on the 5' and 3" end, respectively (Fig. 2). The reconstituted pGEM-T Easy plasmid was clone into DH5α competent cells and named pGPhleo.

Protoplast isolation and fungal transformation

Transformation of *A. alternata* wild type with the disruption constructs was carried out as previously described (2) with some modifications. For protoplast isolation, fungal mycelial plugs harvested from a V-8 culture plate were inoculated into 50 mL GYB medium and grown in the dark for two days with shaking at 200 rpm at 25°C. The mycelia were harvested by centrifugation at 2706 × g and washed three times with a protoplast salt solution (0.7 M KCl, 0.2 M $CaCl_2$). The mycelium mass was incubated in 20 mL of 10 mg/mL kitalase (Wako Chemicals USA Inc., Richmond, VA) dissolved in the protoplast salt solution for 3-4 hours at 25°C with constant shaking at 80 rpm. The digested protoplasts were filtered through cheesecloth and a 60 micron mesh-screen (TWP Inc. Berkeley, CA) and spun at 1730 × g for 10 min at 4°C. The supernatant was decanted and the protoplasts were washed with 10 mL of protoplast salt solution followed by centrifugation at 1730 × g for 10 min at 4°C. This last step was repeated twice. The protoplasts were washed with 10 mL of STC buffer (1 M sorbitol, 50 mM Tris pH 8.0, 50 mM CaCl) and spun at 1730 × g for 10 min at

4°C. The supernatant was decanted and the protoplasts were suspended in fresh STC buffer at 10^8 protoplasts per mL.

For transformation, 80 µL of protoplasts were gently mixed with 2-3 µg of either pGHyg DNA or pGPhleo DNA. The protoplasts were incubated with the DNA on ice for 30 min, followed by the addition of 500 µL of 40 % PEG 4000 (in STC) and a further 30 min incubation at room temperature. Five mL of liquid RM was added to the transformation mix, and the protoplasts were allowed to regenerate in the dark overnight at 28°C. The regenerated protoplasts were plated on solid RM with either 150 µg/mL hygromycin or 10 µg/mL phleomycin. Transformants that appeared after 2-3 days on selective media were transferred onto solid V-8 medium containing 250 µg/mL hygromycin or 20 µg/mL phleomycin. Successful transformants were named in sequential order with the prefix Mt (for *MtDH* minus) or Mp (for *MPDH* minus) according to the gene disrupted.

To create a double knock- out strain, protoplasts of a *MPDH*-minus transformant (Mp-14) were transformed with the pGHyg construct as described above. The regenerated protoplasts were plated on solid RM with both 150 µg/mL hygromycin and 10 µg/mL phleomycin. Transformants that appeared after 2-3 days on selective media were transferred onto solid V-8 medium containing both 250 µg/mL hygromycin and 20 µg/mL phleomycin. Successful transformants were named in sequential order with the prefix B (for both *MtDH* and *MPDH* minus).

Screening for *MtDH* and *MPDH* disruptants using PCR

Mutants were grown in potato dextrose broth and DNA was isolated as described above. Amplification conditions used to screen for *MtDH* disruption were 94°C for one min,

followed by 94°C for 45 s, 63°C for 45 s, 32 cycles at 70°C for 2 min 45 s, and 72°C for 8 min with primers shown in Table 2. The expected sizes for the wild type and disrupted *MtDH* were 826 bp and 2755bp, respectively. PCR amplification conditions used to screen for *MPDH* transformants were 94°C for one min, followed by 94°C for 45 s, 62°C for 45 s, 32 cycles at 70°C for 4 min, and 72°C for 8 min with primers shown in Table 2. The expected sizes for wild type and disrupted *MPDH* were 2188bp and 3579bp, respectively. Mutants Mt-12, Mp-14 and B-10 were selected for further study based on results from PCR screens.

Enzyme assays of disruption mutants

Three-day old cultures of the wild type strain, Mt-12, Mp-14, and B-10 were grown in GYB medium, harvested, washed with sterile distilled water, submerged in liquid nitrogen for five minutes and stored at -80°C until needed. Extraction procedures for cell-free extracts were carried out at 4°C. Enzyme assays were performed as described above.

In vitro growth characteristics on different carbon sources

Cultures of wild type strain, Mt-12, Mp-14, and B-10 (4×10^5 spores mL^{-1} of each) were grown in 50 mL flasks containing liquid minimal medium with 110 mM fructose, sorbitol, mannitol, or glucose as a sole carbon source for seven days with shaking at 200 rpm at 25°C. The mycelium was filtered, washed, and immediately placed in liquid nitrogen and then lyophilized. The dry weight of each culture was recorded.

Mannitol analyses

To check for mannitol production, fungal cultures of wild type as well as single and double gene knockouts (i.e., wild type, Mt-12, Mp-14, and B-10) were grown on Glassbrook minimal medium that consisted of a balanced salt solution with glucose as a sole carbon source for five days. Cultures were filtered, washed with distilled water, and lyophilized.

Samples were submitted to the Metabolomics and Proteomics Laboratory (MPL) at North Carolina State University for mannitol analysis by gas chromatography-mass spectrometry (GC-MS). In brief, two to six milligrams of dry, finely powdered fungal samples were suspended in 250 µL of N,N-dimethyltrimethylsilylamine (TMS-DMA) and 750 µL of solvent (acetonitrile:benzene:tetrahydrofuran, 3:1:1 by volume) and incubated at approximately 60°C for one hour to convert mannitol to the trimethylsilyl (TMS) derivative. The resulting extracts were centrifuged, transferred to 2-mL glass instrument vials and analyzed by GC-MS without further cleanup. GC-MS was performed with an Agilent 6890 (Agilent Technologies, Inc. Palo Alto, CA) gas chromatograph coupled to an Agilent 5973 mass-selective detector. Chromatographic separations were achieved with a Thermo TR-50MS (50 % phenyl polysilphenylene-siloxane) column (30 m length; 0.25 mm inside diameter; 0.25 µm film thickness). Helium, the carrier gas, was set at a constant flow of 1.2 mL per minute (linear velocity of 40 cm/s). One microliter of extract was injected into a split/splitless injector operated with a 100:1 split ratio at a constant temperature of 250°C. The column oven temperature was programmed for a 50°C initial temperature with a 10°C/min ramp to a final temperature of 350°C.

Data were quantified by comparing peak areas obtained for TMS-mannitol in the fungal samples with a series of mannitol reference standards analyzed concurrently with the

fungal extracts. The chromatographic data were processed using Agilent's ChemStation software (Agilent Technologies, Inc.).

Results

Enzyme Assays

Extracts from wild type *A. alternata* showed activity for both MtDH and MPDH. For MtDH, activity was confirmed in both directions, for the conversion of mannitol to fructose (Fig. 3-A) as well as fructose to mannitol (Fig. 3-B). No MtDH activity was seen using NAD(H) as a cofactor (data not shown). The specific activity for the fructose to mannitol reaction was 4748 mU mg^{-1} of protein and for mannitol to fructose was 1033 mU mg^{-1} of protein (mean of three independent experiments). These results confirmed that *A. alternata* has an NADP-dependent mannitol dehydrogenase, which is capable of both mannitol synthesis and degradation. Assays of MPDH activity also documented activity in both directions, for the conversion of mannitol 1-phosphate to fructose 6-phosphate (Fig. 4-A) as well as fructose 6-phosphate to mannitol 1-phosphate (Fig. 4-B). No MPDH activity was seen using NADP(H) as a cofactor (data not shown). The specific activity for the fructose 6-phosphate to mannitol 1-phosphate reaction was 183 mU mg^{-1} of protein and 2216 mU mg^{-1} of protein for the reverse reaction (mean of three independent experiments). These results confirmed that *A. alternata* also has an NAD-dependent mannitol 1-phosphate 5-dehydrogenase, which is capable of mannitol 1-phosphate synthesis and degradation. These results agree with the proposed activities in the published mannitol cycle (Fig. 1,(17))

Gene isolation

PCR using the degenerate primers followed by inverted PCR yielded a 2091bp fragment. This fragment contained an ORF encoding a protein with strong similarity to the MtDH proteins from *C. fulvum*, *A. nidulans*, and *Gibberella zeae* (accession # AAK67169, AA62170, and AAP33281, respectively). Southern analysis showed the presence of only one copy of the gene (data not shown). The amino acid sequence of the *A. alternata* MtDH shared 74 % identity (82 % positives) with that of *C. fulvum,* 71 % identity (81 % positives) with that of *A. nidulans* and 77 % (66 % positives) with that of *G. zeae* (Fig.5). The *A. alternata MtDH* sequence was submitted to GeneBank (accession # AF541874).

A 2999bp fragment was also isolated by PCR using the degenerate primers followed by inverted PCR. This fragment contained an ORF encoding a protein with strong similarity to the mannitol 1-phosphate 5-dehydrogenase published for *A. niger*, *P. brasiliensis*, and *S. nodorum* (accession # AAL89587, AAO47089, and AAT11122, respectively). Southern analysis showed the presence of only one copy of the gene (data not shown). The encoded amino acid sequence shared 87 % identity (92 % positives) with *S. nodorum*, 60 % identity (70 % positives) with that of *A. niger*, and 63 % identity (73% positives) with *P. brasiliensis* (Fig. 6). *A. alternata MPDH* sequence was submitted to GeneBank (accession # AY364263).

Gene disruption

Gene disruption constructs as shown in Fig. 2 were transformed into wild type *A. alternata.* Transformation experiments yielded 50 transformants with the pGHyg (*MtDH* disruption) construct and 20 with the pGPhleo (*MPDH* disruption) construct. Of the first 20

transformants screened by PCR, six were disrupted for *MtDH* and four for *MPDH*. For *MtDH,* mutants Mt-12, Mt-14, Mt-15, Mt-10, Mt-16, and Mt-17 showed replacement of the wild type gene with the disrupted version, while Mt-18, Mt-13, and Mt-9 had ectopic integration with the wild type version intact (Fig. 7-A). For *MPDH,* mutants Mp-10, Mp-14, Mp-18, and Mp-19 showed gene replacement, while Mp-21 showed ectopic integration (Fig. 7-B). To create a strain disrupted for both genes, protoplasts were isolated from the *MPDH* disruption mutant Mp-14 and were transformed with the pGHyg construct. Of the first 12 transformants screened by PCR, mutants B-6, B-7, B-8, B-10, and B-11 showed disruption for *MtDH*, while B-3 and B-4 showed ectopic integration (Fig. 7-A). Mutants Mt-12, Mp-14, and B-10 were chosen for further analysis.

Enzyme assays of disruption mutants

Mutants Mt-12, Mp-14 and B-10 were tested for MtDH and MPDH activity. In contrast to wild type, disruption of the *MtDH* gene resulted in the inability of cell-free extracts of mutants Mt-12 and B-10 to reduce fructose (Fig. 8-A) or oxidize mannitol (data not shown) using NADPH as a cofactor. Similarly, disruption of the *MPDH* gene resulted in the inability of cell-free extracts of mutants Mp-14 and B-10 to reduce fructose 6-phosphate (Fig. 8-B) or oxidize mannitol 1-phosphate (data not shown) using NAD(H) as a cofactor. These results confirmed the identity of the two genes, and documented the lack of additional copies or alternate genes encoding these enzymes.

Mannitol analyses

Mannitol content was determined by GC-MS for wild type *A. alternata* and for mutants Mt-12, Mp-14, and B-10 (Fig. 9). The mannitol content for wild type was found to be 110 μg mg^{-1} dried weight. Disruption of MtDH (Mt-12) reduced the mannitol content to 72.3 μg mg^{-1} dried weight. However, disruption of MPDH (Mp-14) reduced the mannitol levels to 12.6 μg mg^{-1} dried weight, suggesting that this enzyme is responsible for most of the mannitol produced by the fungus. Disruption of both MtDH and MPDH enzymes in mutant B-10 resulted in the inability of the fungus to make mannitol.

In vitro growth characteristics on different carbon sources

As the mannitol cycle was propose to regulate coenzymes and storage of reducing power, wild type, Mt-12, Mp-14, and B-10 were grown in minimal medium with nitrate and different carbon sources to see if the absence of a mannitol cycle produced any growth differences. Wild type, Mt-12, Mp-14, and B-10 were all able to grow in minimal medium supplemented with fructose, sorbitol, mannitol or glucose as a sole carbon source (Fig. 10), although Mp-14 and B-10 had less growth on mannitol. The ability of B-10 to grow on mannitol as a sole carbon source suggests that the fungus contains other enzymes that can utilize mannitol as a substrate.

Discussion

The goals of this research were to confirm the proposed fungal mannitol cycle by genetic analysis. Hult and Gatenbeck (17) first proposed the cycle in 1978. Since that time, enzyme activity and gene sequences for the proposed biosynthetic enzymes have been

reported from diverse fungi (Table 1), however, gene disruption experiments have only been conducted with MPDH (40, 44). In this work, we verified the enzymatic activities of both MtDH and MPDH in *A. alternata*, isolated the genes, and confirmed their function through gene disruption analysis. This is the first report in which both enzymes involved in mannitol metabolism have been disrupted.

We confirmed the findings of Hult and Gatenbeck (17) that *A. alternata* contains an NADP-dependent MtDH capable of reducing fructose and oxidizing mannitol. The amino acid sequence of the *A. alternata* MtDH was very similar to the ones published for other Ascomycete fungi (e.g., *C. fulvum*, *G. zeae*, and *A. nidulans*). While not very similar to MtDH enzymes from basidiomycetes (e.g., *U. fabae*, and *A. bisporus*), the sequences still shared common conserved domains (Fig. 5). *A. alternata's* protein sequence contains a coenzyme binding site at amino acid 26 (GXXXGXG) (24) and a short-chain dehydrogenases/reductases family signature at amino acids 161-189 ([LIVSPADNK]-x(12)-Y-[PSTAGNCV]-[STAGNQCIVM]-[STAGC]-K-{PC}-[SAGFYR]-[LIVMSTAGD]-x-{K}-[LIVMFYW]-x(2)-{YR}-[LIVMFYWGAPTHQ]-[GSACQRHM]) (16).

Our assays also confirmed the presence of an NAD-dependent MPDH capable of interconverting fructose 6-phosphate and mannitol 1-phosphate. The amino acid sequence of the *A. alternata* MPDH was very similar to those of *P. nodorum*, *A. niger*, and *P. brasiliensis* (Fig. 6). The protein sequence for MPDH contains a coenzyme binding site at amino acid 11 (GXGXXG) (24) and a mannitol dehydrogenase signature at amino acids 154-165 [LIVMY]-x-[FS]-x(2)-[STAGCV]-x-V-D-R-[IV]-x-[PS] (16).

Using the sequences isolated, two constructs were made and used for targeted gene disruption, yielding both single and double mutants for each gene. Disruption mutants

lacked enzyme activity for the respective genes, confirming their function. These results also support our Southern analysis results that *A. alternata* contains single copies of these genes (data not shown).

The mutants were assayed for mannitol content by GC-MS. The mannitol content for wild type was found to be 110 µg mg^{-1} dried weight. The 11.2 % of mannitol per mg fungal dry weight is close to the values reported (9-15 %) for *A. alternata* (17) and other fungi (26). Disruption of MtDH (Mt-12) reduced the mannitol content to by approximately 30-35 % of that of wild type. However, disruption of MPDH (Mp-14) reduced the mannitol levels by approximately 85-90 % of wild type, suggesting that this is the enzyme responsible for most of the mannitol produced by the fungus. This finding agrees with those of Ruijter et al., (40) and Solomon et al., (44) on *A. niger* and *S. nodorum,* respectively. Disruption of both enzymes diminished the mannitol content to levels not detectable by the methods used (Fig. 9). These results demonstrate that MtDH and MPDH are the only mannitol biosynthetic enzymes in *A. alternata*, and thus confirm the original results of Hult and Gatenbeck (17).

Although both MtDH and MPDH are capable of both mannitol synthesis and degradation, the mannitol cycle as proposed argued that MPDH was the primary biosynthetic enzyme and MtDH the primary catabolic enzyme. Our results with growth of the mutants on mannitol as a sole carbon source do not support this hypothesis. Mt-12 (MtDH disruptant) grew normally on medium with mannitol as a sole carbon source (Fig 10). It seems puzzling that the fungus was able to readily utilize mannitol in the absence of MtDH if the mannitol cycle functions as proposed. Enzymes with the ability to phosphorylate mannitol have been studied in fungi (51, 25). It is possible that mannitol is phosphorylated and then utilized through the enzyme MPDH. It has been shown that growth of *Aspergillus* species and

Sphaerosporella brunnea on mannitol causes a decrease in both MtDH and mannitol 1-phosphatase activity, and an increase in MPDH activity (25, 37, 47). These results suggest that MPDH can both synthesize and degrade mannitol 1-phosphate *in vivo.* Mp-14 (MPDH disruptant) was able to grow on mannitol as a sole carbon source, but not as well as the wild type or Mt-12. Mannitol may be down regulating MtDH as seen in *A. candidus* (47) and *S. brunnea* (37), or MPDH may have more of a degradative role than initially proposed, thus explaining the limited growth. The ability of mutant B-10 (disrupted in both genes) to grow on mannitol as a sole carbon source was completely unexpected. This result suggests that the fungus contains other enzymes that utilize mannitol as a substrate, such as ribitol 5-phosphate 2-dehydrogenase (EC 1.1.1.137), xylitol dehydrogenase (EC 1.1.1.9) and glycerol dehydrogenase (EC 1.1.1.72) (21, 41).

As the mannitol cycle was propose to regulate coenzymes and storage of reducing power, wild type, Mt-12, Mp-14, and B-10 were grown in minimal media with nitrate and different carbon sources to see if the absence of a mannitol cycle produced any growth differences. Growth on nitrate would require large amounts of NAPDH, which is oxidized to $NADP^+$ as nitrate is converted to ammonium. However, all the strains were able to grow in the minimal medium supplemented with fructose, sorbitol, mannitol or glucose as a sole carbon source (Fig. 10) suggesting that the mannitol cycle is not necessary for NADPH generation. This result is in agreement with other studies which have shown that the primary source of NADPH in fungi is the pentose phosphate pathway (42).

Mannitol has been suggested to play a role as a storage compound in fungi and to be important in spore germination. Interestingly, the spores from the mannitol mutants generated in our research were able to germinate and grow in culture (Fig. 10) and were able

to germinate *in planta* and infect tobacco (Vélez and Daub, unpublished results). Presumably, other carbohydrates are able to substitute for mannitol as a storage compound. Chromatograms from our mannitol analyses reveled that as mannitol levels decreased in the different mutants, the disaccharide content increased (data not shown). We did not detect any phenotypic differences in spores, mycelia, or colony morphology of the different mutants as compared to wild type (data not shown).

In summary, we used a genetic analysis to study mannitol metabolism in *A. alternata*. Our results confirm much of the work published by Hult and Gatenbeck in 1978 from studies of cell-free extracts of the fungus. Our studies confirm the presence of both MtDH and MPDH in the fungus, their activity as proposed, and the cofactor requirements. Further, we showed that disruption of both genes is necessary to block mannitol production. By contrast, our results do not support the functioning of a mannitol cycle in *A. alternata* Both production and degradation of mannitol were more severely impacted by the loss of MPDH, with MtDH having a more limited impact. We conclude that mannitol biosynthesis and degradation in *A. alternata* does not involve a functional cycle and can occur through either enzyme, with MPDH as the most important.

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Figure 1. Proposed mannitol cycle in fungi (17). HK= hexokinase, MPDH = mannitol 1-phosphate 5-dehydrogenase (EC 1.1.1.17), M1Pse= mannitol 1-phosphatase (EC 3.1.3.22), P_i= inorganic phosphate, MtDH = mannitol dehydrogenase (EC 1.1.1.138), mannitol 1-P =mannitol 1-phosphate, fructose 6-P = fructose 6-phosphate

Figure 2. Construction of pGHyg (A) and pGPhleo (B) for gene disruption. For both constructs, sequences were deleted from the *MtDH* and *MPDH* genes by restriction enzyme digestion as shown. The *hph* (hygromycin resistance) gene fragment was ligated into the remaining *MtDH* sequences after digestion with *EcoO1091I* and *Acc65I*. The *ble* (phleomycin resistance) gene fragment was ligated into the remaining *MPDH* sequences after digestion with *EheI*. Both marker genes are under the control of a constitutive fungal promoter and terminator. The lines under each figure indicate the site of the primers and size of the fragment amplified by PCR to show disruption.

Figure 3. MtDH activity in *A. alternata* wild type strain. Enzymatic activity assayed as the change in NADPH concentration following addition of substrate. (A) conversion of mannitol to fructose; (B) conversion of fructose to mannitol. Arrows indicate addition of substrate: (A) mannitol; (B) fructose.

Figure 4. MPDH activity in *A. alternata* wild type strain. Enzymatic activity assayed as the change in NADH concentration following addition of substrate. (A) conversion of mannitol 1-phosphate to fructose 6-phosphate; (B) conversion of fructose 6-phosphate to mannitol 1-

phosphate. Arrows indicate addition of substrate: (A) mannitol 1-phosphate; (B) fructose 6-phosphate).

Figure 5. MtDH amino acid sequence comparison of *Cladosporium fulvum* (AAK67169) *Gibberella zeae* (AAP33281), *Aspergillus nidulans* (EAA62170), and *Alternaria alternata* (AAN28666), *Uromyces fabae* (AAB39878), and *Agaricus bisporus* (O93868). Alignment was done using default parameters on ClustalW (http://www.ebi.ac.uk/Tools/). "*" means that the residues in that column are identical in all sequences in the alignment; ":" means that conserved substitutions have been observed; "." means that semi-conserved substitutions are observed. The NADP binding motif and the short-chain dehydrogenases/reductases family signature (16) are shown in boxes.

Figure 6. MPDH amino acid sequence comparison of *Aspergillus fumigatus* (EAL93361), *Aspergillus niger* (AAL89587), *Paracoccidioides brasiliensis* (AAO47089), *Magnaporthe grisea* (AAX07705), *Phaeosphaeria nodorum* (AAT11122), and *Alternaria alternata* (AAQ63948). Alignment was done using default parameters on ClustalW (http://www.ebi.ac.uk/Tools/). "*" means that the residues in that column are identical in all sequences in the alignment; ":" means that conserved substitutions have been observed; "." means that semi-conserved substitutions are observed. The NAD binding motif and the mannitol dehydrogenases signature (16) are shown in boxes.

Figure 7. Screening for *MtDH* and *MPDH* disruption mutants using PCR. Brackets across the top indicate strains transformed with the MtDH disruption construct, MPDH disruption construct, and both constructs. A. Amplification of *MtDH* sequences. The expected sizes for the wild type (black arrow) and disrupted *MtDH* (white arrow) were 826 bp and 2755bp, respectively. B. Amplification of *MPDH* sequences. The expected sizes for wild type (black arrow) and disrupted *MPDH* (black arrow) were 2188bp and 3579bp, respectively. Mutants Mt-12, Mt-14, Mt-15, Mt-10, Mt-16, and Mt-7 showed gene replacement and disruption of *MtDH*. Mutants Mp10, Mp-14, Mp-18, and Mp-19 showed gene replacement and disruption of *MPDH*. Mutants B-6, B-7, B-8, B-10, and B-11 were disrupted for both *MtDH* and *MPDH*. Other transformants showed ectopic integration with the wild type copies intact.

Figure 8. Enzyme activities in wild type *A. alternata* and disruption mutants. (A) MtDH activity assay, measuring the decrease in NADPH upon addition of fructose (arrow). Solid line = wild type; dashed line = *MtDH* disruption mutant Mt-12; dotted line = B-10 mutant disrupted in both genes. (B) MPDH activity assay, measuring the decrease in NADH upon the addition of fructose-6-phosphate (arrow). Solid line = wild type; dashed line = *MPDH* disruption mutant Mp-14; dotted line = B-10 mutant disrupted for both genes.

Figure 9. GC-MS chromatogram of extracts from the *A. alternata* wild type strain, the Mt-12 *MtDH* disruption mutant, the Mp-14 *MPDH* disruption mutant, and the B-10 mutant disrupted for both genes as compared to a mannitol standard (retention time of 11.42 min). Disruption of *MPDH* had a more severe impact on mannitol production than did disruption of *MtDH*. The mutant disrupted in both genes was unable to synthesize mannitol.

Figure 10. Growth of wild type *A. alternata* and mannitol enzyme mutants in minimal media supplemented with 110 mM different carbon sources. Mt-12, disrupted for *MtDH*; Mp-14, disrupted for *MPDH*; B-10, disrupted for both genes. Mt-12 grew equally well on all carbon sources, however both Mp-14 and B-10 mutants grew poorly on mannitol as a sole carbon source.

Table 1. MtDH and MPDH enzyme activities reported in fungi

Organism	MtDH: Cofactor[1]	MPDH: Cofactor[2]	Sequence Reported MtDH/MPDH[3]	Phyla[4]	Reference
Alternaria alternata	NADP(H)	NAD(H)	AAN28666/AAQ63948	A	(17) this work
Aspergillus candidus	NADP(H)	NAD(H)	N/N	A	(47)
Aspergillus fumigatus		NAD(H)	N	A	(4)
Aspergillus nidulans	NADP(H)	NAD(H)	N	A	(14, 42)
Aspergillus niger		NAD(H)	AAL89587	A	(4, 18, 22)
Aspergillus oryzae	NADP(H)	NAD(H)	N/N	A	(4, 18)
Aspergillus parasiticus	NADP(H)	NAD(H)	N/N	A	(13)
Botrytis cinerea	NADP(H)	NAD(H)	N/N	A	(18)
Candida magnoliae	NADP(H)		X	A	(24)
Ceratocystis multiannulata	NADP(H)		N	A	(18)
Cephalosporium chrysogenum	NADP(H)		N	A	(3)
Cenococcum graniforme	NADP(H)	NAD(H)	N/N	A	(29)
Chaetomium globosum	NAD(H)		N	A	(1)
Chaetomium thermophile var. dissitum		NAD(H)	N	A	(4)
Cladosporium cladosporioides	NADP(H)		N	A	(18)
Cladosporium fulvum	NADP(H)		AF387300	A	(35)
Coccidioides immitis		NAD(H)	N	A	(27)
Dendryphiella salina	NADP(H)		N	A	(15)
Diplodia viticola	NADP(H)		N	A	(48)
Geotrichum candidum	NADP(H)		N	A	(8)
Gibberella zeae	NADP(H)		AAP33281	A	(18, 50)
Humicola lanuginosa		NAD(H)	N	A	(4)
Malbranchae pulchella var. sulfurea		NAD(H)	N	A	(4)
Neurospora crassa	NADP(H)		N	A	(18)
Penicillium chrysogenum	NADP(H)	NAD(H)	N/N	A	(4, 5)
Penicillium cyclopium			N	A	(4)
Penicillium duponti		NAD(H)	N	A	(4)
Penicillium frequentans	NADP(H)	NAD(H)	N/N	A	(18)
Penicillium islandicum	NADP(H)	NAD(H)	N/N	A	(18)
Penicillium notatum		NAD^+/H	N	A	(4)
Penicillium urticae		NAD(H)	N	A	(4)
Pyrenochaeta terrestris		NAD(H)	N	A	(55)
Piricularia oryzae	NADP(H)	NAD(H)	N/N	A	(56)
Saccharomyces cerevisiae	NAD(H)		U18795	A	(36)
Sclerotinia sclerotiorum	NADP(H)	NAD(H)	N/N	A	(53)
Sphaerosporella brunnea	NADP(H)	NAD(H)	N/N	A	(37)
Stagonospora nodorum		NAD(H)	AAT84078	A	(44)
Thermomyces lanuginosus	NADP(H)	NAD(H)	N/N	A	(18)
Trichothecium roseum	NADP(H)	NAD(H)	N/N	A	(18)

Table 1. Continuation

Organism	MtDH: Cofactor[1]	MPDH: Cofactor[2]	Sequence Reported MtDH/MPDH[3]	Phyla[4]	Reference
Agaricus bisporus	NADP(H)		AAC79985	B	(18, 32, 33, 46)
Agaricus campestris	NADP(H)		N	B	(12)
Amanita muscaria	NAD(H)		N	B	(37)
Armillariella mellea	NAD(H)		N	B	(37)
Chosdrosterium purpureum	NAD(H)		N	B	(37)
Cryptococcus neoformans		NAD(H)	AAG09209	B	(49)
Heterobasidion annosum	NAD(H)		N	B	(37)
Laccaria laccata	NADP(H)		N	B	(37)
Lentinus edodes	NADP(H)		N	B	(23)
Marasmius scorodonius	NAD(H)		N	B	(37)
Melampsora lini	NADP(H)		N	B	(11)
Mycena metata	NAD(H)		N	B	(37)
Piloderma croceum	NADP(H)	NAD(H)	N/N	B	(37, 38)
Pleurotus ostreatus	NADP(H)	NAD(H)	X	B	(7)
Schizophyllum commune	NAD(H)		N	B	(34)
Suillus bovines	NADP(H)		N	B	(37)
Suillus variegates	NADP(H)		N	B	(37)
Uromyces fabae	NADP(H)		O00058	B	(52)
Absidia glauca	NAD(H)	NAD(H)	N/N	Z	(51)
Mucor lusitanicus	NADP(H)		N	Z	(18)

[1] Notation of co-factor indicates presence of MtDH activity

[2] Notation of co-factor indicates presence of MPDH activity

[3] N= no gene or sequence reported; X = gene recovery reported but no sequence provided; NCBI Accession numbers provided for reported sequences

[4] A: Ascomycota, B: Basidiomycota, Z: Zygomycota

Table 2. PCR primers used for experiments

Gene Experiment	Forward (5' to 3')	Reverse (5' to 3')
MtDH degenerate	GGCAAGATCGACGCCTTYATHGCNAA	TGGCGACGTTGTAGGAGGTYTGYTCYTG
MtDH inverted	GTCATCACCTCCTCCATGTC	CAACGGTAGCGTCGAGGATA
MtDH with *Pfu*	ATCCGCTTGAGACGTTTCG	CGGCTGAACGATGGTGAT
MPDH degenerate	CAACATCGGCCGGGGNTTYGTNGC	TGGTCGGGGACGATCCKRTCDATNGC
MPDH inverted	AAGCCGGATGTTAGGGT	TCTTGGTCTCTTCGCTCA
MPDH with *Pfu*	GATCCCGAGGTACTTATGCG	CGATGTTGTGGATATCGTCTCT
ble gene pBCPhleo	AATTCCCTTGTATCTCTACACA	ACATCGAACTGGATCTCAA
MtDH disruptants	ATAATCGCCACAATGCCC	CGGAATGACACGTTTACCTG
MPDH disruptants	GATCCCGAGGTACTTATGCG	GCTACCTCCCTGGACCTT

Figure 1.

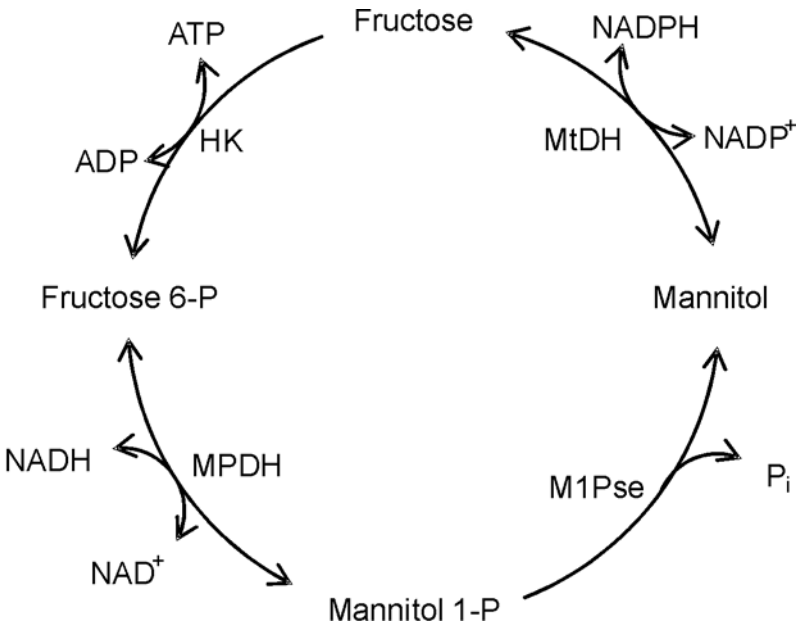

Fructose
ATP
ADP
HK
Fructose 6-P
NADPH
MtDH
NADP+
Mannitol
NADH
MPDH
NAD+
Mannitol 1-P
M1Pse
Pi

Figure 2.

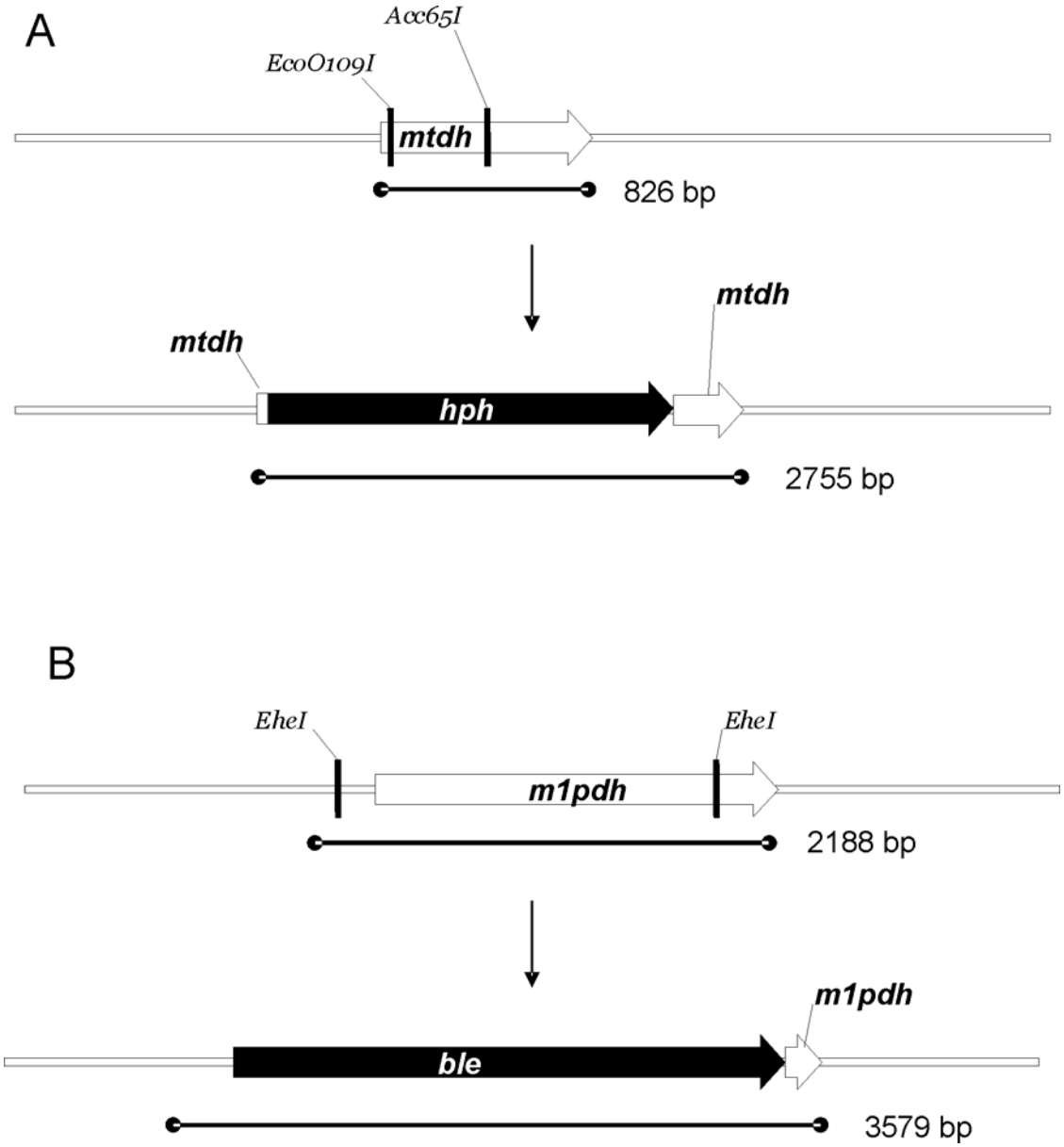

A
Acc65I
EcoO109I
mtdh
826 bp
mtdh
mtdh
hph
2755 bp
B
EheI
EheI
m1pdh
2188 bp
m1pdh
ble
3579 bp

Figure 3

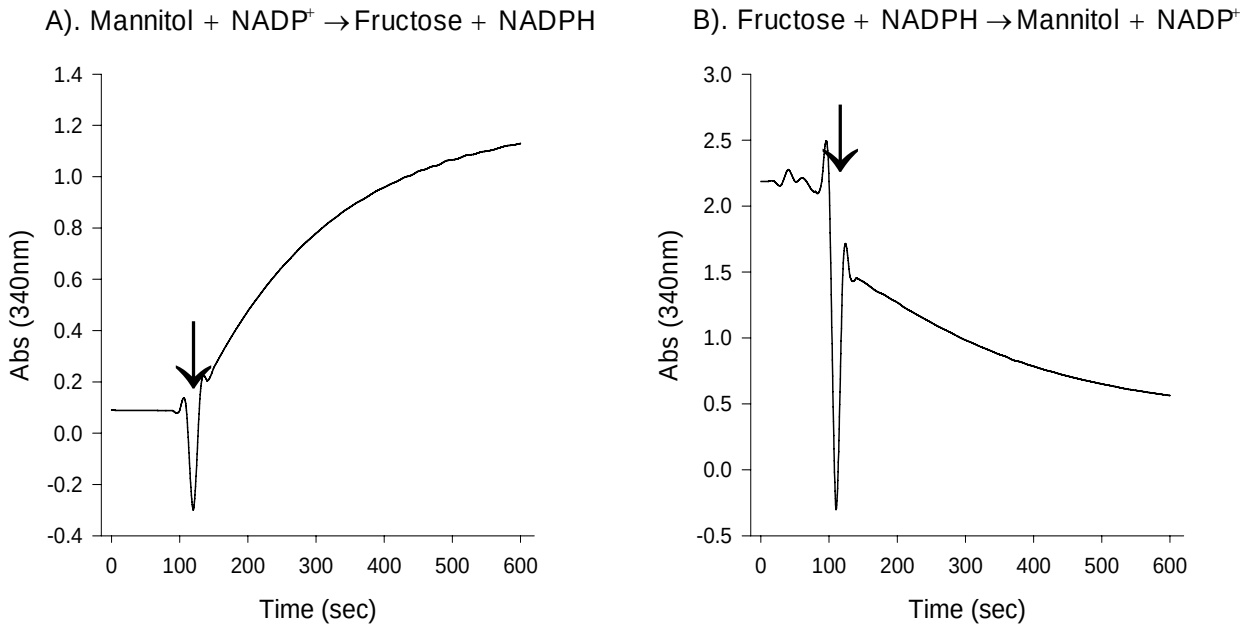

A). Mannitol + NADP+ → Fructose + NADPH
Abs (340nm)
Time (sec)
B). Fructose + NADPH → Mannitol + NADP+
Abs (340nm)
Time (sec)

Figure 4

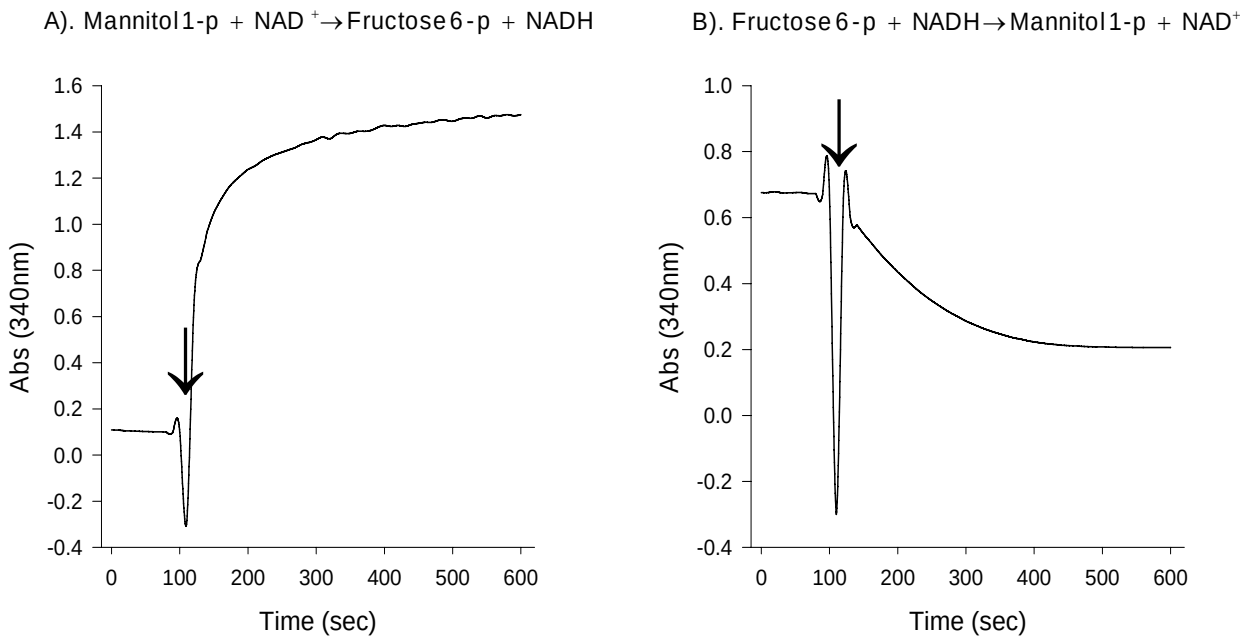

A). Mannitol 1-p + NAD+ → Fructose 6-p + NADH
B). Fructose 6-p + NADH → Mannitol 1-p + NAD+
Abs (340nm)
Time (sec)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
-0.4
0
100
200
300
400
500
600

Figure 5

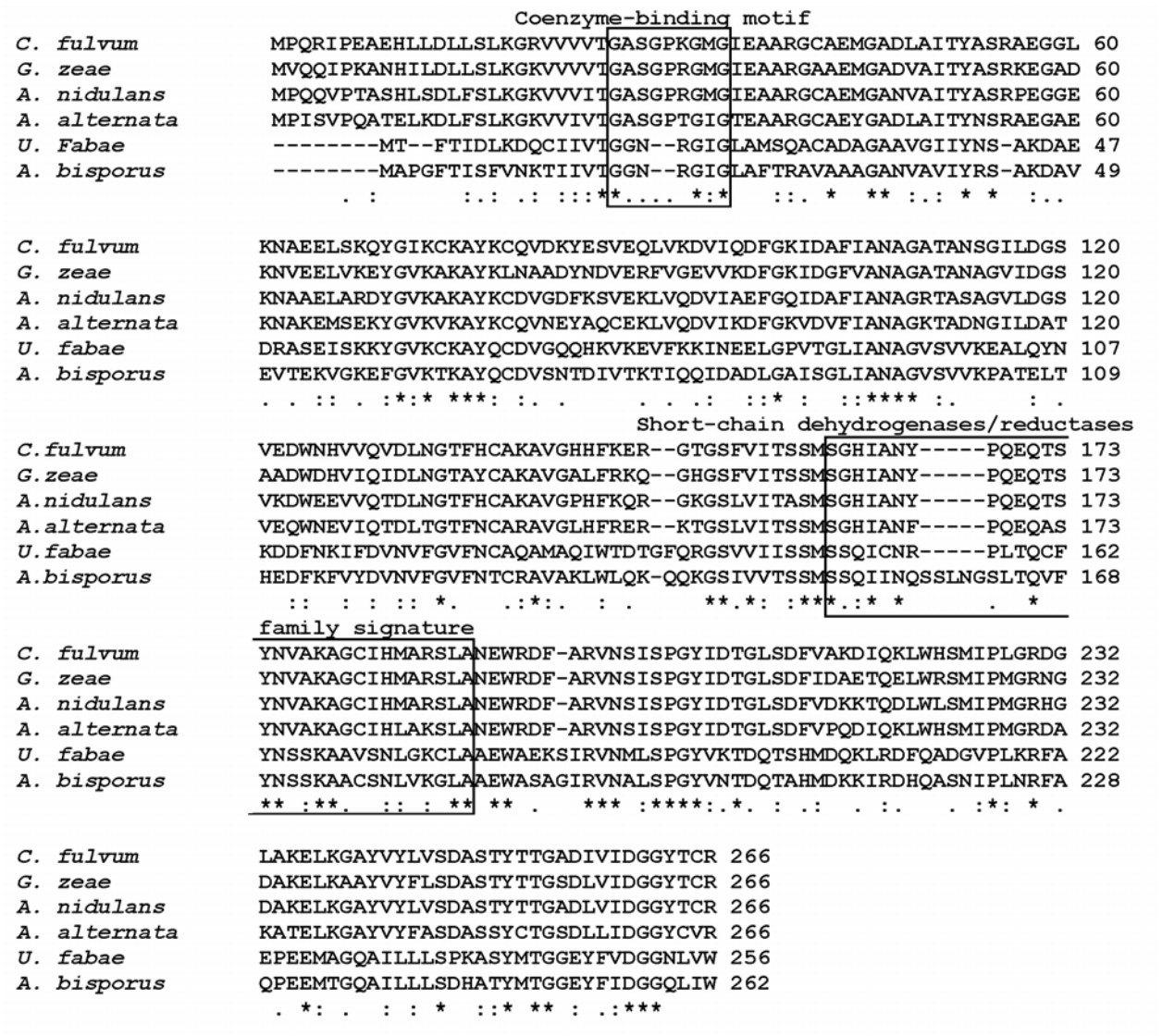

Coenzyme-binding motif
C. fulvum MPQRIPEAEHLLDLLSLKGRVVVVTGASGPKGMGIEAARGCAEMGADLAITYASRAEGGL 60
G. zeae MVQQIPKANHILDLLSLKGKVVVVTGASGPRGMGIEAARGAAEMGADVAITYASRKEGAD 60
A. nidulans MPQQVPTASHLSDLFSLKGKVVVITGASGPRGMGIEAARGCAEMGANVAITYASRPEGGE 60
A. alternata MPISVPQATELKDLFSLKGKVVIVTGASGPTGIGTEAARGCAEYGADLAITYNSRAEGAE 60
U. Fabae --------MT--FTIDLKDQCIIVTGGN--RGIGLAMSQACADAGAAVGIIYNS-AKDAE 47
A. bisporus --------MAPGFTISFVNKTIIVTGGN--RGIGLAFTRAVAAAGANVAVIYRS-AKDAV 49
KNAEELSKQYGIKCKAYKCQVDKYESVEQLVKDVIQDFGKIDAFIANAGATANSGILDGS 120
KNVEELVKEYGVKAKAYKLNAADYNDVERFVGEVVKDFGKIDGFVANAGATANAGVIDGS 120
KNAAELARDYGVKAKAYKCDVGDFKSVEKLVQDVIAEFGQIDAFIANAGRTASAGVLDGS 120
KNAKEMSEKYGVKVKAYKCQVNEYAQCEKLVQDVIKDFGKVDVFIANAGKTADNGILDAT 120
DRASEISKKYGVKCKAYQCDVGQQHKVKEVFKKINEELGPVTGLIANAGVSVVKEALQYN 107
EVTEKVGKEFGVKTKAYQCDVSNTDIVTKTIQQIDADLGAISGLIANAGVSVVKPATELT 109
Short-chain dehydrogenases/reductases
C.fulvum VEDWNHVVQVDLNGTFHCAKAVGHHFKER--GTGSFVITSSMSGHIANY-----PQEQTS 173
G.zeae AADWDHVIQIDLNGTAYCAKAVGALFRKQ--GHGSFVITSSMSGHIANY-----PQEQTS 173
A.nidulans VKDWEEVVQTDLNGTFHCAKAVGPHFKQR--GKGSLVITASMSGHIANY-----PQEQTS 173
A.alternata VEQWNEVIQTDLTGTFNCARAVGLHFRER--KTGSLVITSSMSGHIANF-----PQEQAS 173
U.fabae KDDFNKIFDVNVFGVFNCAQAMAQIWTDTGFQRGSVVIISSMSSQICNR-----PLTQCF 162
A.bisporus HEDFKFVYDVNVFGVFNTCRAVAKLWLQK-QQKGSIVVTSSMSSQIINQSSLNGSLTQVF 168
family signature
C. fulvum YNVAKAGCIHMARSLANEWRDF-ARVNSISPGYIDTGLSDFVAKDIQKLWHSMIPLGRDG 232
G. zeae YNVAKAGCIHMARSLANEWRDF-ARVNSISPGYIDTGLSDFIDAETQELWRSMIPMGRNG 232
A. nidulans YNVAKAGCIHMARSLANEWRDF-ARVNSISPGYIDTGLSDFVDKKTQDLWLSMIPMGRHG 232
A. alternata YNVAKAGCIHLAKSLANEWRDF-ARVNSISPGYIDTGLSDFVPQDIQKLWHSMIPMGRDA 232
U. fabae YNSSKAAVSNLGKCLAAEWAEKSIRVNMLSPGYVKTDQTSHMDQKLRDFQADGVPLKRFA 222
A. bisporus YNSSKAACSNLVKGLAAEWASAGIRVNALSPGYVNTDQTAHMDKKIRDHQASNIPLNRFA 228
C. fulvum LAKELKGAYVYLVSDASTYTTGADIVIDGGYTCR 266
G. zeae DAKELKAAYVYFLSDASTYTTGSDLVIDGGYTCR 266
A. nidulans DAKELKGAYVYLVSDASTYTTGADLVIDGGYTCR 266
A. alternata KATELKGAYVYFASDASSYCTGSDLLIDGGYCVR 266
U. fabae EPEEMAGQAILLLSPKASYMTGGEYFVDGGNLVW 256
A. bisporus QPEEMTGQAILLLSDHATYMTGGEYFIDGGQLIW 262

Figure 6

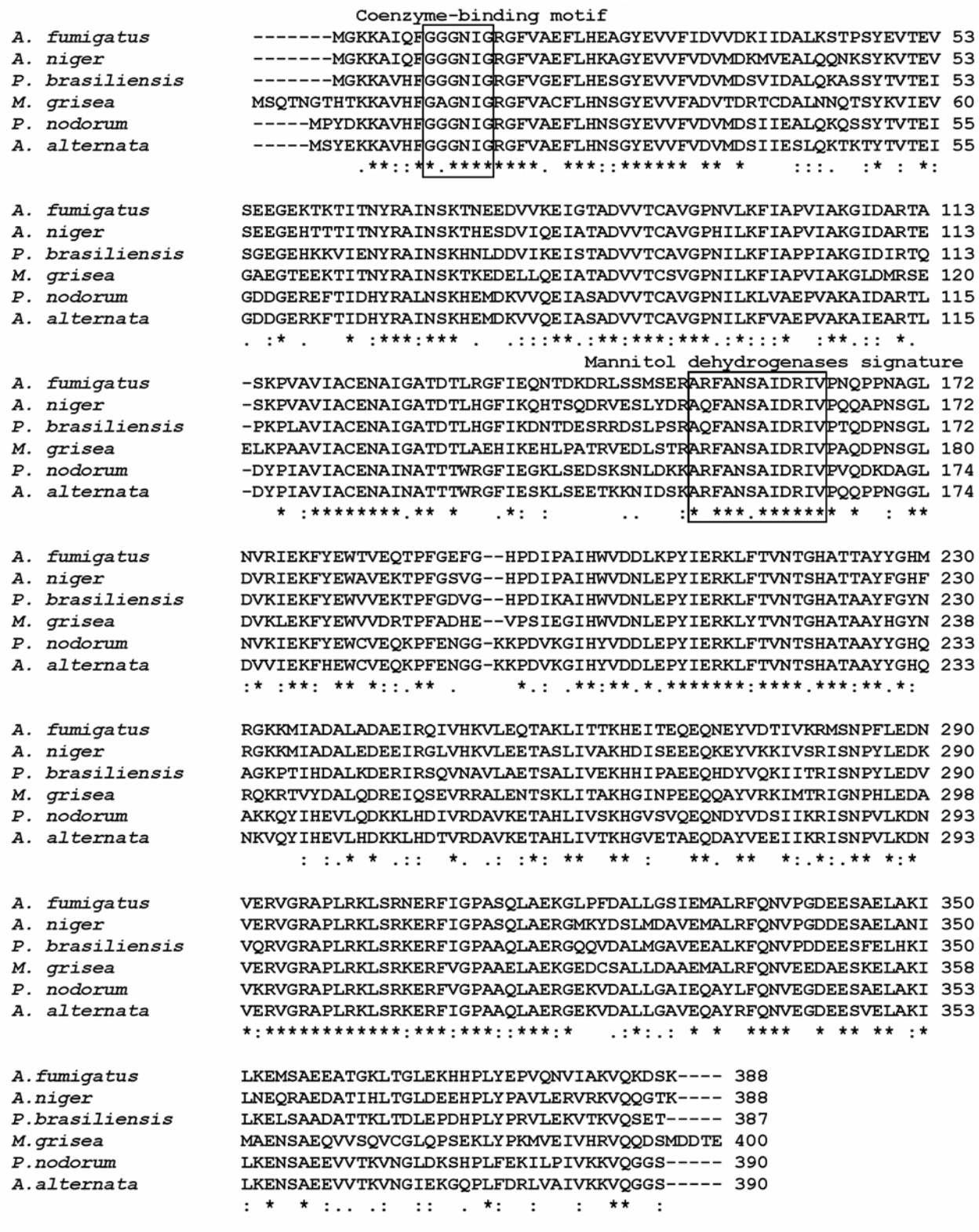

Coenzyme-binding motif
A. fumigatus -------MGKKAIQFGGGNIGRGFVAEFLHEAGYEVVFIDVVDKIIDALKSTPSYEVTEV 53
A. niger -------MGKKAIQFGGGNIGRGFVAEFLHKAGYEVVFVDVMDKMVEALQQNKSYKVTEV 53
P. brasiliensis -------MGKKAVHFGGGNIGRGFVGEFLHESGYEVVFVDVMDSVIDALQKASSYTVTEI 53
M. grisea MSQTNGTHTKKAVHFGAGNIGRGFVACFLHNSGYEVVFADVTDRTCDALNNQTSYKVIEV 60
P. nodorum -----MPYDKKAVHFGGGNIGRGFVAEFLHNSGYEVVFVDVMDSIIEALQKQSSYTVTEI 55
A. alternata -----MSYEKKAVHFGGGNIGRGFVAEFLHNSGYEVVFVDVMDSIIESLQKTKTYTVTEI 55
A. fumigatus SEEGEKTKTITNYRAINSKTNEEDVVKEIGTADVVTCAVGPNVLKFIAPVIAKGIDARTA 113
A. niger SEEGEHTTTITNYRAINSKTHESDVIQEIATADVVTCAVGPHILKFIAPVIAKGIDARTE 113
P. brasiliensis SGEGEHKKVIENYRAINSKHNLDDVIKEISTADVVTCAVGPNILKFIAPPIAKGIDIRTQ 113
M. grisea GAEGTEEKTITNYRAINSKTKEDELLQEIATADVVTCSVGPNILKFIAPVIAKGLDMRSE 120
P. nodorum GDDGEREFTIDHYRALNSKHEMDKVVQEIASADVVTCAVGPNILKLVAEPVAKAIDARTL 115
A. alternata GDDGERKFTIDHYRAINSKHEMDKVVQEIASADVVTCAVGPNILKFVAEPVAKAIEARTL 115
Mannitol dehydrogenases signature
A. fumigatus -SKPVAVIACENAIGATDTLRGFIEQNTDKDRLSSMSERARFANSAIDRIVPNQPPNAGL 172
A. niger -SKPVAVIACENAIGATDTLHGFIKQHTSQDRVESLYDRAQFANSAIDRIVPQQAPNSGL 172
P. brasiliensis -PKPLAVIACENAIGATDTLHGFIKDNTDESRRDSLPSRAQFANSAIDRIVPTQDPNSGL 172
M. grisea ELKPAAVIACENAIGATDTLAEHIKEHLPATRVEDLSTRARFANSAIDRIVPAQDPNSGL 180
P. nodorum -DYPIAVIACENAINATTTWRGFIEGKLSEDSKSNLDKKARFANSAIDRIVPVQDKDAGL 174
A. alternata -DYPIAVIACENAINATTTWRGFIESKLSEETKKNIDSKARFANSAIDRIVPQQPPNGGL 174
A. fumigatus NVRIEKFYEWTVEQTPFGEFG--HPDIPAIHWVDDLKPYIERKLFTVNTGHATTAYYGHM 230
A. niger DVRIEKFYEWAVEKTPFGSVG--HPDIPAIHWVDNLEPYIERKLFTVNTSHATTAYFGHF 230
P. brasiliensis DVKIEKFYEWVVEKTPFGDVG--HPDIKAIHWVDNLEPYIERKLFTVNTGHATAAYFGYN 230
M. grisea DVKLEKFYEWVVDRTPFADHE--VPSIEGIHWVDNLEPYIERKLYTVNTGHATAAYHGYN 238
P. nodorum NVKIEKFYEWCVEQKPFENGG-KKPDVKGIHYVDDLEPYIERKLFTVNTSHATAAYYGHQ 233
A. alternata DVVIEKFHEWCVEQKPFENGG-KKPDVKGIHYVDDLEPYIERKLFTVNTSHATAAYYGHQ 233
A. fumigatus RGKKMIADALADAEIRQIVHKVLEQTAKLITTKHEITEQEQNEYVDTIVKRMSNPFLEDN 290
A. niger RGKKMIADALEDEEIRGLVHKVLEETASLIVAKHDISEEEQKEYVKKIVSRISNPYLEDK 290
P. brasiliensis AGKPTIHDALKDERIRSQVNAVLAETSALIVEKHHIPAEEQHDYVQKIITRISNPYLEDV 290
M. grisea RQKRTVYDALQDREIQSEVRRALENTSKLITAKHGINPEEQQAYVRKIMTRIGNPHLEDA 298
P. nodorum AKKQYIHEVLQDKKLHDIVRDAVKETAHLIVSKHGVSVQEQNDYVDSIIKRISNPVLKDN 293
A. alternata NKVQYIHEVLHDKKLHDTVRDAVKETAHLIVTKHGVETAEQDAYVEEIIKRISNPVLKDN 293
A. fumigatus VERVGRAPLRKLSRNERFIGPASQLAEKGLPFDALLGSIEMALRFQNVPGDEESAELAKI 350
A. niger VERVGRAPLRKLSRKERFIGPASQLAERGMKYDSLMDAVEMALRFQNVPGDDESAELANI 350
P. brasiliensis VQRVGRAPLRKLSRKERFIGPAAQLAERGQQVDALMGAVEEALKFQNVPDDEESFELHKI 350
M. grisea VERVGRAPLRKLSRKERFVGPAAELAEKGEDCSALLDAAEMALRFQNVEEDAESKELAKI 358
P. nodorum VKRVGRAPLRKLSRKERFVGPAAQLAERGEKVDALLGAIEQAYLFQNVEGDEESAELAKI 353
A. alternata VERVGRAPLRKLSRKERFIGPAAQLAERGEKVDALLGAVEQAYRFQNVEGDEESVELAKI 353
A.fumigatus LKEMSAEEATGKLTGLEKHHPLYEPVQNVIAKVQKDSK---- 388
A.niger LNEQRAEDATIHLTGLDEEHPLYPAVLERVRKVQQGTK---- 388
P.brasiliensis LKELSAADATTKLTDLEPDHPLYPRVLEKVTKVQSET----- 387
M.grisea MAENSAEQVVSQVCGLQPSEKLYPKMVEIVHRVQQDSMDDTE 400
P.nodorum LKENSAEEVVTKVNGLDKSHPLFEKILPIVKKVQGGS----- 390
A.alternata LKENSAEEVVTKVNGIEKGQPLFDRLVAIVKKVQGGS----- 390

Figure 7

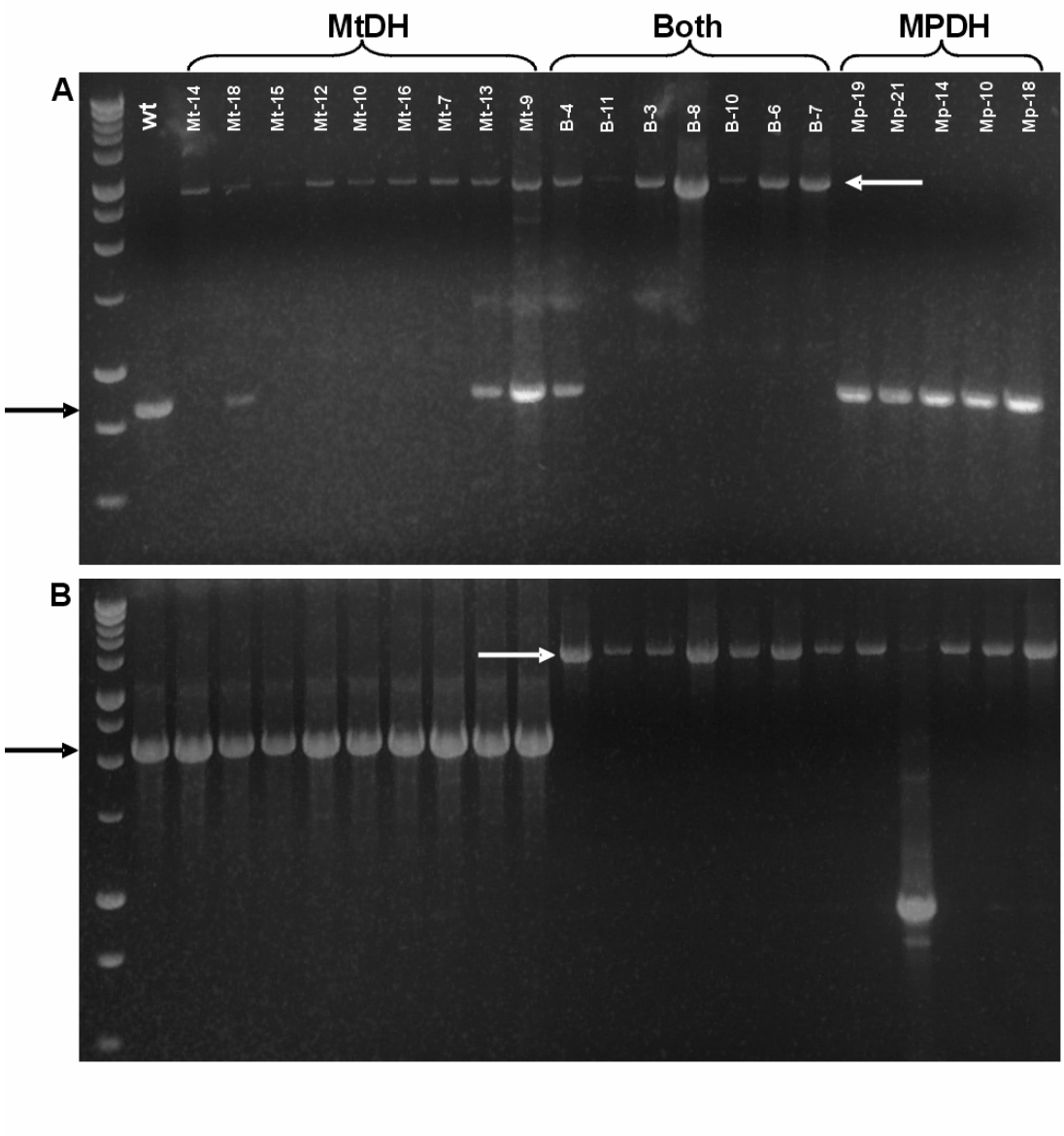

MtDH
Both
MPDH
A
wt
Mt-14
Mt-18
Mt-15
Mt-12
Mt-10
Mt-16
Mt-7
Mt-13
Mt-9
B-4
B-11
B-3
B-8
B-10
B-6
B-7
Mp-19
Mp-21
Mp-14
Mp-10
Mp-18
B

Figure 8

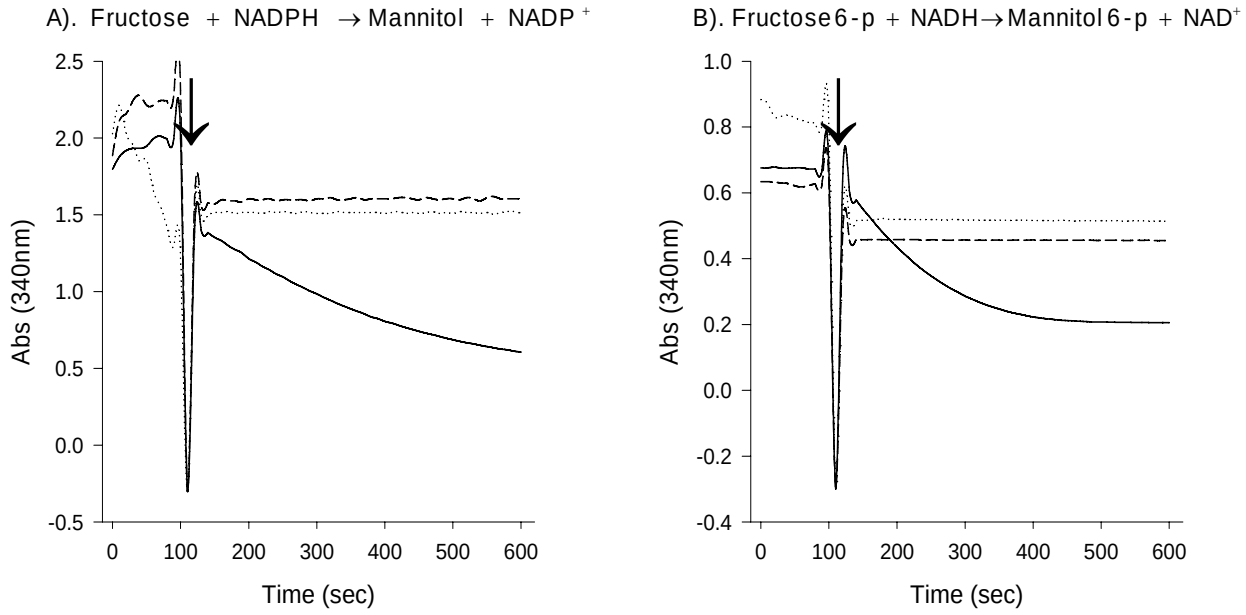

A). Fructose + NADPH → Mannitol + NADP +
Abs (340nm)
Time (sec)
B). Fructose 6-p + NADH → Mannitol 6-p + NAD+
Abs (340nm)
Time (sec)

Figure 9

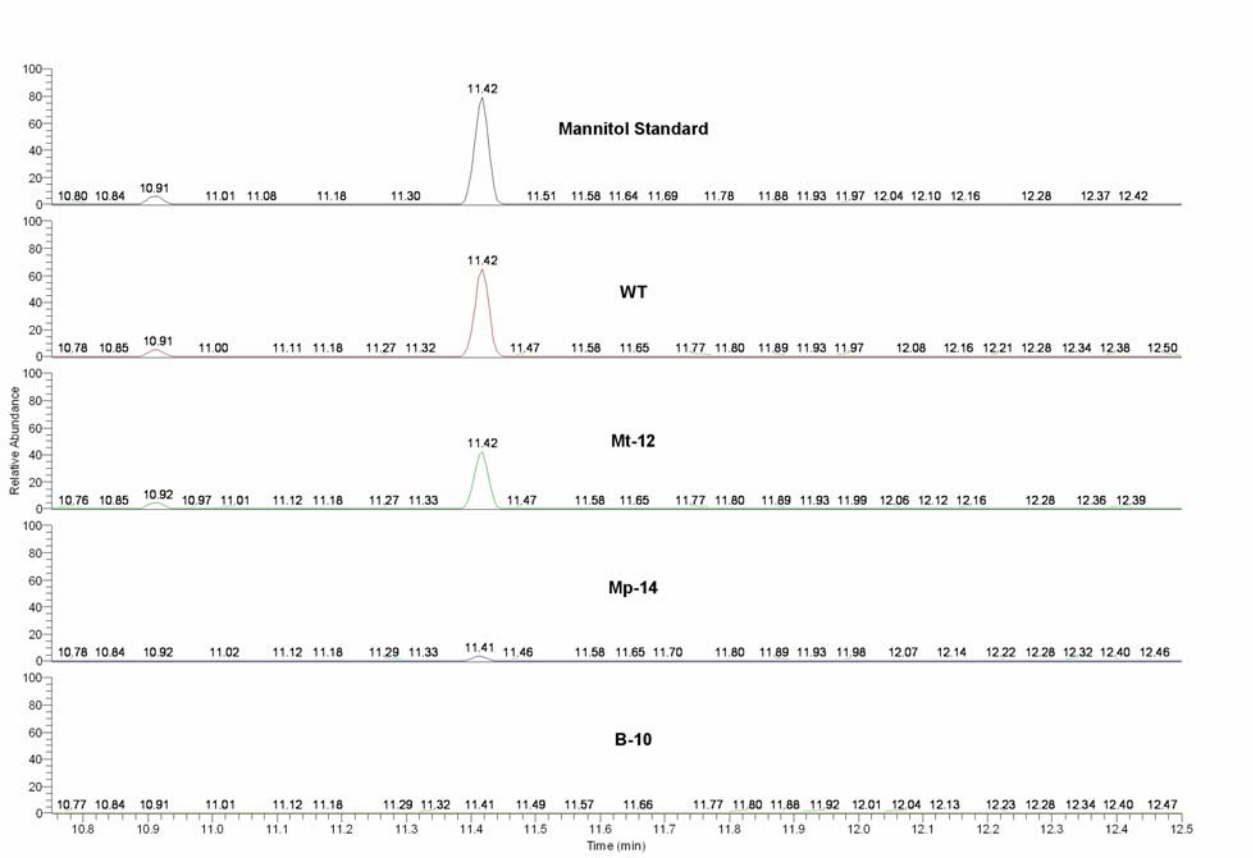
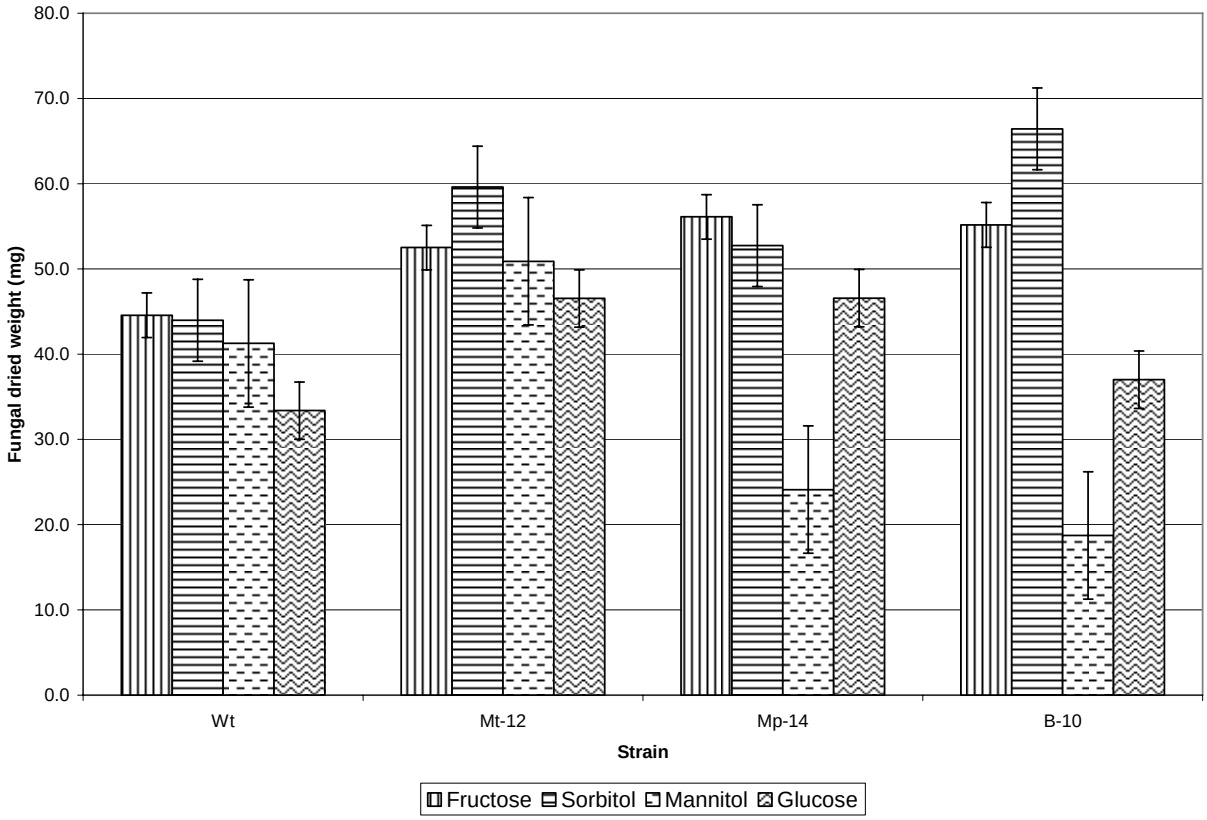

Mannitol Standard
WT
Mt-12
Mp-14
B-10
Relative Abundance
Time (min)

Figure 10


80.0
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
Fungal dried weight (mg)
Wt
Mt-12
Mp-14
B-10
Strain
Fructose
Sorbitol
Mannitol
Glucose

CHAPTER 3: Mannitol biosynthesis is required for pathogenicity of *Alternaria alternata*

Heriberto Vélez[a] and Margaret E. Daub[b*]

[a] Department of Plant Pathology, NC State University, Raleigh, NC 27695 USA

[b] Department of Botany, NC State University, Raleigh, NC 27695 USA

[*]Corresponding author

Corresponding Author: Margaret E. Daub Department of Botany, Campus Box 7612, 2214 Gardner Hall, NC State University, Raleigh, NC 27695 USA

Telephone: 1-919-513-3807, Fax: 1-919-515-3436, Email: margaret_daub@ncsu.edu

Abstract

Mannitol is produced by many fungi as a carbohydrate reserve. Recently, a role for mannitol in antioxidant defense has been supported by experiments with *Saccharomyces cerevisiae*, *Cryptococcus neoformans*, *Uromyces fabae* and *Alternaria alternata*. In a previous work, we confirmed the presence of the two mannitol biosynthetic enzymes, mannitol dehydrogenase (MtDH) and mannitol 1-phosphate 5-dehydrogenase (MPDH), in the fungus *A. alternata* and created mutants deficient in these enzymes. These studies showed that mutants deficient in MPDH or MtDH alone produced approximately 11% and 66%, respectively, of wild type levels, and that a double mutant deficient in both enzymes did not produce any mannitol. These mutants were used to investigate the role of mannitol in pathogenicity of *A. alternaria* on its host, tobacco. Spore germination assays *in vitro* demonstrated that conidia of all mutants were viable and germinated normally. Tobacco leaves were inoculated with 200 µl droplets of conidia and the severity of resulting lesions were scored from 0 to 4, with 0 being no visible symptoms to 4 for a large necrotic and chlorotic lesion typical of severe Brown Spot infection. Severity of lesions caused by the MtDH disruptant was not significantly different from that of the wild type, with mean severity ratings of 3.1 and 2.9, respectively. By contrast, the MPDH disruptant and the double mutant caused significantly less disease, with mean lesion severity ratings averaging 1.5 and 1.1, respectively. Microscopy analysis indicated that the double mutant was unaffected in the ability to germinate and produce appressoria on tobacco leaves. Histochemical staining for H_2O_2 showed that both the wild type strain and the double mutant were able to elicit a defense response from the host, indicating that both penetrated and infected the host. Quantitative PCR studies showed that genes for both enzymes were up-regulated in the presence of tobacco extracts, with *MPDH*

having a stronger response. We conclude that mannitol biosynthesis is required for pathogenesis of *A. alternata* on tobacco, but is not required for normal spore germination either *in vitro* or *in planta* or for initial infection.

Keywords: mannitol metabolism, reactive oxygen species, antioxidant, 3,3'-Diaminobenzidine tetrahydrochloride, plant disease

Introduction

Mannitol is purported to have role in fungi as a storage or translocated carbohydrate (Lewis and Smith, 1967). For example, in *Aspergillus oryzae* and *Aspergillus niger* mannitol found in the conidia is metabolized at a very early stage during germination (Horikoshi et al., 1965; Witteveen and Visser, 1995). Studies with *Aspergillus nidulans* using ^{13}C-labeled carbon showed that mannitol, arabitol, erythritol and glycerol were present and were the first carbon sources to be depleted upon starvation, strengthening their role as storage compounds (Dijkema et al., 1985). More recently, it has been suggested that mannitol may play a role as an antioxidant in host-pathogen interactions, as mannitol has been shown to quench reactive oxygen species (ROS) both *in vitro* and *in vivo* (Smirnoff and Cumbes, 1989; Chaturvedi et al., 1997; Voegele et al., 2005).

The hypothesis for a role for mannitol in host-pathogen interactions has come from direct and indirect observations. Mannitol was reported to accumulate in liver tissue and blood of rats infected with aspergillosis (Wong, et al., 1989), and mutants of *Cryptococcus neoformans* that produced less mannitol were found to be less virulent than wild type (Chaturvedi et al., 1996[a]). It was suggested that *C. neoformans* produced and secreted mannitol to protect itself against oxidative killing mechanisms of phagocytic cells (Chaturvedi et al., 1996[b]). Another study expressed a bacterial mannitol biosynthetic enzyme (mannitol 1-phosphate dehydrogenase) in a glycerol-deficient strain of *Saccharomyces cerevisiae* resulting in mannitol production. Mannitol-producing transformants and wild type *S. cerevisiae* were less sensitive to hydroxyl radicals (HO·) and other reactive oxygen intermediates than the glycerol-deficient mutants, suggesting that mannitol could substitute for glycerol to protect *S. cerevisiae* from oxidative damage (Chaturvedi et al., 1997).

Voegele et al., (2005) identified the mannitol biosynthetic enzyme mannitol dehydrogenase (MtDH) in the rust fungus *Uromyces fabae* and showed that it localized to the lumen of haustoria and in the lumen of uredospores. They suggested that mannitol was acting as a storage compound and was found in sufficient quantities to function as a scavenger of reactive oxygen species (ROS). Mannitol was found in intercellular fluids of tomato leaves infected with virulent races of *Cladosporium fulvum,* but not when leaves were inoculated with avirulent races (Joosten et al., 1990), suggesting that it was required for successful pathogenesis.

We have been studying the possible role of mannitol in disease caused by *Alternaria alternata,* a fungal pathogen of tobacco (*Nicotiana tabacum* L). In 1998, Jennings et al., discovered that tobacco, which does not synthesize mannitol, has an endogenous mannitol dehydrogenase (MTD; EC 1.1.1.255; 1-oxidoreductase which converts mannitol to mannose) that is induced by fungal colonization and other inducers of the plant defense responses including fungal elicitor, salicylic acid, and 2,6-dichloroisonicotinic acid. They concluded that upon infection, the fungus produces mannitol to quench ROS produced by plant defense responses, and the plant induced MTD to convert the fungal mannitol into mannose and thus allow for ROS-mediated plant defenses to be effective against the fungus. To test this hypothesis, our lab transformed tobacco to constitutively express a celery MTD gene, and inoculated the transgenic plants with *A. alternata* in a detached leaf assay (Jennings et al., 2002). The transgenic plants expressing the MTD enzyme had enhanced resistance, supporting the hypothesis that mannitol is required for successful infection of tobacco by *A. alternata*.

In *A. alternata*, mannitol was proposed to be produced through a mannitol cycle, initially identified by Hult and Gatenbeck in 1978 (Hult and Gatenbeck, 1978) from studies of cell-free extracts. In this cycle, fructose 6-phosphate is converted to mannitol 1-phosphate using the cofactor NADH by the enzyme mannitol 1-phosphate 5-dehydrogenase (MPDH; EC 1.1.1.17). The phosphate group is removed by a phosphatase (EC 3.1.3.22) presumed to be specific for mannitol 1-phosphate (Yamada et al., 1961; Ramstedt et al., 1986) producing inorganic phosphate and mannitol. Mannitol is then utilized by the enzyme mannitol dehydrogenase (MtDH; EC 1.1.1.138), a 2-oxidoreductase that catalyzes the conversion of mannitol to fructose using the cofactor NADPH. To complete the cycle, fructose would be phosphorylated to fructose 6-phosphate by a hexokinase (EC 2.7.1.1).

In previous work (Vélez et al, submitted), we confirmed the presence of both MtDH and MPDH in a tobacco isolate of *A. alternata*. A degenerate primer strategy was used to isolate the encoding genes, and gene disruption was used to generate mutants deficient in each enzyme and in both enzymes. GC-MS experiments showed that a mutant deficient in both enzymes produced no mannitol; however mutants deficient in MPDH or MtDH alone produced approximately 11% and 66%, respectively, of wild type levels, indicating that both enzymes were able to synthesize mannitol. Growth of the mutants on mannitol as a sole carbon source indicated as well that both enzymes can degrade mannitol and suggested that MPDH may have more of a degradative role than initially proposed. Our results confirmed much of the work reported by Hult and Gatenbeck (1978), but also documented the absence of a functional mannitol cycle in the fungus *A. alternata*, as mannitol could be made or degraded by both enzymes. Our work was the first study where both MTDH and MPDH were disrupted in a fungus to produce mannitol-deficient mutants.

The goal of this work was to confirm the conclusion of Jennings et al., (2002) that mannitol production is required for successful pathogenesis by *A. alternata.* Here we report results of infection studies of our mutants on tobacco. We show that mannitol is required for successful pathogenesis, but is not required for spore germination or initial infection. Further, we show that the biosynthetic genes are up-regulated in response to tobacco leaf extracts, documenting the importance of this pathway in plant pathogenesis.

Results

Mannitol minus strains

Mutants for MtDH (Mt-12), MPDH (Mp-14), and the double mutant (B-10) were created according to Vélez et al., (submitted). Briefly, a degenerate primer strategy was used to isolate the encoding genes, and disruption constructs were made for each gene using marker genes encoding hygromycin B (pGHyg for *MtDH* disruption) or phleomycin resistance (pGPhleo for *MPDH* disruption). Transformation experiments yielded disruption mutants for both genes. To create a double mutant, protoplasts were isolated from the *MPDH* disruption mutant (Mp-14) and were transformed with the pGHyg construct. PCR analysis was used to confirm disruption. Enzyme assays demonstrated a lack of enzymatic activity, while GC-MS experiments showed that B-10 (disrupted for both genes) did not produce mannitol. Mutants Mp-14 (disrupted for MPDH) and Mt-12 (disrupted for MtDH) produced approximately 11 % and 66%, respectively, of wild type levels (Vélez et al., submitted). Other than mannitol production and reduced ability to grow on mannitol as a sole carbon source, the mutants show no other phenotypic differences from wild type. They

are not affected in growth on a diversity of media, and grow and sporulate normally in culture.

Spore germination

Since previous reports have shown that mannitol is a storage carbohydrate involved in spore germination, a spore germination assay was conducted to verify that the spores of the mannitol deficient mutants were viable. Spores of the wild type, and Mt-12, Mp-14, and B-10 mutants were plated on glass slides coated with minimal medium. After 24 hours, the cultures were stained with lactophenol aniline blue. Spores were scored as germinated if the germ tube equaled or exceeded the length of the spore. Results are shown in Table 1 and Fig. 1. All mutants germinated as well or better than wild type, indicating that mannitol is not needed for spore germination in *A. alternata.*

Pathogenicity assays

Tobacco leaves were inoculated using a detached leaf assay as described by Spurr (1973). Squares (5 x 5 cm) were cut from leaves harvested from 12-week-old tobacco plants (cv. 'Burley 21') grown in the greenhouse. The squares were placed abaxial side up in a moist chamber and were inoculated with twenty 10 µL-drops from a spore suspension (1 x 10^5 spores mL^{-1}). After 7 days, lesions were scored from 0 to 4, with 0 = no visible symptoms, 1 = pin-needle size lesions, 2 = pin needle size lesions coalescing, 3 = coalesced necrotic lesion, and 4= large necrotic and chlorotic lesion typical of Brown Spot symptoms (Fig. 2A). All inoculations yielded some symptoms, and no inoculation points for any strain were scored as 0 (Fig. 2B). Infections caused by the wild type strain and the MtDH (Mt-12)

and MPDH (Mp-14) single mutants ranged from 1-4 whereas infections caused by the double mutant (B-10) ranged from 1-3. Overall, Mt-12 caused as much disease as did the wild type isolate. Mean lesion ratings for wild type and Mt-12 were 2.9 and 3.1, respectively. Also, 89 % and 94 % of the wild type and Mt-12 infections, respectively, were in the most severe categories of 2, 3 and 4. By contrast, the majority of the lesions caused by Mp-14 and B-10 were category 1, with few or no lesions scored as category 4. Overall, mean lesion severity ratings for B-10 and Mp-14 were 1.1 and 1.5, respectively. Only 36 % and 9 % of the Mp-14 and B-10 lesions, respectively, were rated in categories 2, 3 and 4. Statistical analysis showed a significant difference between wild type and Mp-14 ($p \leq 0.0001$), and between wild type and B-10 ($p \leq 0.0001$). There was no a significant difference between wild type and Mt-14 ($p \leq 0.1835$).

In planta sporulation

Our mutants are unaffected in sporulation in culture, however, Solomon et al., (2005) previously reported that the enzyme MPDH was necessary for sporulation of the fungus *Stagonospora nodorum* on wheat plants. They found that pycnidia were present in lesions caused by a wild type isolate, but not by a MPDH mutant of *S. nodorum*. We took advantage of the fact that *A. alternata* can sporulate in either side of the leaf surface to test whether or not knocking either *MtDH*, *MPDH* or both genes suppressed sporulation *in planta.* The abaxial side of detached leaf pieces were inoculated with six 10 µL-drops of a spore suspension ($3x10^5$ spores mL^{-1}) and incubated as described above for the pathogenicity assay. Lesion development was monitored, and spores were recovered from lesions on the adaxial side of the leaf using 10 µl drops of water. Seven days after inoculation, spores were

recovered from lesions caused by wild type, Mp-14, and Mt- 12 (data not shown). B-10, the double mutant, also sporulated *in planta*, but only after 5-7 weeks. Thus, mannitol production is not necessary for sporulation of *A. alternata* either in culture or *in planta*; however, there was a significant delay for *in planta* sporulation of the double mutant.

Histochemical detection of H_2O_2

Histochemical staining with 3,3'-diaminobenzidine tetrahydrochloride (DAB), which polymerizes and produces a brown color in the presence of H_2O_2, was used to detect a host defense response at the infection site. Stem cuttings, each containing two leaves, were allowed to take up a DAB solution for two hrs prior to excising the leaves and inoculating with 10 µL-drops of wild type and B-10 spore suspension (2×10^5 spores mL^{-1}). To visualize infection, leaf tissue surrounding the inoculation sites was cut out, cleared, and stained with lactophenol aniline blue to visualize hyphae. Results are shown in Fig. 3. Spores from both the wild type and B-10 were able to germinate on the surface of tobacco leaves, producing germ tubes from which appressoria developed. No differences were noted between the isolates in the frequency of spore germination or appressorium formation. In both cases, brown staining, indicative of the reaction of DAB with H_2O_2, was seen, indicating that both isolates penetrated the leaf and induced a defense response by the host. No differences were noted in the intensity or extent of DAB staining of leaf tissue inoculated with the two isolates.

Gene regulation

Jennings et al., (1998) observed that when *A. alternata* was grown in culture medium amended with tobacco leaf extracts, the fungus responded by making and excreting mannitol.

We used quantitative reverse transcription PCR (Q-PCR) to determine if *MtDH* and *MPDH* were regulated by tobacco leaf extracts. Results are shown in Figure 4. Transcript of both *MtDH* and *MPDH* started to increase by 30 min after the addition of tobacco extract, reaching 2.5 to almost 4-fold higher by 2 hr. Response of the genes in the disruption mutants was similar to that in wild type. No increase in transcript for *MtDH* and *MPDH* was seen if cultures were treated with water instead of tobacco leaf extract (data not shown).

Discussion

The polyol mannitol has been shown to quench ROS both *in vitro* and *in vivo* (Smirnoff and Cumbes, 1989; Chaturvedi et al., 1997; Voegele et al., 2005). Recently, a role for mannitol in antioxidant defense has also been supported by experiments with *S. cerevisiae* (Chaturvedi et al., 1997), *A. alternata* (Jennings et al., 1998; Jennings et al., 2002), and the rust fungus *U. fabae* (Voegele et al., 2005). Jennings et al., (1998) hypothesized that the fungus *A. alternata* secreted mannitol to quench ROS during infection of tobacco plants. In response, the plant would express a mannitol dehydrogenase that would convert the mannitol of fungal origin into mannose and allow the ROS-mediated plant defense response to be effective against the fungus. Transgenic tobacco plants expressing a MTD from celery were created and shown to have enhanced resistance to *A. alternata* (Jennings et al., 2002). The purpose of our present work was to create a strain of *A. alternata* unable to make mannitol and assay its pathogenicity on tobacco to further evaluate this hypothesis.

Our previous work (Vélez et al., submitted) confirmed the presence of the mannitol biosynthetic enzymes MtDH and MPDH in *A. alternata.* These genes were disrupted through targeted gene disruption. Mutants for each gene and a double mutant were

confirmed as lacking enzyme activity and were assayed for mannitol content by GC-MS. Mannitol production was reduced by almost 90% in the Mp-14 mutant (*MPDH* minus) and by almost 35% in the Mt-12 mutant (*MtDH* minus). No mannitol was produced by the double mutant (B-10). Our results confirmed the identity of the two genes, and documented the lack of additional copies or alternate genes encoding these enzymes (Vélez et al., submitted). This study provided the mannitol-deficient mutants used here to determine the role of mannitol in fungal pathogenicity of plants.

Mannitol has been suggested to play a role as a storage compound in fungi and to be important in spore germination. However, our spore germination assay verified that the spores of both the single mutants and the double mutant were viable and were able to germinate normally (Fig. 1, Table 1). Chromatograms from our mannitol analyses reveled that as mannitol levels decreased in the different mutants, the disaccharide content increased (Fig. 2). This result is consistent with Solomon et al. (2005), who saw an increase in trehalose when the *MPDH* gene was disrupted in *S. nodorum*, suggesting that other carbon sources are able to substitute for mannitol as a storage compound in the spores.

To evaluate the contribution of mannitol in plant-pathogen interactions, wild type *A. alternaria* and mutants Mt-12, Mp-14 and the double mutant B-10 were used to infect tobacco leaves. Severity of infection was strongly correlated with mannitol content. No significant differences were found in disease severity between the wild type isolate and the MtDH mutant, which is only moderately altered in mannitol production. By contrast, the MPDH mutant (which produces only 11% of wild type mannitol levels) and the mannitol-deficient B-10 double mutant were significantly reduced in disease severity (Fig. 3). These

results indicate that mannitol is required for normal disease development by *A. alternata* on tobacco.

Solomon et al., (2005) previously reported that the enzyme MPDH was necessary for sporulation of the fungus *S. nodorum* on wheat plants. In contrast to *S. nodorum*, our inoculation experiments showed that both Mp-14 (missing MPDH) and Mt-12 (missing MtDH) were able to sporulate *in planta*. With the double mutant, sporulation also was observed, but not until 5 weeks after inoculation. As this is a detached leaf assay, sporulation at 5 weeks likely represents saprophobic growth rather than infection. Thus we have no evidence that mannitol is required for sporulation by *A. alternata*, either in culture or *in planta*.

The precise function for mannitol in disease development is not clear. Our results showed that the B-10 double mutant was able to germinate, produce appressoria, and penetrate leaf tissue and induce a host response as effectively as the wild type (Fig. 4), thus mannitol must function after the initial penetration process. Our hypothesis was that mannitol is required to quench ROS produced during the host defense response. To test this hypothesis, we used DAB histochemical staining to determine if the reduction in pathogenicity was related to the mutants' inability to quench ROS. DAB has been used for *in vivo* and *in situ* localized accumulation of H_2O_2 during *Erysiphe graminis* f. sp. *hordei*-barley interaction, as well as the *Peronospora parasitica*-Arabidopsis interaction (Thordal-Christensen et al., 1997; Aviv et al., 2002). In the presence of H_2O_2, DAB polymerizes and produces a brown color. If mannitol is being produced by *A. alternata* to quench ROS, it would be expected that in the absence of mannitol, an increase the intensity of the DAB staining would be seen. No differences in the intensity of the staining were seen, however,

this method is not quantitative. Necrotrophic pathogens like *Botrytis cinerea*, *Sclerotinia sclerotiorum* and *Exserohilum turcicum* take advantage of the ROS from the plant to facilitate their infection (Govrin et al., 2000; Keissar et al., 2002). As *A. alternata* is also a necrotrophic pathogen (Lucas, 1975), it would seem counter-productive to quench ROS. Quenching of ROS remains a strong possibility for the requirement for mannitol in pathogenicity, however, definitive proof of this hypothesis will require further investigation.

Since mannitol is required for disease, we hypothesized that the biosynthetic genes would be regulated during infection. Jennings and collaborators saw an increase in the levels of mannitol when *A. alternata* was grown in malt medium amended with tobacco extract (Jennings et al., 1998). In agreement with their results, our Q-PCR results showed that both *MtDH* and *MPDH* transcript increased after exposure of the cultures to tobacco leaf extract (Fig. 5), suggesting that these genes are regulated by plant compounds and supporting the genes' importance in the pathogenicity of the fungus. In addition to regulation by tobacco leaf extracts, the genes and proteins are likely regulated in other ways. Both the *MPDH* and *MtDH* gene sequences contain putative CreA binding sites, 5'-SYGGRG-3' (Cubero and Scazzocchio 1994), suggesting regulation by carbohydrates. However, we conducted Q-PCR studies to evaluate the expression of the genes in response to different carbon sources, and they did not show significant effects (data not shown). In addition, both MPDH and MtDH protein sequences show putative sites for phosphorylation by cAMP-dependent protein kinase A, protein kinase C, and casein kinase I, suggesting regulation at the protein level.

In summary, we have shown that mannitol is required for normal disease development by *A. alternata* on tobacco. Although mannitol-deficient mutants germinate, form appressoria, and penetrate the leaf to induce a host response, normal disease

development and symptom expression does not occur. In support of these findings, we showed that both mannitol biosynthetic genes are up-regulated in response to tobacco extracts. In contrast to reports from other systems, mannitol in *A. alternata* is not required for conidiation or for spore germination. Histochemical staining did not show differences in host ROS the leaf, thus it remains unclear if the function of mannitol is to serve as a quencher of ROS or if it has another essential function in disease development. Based on these results, transformation of plants to express a MTD (EC 1.1.1.255) by genetic engineering may provide resistance to *A. alternata* and perhaps other mannitol secreting fungi.

Methods and Materials

Strains and media

All studies utilized *A. alternata* strain A5, isolated from *A. alternaria*-infected tobacco in Oxford, NC (provided by H. Spurr Jr, Oxford, NC) (Spurr Jr, 1973), and mannitol-deficient mutants Mt-12, Mp-14, and B-10 (Vélez et al., submitted). Richard's minimal medium (RM; 10g KNO_3, 5 g KH_2PO_4, 2.5 g $MgSO_4$, 20 g sucrose, 1 g yeast extract and 15% agar per L) was used for spore germination assays. Cultures were grown on complete medium (CM) for spore production. CM contains 10 g glucose, 2 g peptone, 1 g yeast extract, 1g casamino acids, 0.l% (v/v) trace elements (pH 6.5; 2.2 g $ZnSO_4$, 1.1 g H_3BO_3, 0.5 g $MnCl_2$-$4H_2O$, 0.5 g $FeSO_4$-7 H_2O, 0.17 g $CoCl_2$-6 H_2O, 0.16 g $CuSO_4$-5 H_2O, 0.15g Na_2MoO_4-2 H_2O, 5 g Na_4EDTA in 100mL water), 0.l% (v/v) vitamin supplement (0.01 g biotin, 0.01 g pyridoxine, 0.01 g thiamine, 0.01 g riboflavin, 0.01 g *p*-amino benzoic acid, 0.01 g nicotinic acid in 100mL water), and 5 % (v/v) nitrate salts (120 g $NaNO_3$, 10.4 g KCl, 30.4 g KH_2PO_4, 10.4 g $MgSO_4$-$7H_2O$ in 1 L water) and 15% agar per L)(Talbot et al., 1993). Malt extract medium (15 g malt extract, 3 g peptone, 30 g glucose per L) was used to grow the fungus for gene regulation experiments.

Mannitol-minus strains

Alternaria alternata MtDH- and MPDH-mutant strains (Mt-12 and Mp-14, respectively) were created according to Vélez et al., (submitted). Briefly, *MtDH* and *MPDH* were isolated using degenerate primers and inverted PCR. Both sequences were cloned into pGEM-T Easy (Promega, Madison, WI) using Subcloning Efficiency DH5α Competent Cells (Invitrogen, Carlsbad, CA). For *MtDH,* restriction enzymes were used to digest a 364bp

fragment within the ORF of the gene and replaced by a gene encoding hygromycin resistance (*hph*) flanked by a fungal promoter and terminator. For *MPDH*, restriction enzymes were used to digest a 1324bp fragment from the *MPDH* gene and replaced by the *ble* gene (encoding phleomycin resistance) flanked by a fungal promoter and terminator from the plasmid pBC-Phleo. Protoplasts of *A. alternata* were transformed, and gene disruption strains were confirmed by PCR and by lack of enzyme activity. To create the double gene knock out mutant B-10, protoplasts of the MPDH mutant (Mp-14) were transformed with the *MTDH* disruption construct. Quantifying mannitol by GC-MS found that wild type, Mt-12 and Mp-14 produced 110, 72.3, and 12.6 μg/mg dry weight, respectively. B-10 produced no detectable mannitol.

Spore preparation

Wild type *A. alternata* and mutants Mt-12, Mp-14, and B-10 were grown on CM at 25°C under fluorescent white light for 12 hrs followed by 12 hrs darkness for 7-10 days to induce sporulation. Sterile water was added to plates and the resulting spore suspensions collected. Spores were quantified using a hemocytometer and resuspended in sterile water.

Spore germination

Sterilized microscopes slides (Fisher Scientific, Pittsburgh, PA) were covered with approximately 2 mL of RM medium. Five 10 μL drops from a spore suspension ($1x10^5$ spores ml^{-1}) for each strain were placed on each slide. Three slides were prepared per strain, placed inside a petri plate, and placed in an incubator at 25°C. The next day, a few drops of lactophenol aniline blue stain (Remel Inc., Lenexa, KS) were place on top of each slide to

stain the fungal hyphae. Germinated spores were visualized using an Olympus BX60 microscope (Olympus America Inc., Melville, NY). Conidia were scored as germinated if the germ tube was equal to or longer than the length of the spore.

Pathogenicity assays

For pathogenicity assays, a detached leaf assay of Spurr (1973) was used with some modifications. Ten to 25 cm leaves were harvested from 12-week-old tobacco plants (cv 'Burley 21') grown in the greenhouse. Four 5 x 5 cm-squares were cut from each leaf and placed abaxial side up on a wire mesh, which was suspended above a paper towel impregnated with sterile ionized water in a clear polystyrene box (26.67 x 15.24 x 3.81 cm; Althor Products, Bethel, CT) with a hinged lid. Twenty 10µL-drops of spore suspension (1 x 10^5 spores mL^{-1}) of each strain (wild type, Mt-12, Mp-14, and B-10) were placed on each leaf section and allowed to air-dry. Incubation boxes were then closed and placed in a 21°C incubator with 8 hr day/16 hr night cycle. Symptom formation was monitored, and lesion severity was scored 7 days after inoculation. The numbers of successful infections were scored from 0 to 4, with 0 being uninfected and 4 being a severe necrotic and chlorotic lesion typical of Brown Spot (Fig. 2A). Each box contained 4 leaf pieces, one each inoculated with each strain. Results shown are totals from 17 incubation boxes from two experiments.

In planta sporulation

Two approximately 20 cm-long leaves were harvested from 12-week-old tobacco plants (cv 'Burley 21') grown in the greenhouse. Five 10µL-drops of a spore suspension ($3x10^5$ spores mL^{-1}) from wild type, Mt-12, Mp-14 and B-10 were placed on the abaxial side

of the leaf and allowed to air-dry. The inoculation sites on the leaf were labeled with each strain, and the leaf was placed inoculated side down in the plastic incubation chambers described above for the pathogenicity assays. After lesion development, 10 µL of sterile water was placed on top of the lesion on the adaxial side of the leaf, and then transferred to a microscope slide. The presence or absence of spores was determined using an Olympus BX60 microscope.

Histochemistry and microscopic analysis of infection

DAB (3,3'-Diaminobenzidine tetrahydrochloride; Sigma-Aldrich, St. Louis, MO) staining was carried out as previously described (Aviv et al., 2002) with some modifications. Two mg of DAB were dissolved in 200 µL of 1 M HCl, followed by the addition of 200 µL of sterile water and vortexing. The volume was brought up to 5 mL slowly with sterile water. A stem cutting containing two approximately 20 cm-long leaves was harvested from 12-week-old tobacco plants (cv 'Burley 21') grown in the greenhouse and placed in a small beaker containing the DAB solution. Transpiration of the DAB solution was allowed to occur for 1 hr on the bench top away from strong lighting. After 1 hr, the DAB solution was diluted with an additional 5mL of sterile water, and the leaves allowed to take up the solution for an additional hr. The leaves were cut from the stem and inoculated with five rows of three 10µL-drops of each wild type and B-10 spore suspension (2×10^5 spores mL^{-1}). Leaves were incubated in plastic inoculation chambers as described for the pathogenicity assays. Samples from each inoculation were taken daily for 3 days, cleared in a hot acetic acid:glycerol:ethanol (1:1:5, v/v/v) solution for five min, and then stored in 60% glycerol until examined using an Olympus BX60 microscope. A few drops of lactophenol aniline

blue stain (Remel Inc.) were place on top of the leaf surface to stain fungal hyphae. Pictures were taken with a SPOT RT color camera (Meyer Instruments, Inc., Houston, TX) attached to the Olympus BX60 microscope using the vendor software (SPOT RT v 3.5.6).

Gene regulation

Cultures of wild type, Mt-12, and Mp-14, were grown in 50 mL flasks containing liquid malt medium for three days with shaking at 200 rpm at 25°C. Two-milliliters of tobacco leaf extract prepared by the methods of Jennings et al., (1998) were added to each 50 mL flask. Samples were collected at 30, 45, 60, and 120 min. after the addition of tobacco extract, and the mycelium was filtered, frozen in liquid nitrogen and lyophilized. An untreated sample collected at time 0 was used as a control. RNA was extracted from the lyophilized tissue using TRI reagent (Sigma, St. Louis, MO) according to the manufacturer's instructions. RNA samples were resuspended in RNAsecure (Ambion, Austin, TX) and DNase treated twice with TURBO DNA-*free* (Ambion) for 1 hour at 37°C. The RNA concentration was determined with a Beckman DU Series 650 spectrophotometer (Beckman Instruments, Fullerton CA).

One µg of RNA from each sample was reverse transcribed using random hexamers and multiscribe reverse transcriptase as supplied in the TaqMan Reverse Transcription Reagents kit (Applied Biosystems) in a 50 µL reaction volume. Reverse transcription (RT)-PCR (25°C for 10 min, 48°C for 30 min, 95°C for 5 min) was done in a DNA Engine Opticon 2 Real-Time PCR Detection System (Bio-Rad Laboratories, Inc., Waltham, MA).

Quantitative PCR (Q-PCR) experiments were performed on wild type, Mt-12, and Mp-14 samples collected. Separate Q-PCR reactions were setup using 5 µL cDNA in a 25 µL total volume reaction containing 2X SYBR Green PCR Master Mix (Applied Biosystems,

Foster City, CA) and primers shown in Table 2 (0.9 pmol/μL final concentration) for *MtDH* and *MPDH*. All genes were normalized to the expression of 18S RNA. 18S RNA expression was done using the TaqMan Ribosomal RNA Control Reagents (Applied Biosystems). All Q-PCR reactions were done in triplicate using a DNA Engine Opticon 2 Real-Time PCR Detection System (95°C for 10 min followed by 40 cycles of 95°C for 15 s, 60°C for 1 min).

Statistical analysis

SAS 9.1.3 (SAS Institute Inc., Cary, NC, USA.) was used for the statistical analysis. Boxes were treated as randomized complete blocks, and the data were analyzed using the Cochran-Mantel-Haenszel test and with the PROC GLM procedure of the SAS computer program.

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Figure 1. Spore germination assay. Spores from *A. alternata* wild type (A), MtDH mutant (B), MPDH mutant (C), and the double mutant (D) were plated on a minimal salts medium coating the surface of a microscope slide. At 24 hours germinated spores were visualized using a microscope and counted as germinated if the germ tube equaled or exceeded the length of the spore. No differences were noted in germination rates of the mutants as compared to wild type.

Figure 2. GC-MS chromatogram of mannitol for wild type *A. alternaria* and mutants Mt-12, Mp-14, and B-10. Mutants MtDH-12 and MPDH-14 have less mannitol compared to wild type. No mannitol is seen for B-10. The less mannitol present, the greater the disaccharide sugar content as indicated by the arrows.

Figure 3. Tobacco pathogenicity assay. Detached tobacco leaf sections were inoculated with 10μL-drops of a 1 x 10^5 spores mL^{-1} conidial suspension of *A. alternata* wild type and the Mt-12, Mp-14, and B-10 mutants. A. Rating scale used to measure lesion severity. B. Total numbers of inoculation sites scored at each severity value 7 days after inoculation for each isolate. Statistical analysis indicated that disease severity caused by the wild type strain was significantly different from that of Mp-14 ($p \leq 0.0001$) and B-10 ($p \leq 0.0001$), but was not significantly different from Mt-14 ($p \leq 0.1835$).

Figure 4. Microscopic analysis of infection of tobacco leaf tissue by wild type *A. alternata* (A) and the B-10 double mutant (B). Leaf tissue pre-treated with 3,3'-Diaminobenzidine tetrahydrochloride (DAB) was inoculated with conidia and infection monitored for 24 hrs.

Tissue was stained with lactophenol aniline blue to visualize hyphae. Both the wild type (mannitol producing) and B-10 (mannitol non-producing) spores were able to germinate, produce appressoria (indicated by arrows), and penetrate and elicit a defense response (brown staining) from the host. Bar = 20 μm.

Figure 5. Regulation of *MtDH* and *MPDH* by tobacco leaf extracts. Transcript measured by quantitative RT-PCR and shown relative to expression prior to addition of leaf extracts, which is set at 1. Expression of both *MtDH* and *MPDH* increased in response to the tobacco leaf extract. Data from one of three independent experiments is shown: A) Expression of both genes in wild type B) Expression on MPDH in Mt-12 C) Expression of MtDH in Mp-14.

Table 1. Germination of conidia of *Alternaria alternata* wild type and mannitol mutants on minimal salts medium. Conidia were scored as germinated if the germ tube length equaled or exceeded the length of the spore.

Strain	**Number scored**	**Number germinated**	**%**
Wild Type	910	701	77
MtDH mutant (Mt-12)	928	863	93
MPDH mutant (Mp-14)	970	883	91
Double mutant (B-10)	1236	1112	90

Table 2. Primers used for Q-PCR

Gene	Forward (5' to 3')	Reverse (5' to 3')
MtDH	CGCCATACTTCTCGCTCATCT	CCTCGCCATCACCTACAACTCT
MPDH	ATGCGGTCGATAGCAGAGTTG	AGAGCAAGCTGAGCGAAGAGA

Figure 1

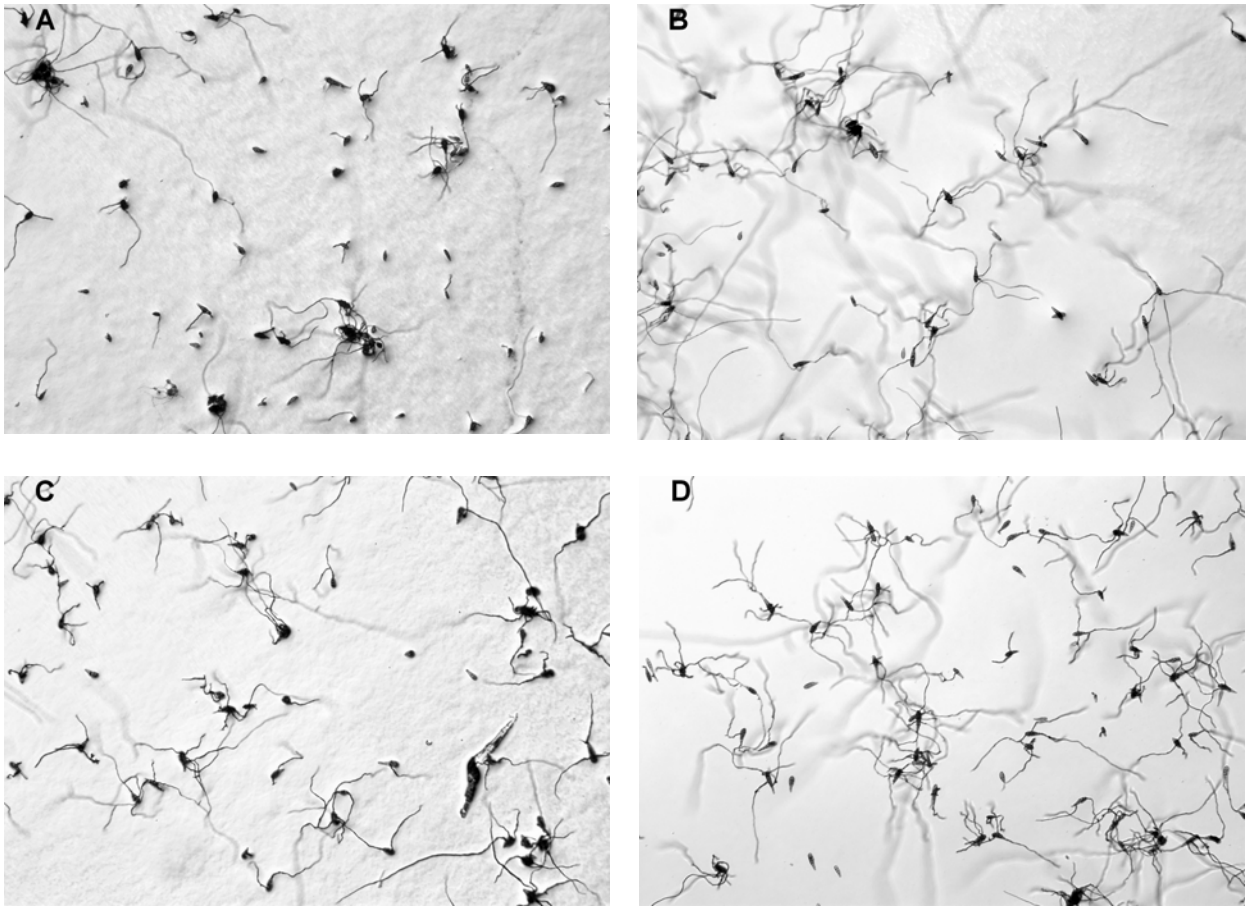


Figure 2

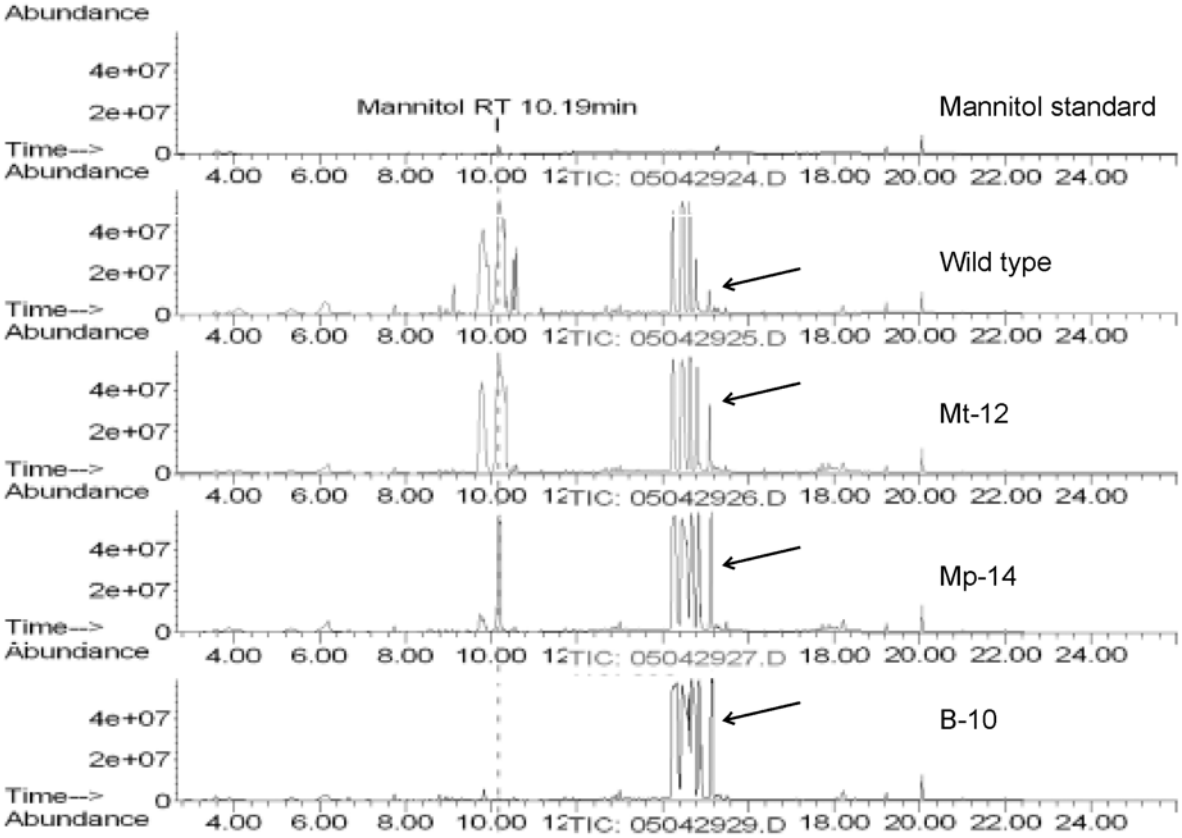

Abundance
Mannitol RT 10.19min
Mannitol standard
Wild type
Mt-12
Mp-14
B-10
Time-->
Abundance
4e+07
2e+07
0
4.00 6.00 8.00 10.00 12
TIC: 05042924.D
TIC: 05042925.D
TIC: 05042926.D
TIC: 05042927.D
TIC: 05042929.D
18.00 20.00 22.00 24.00

Figure 3

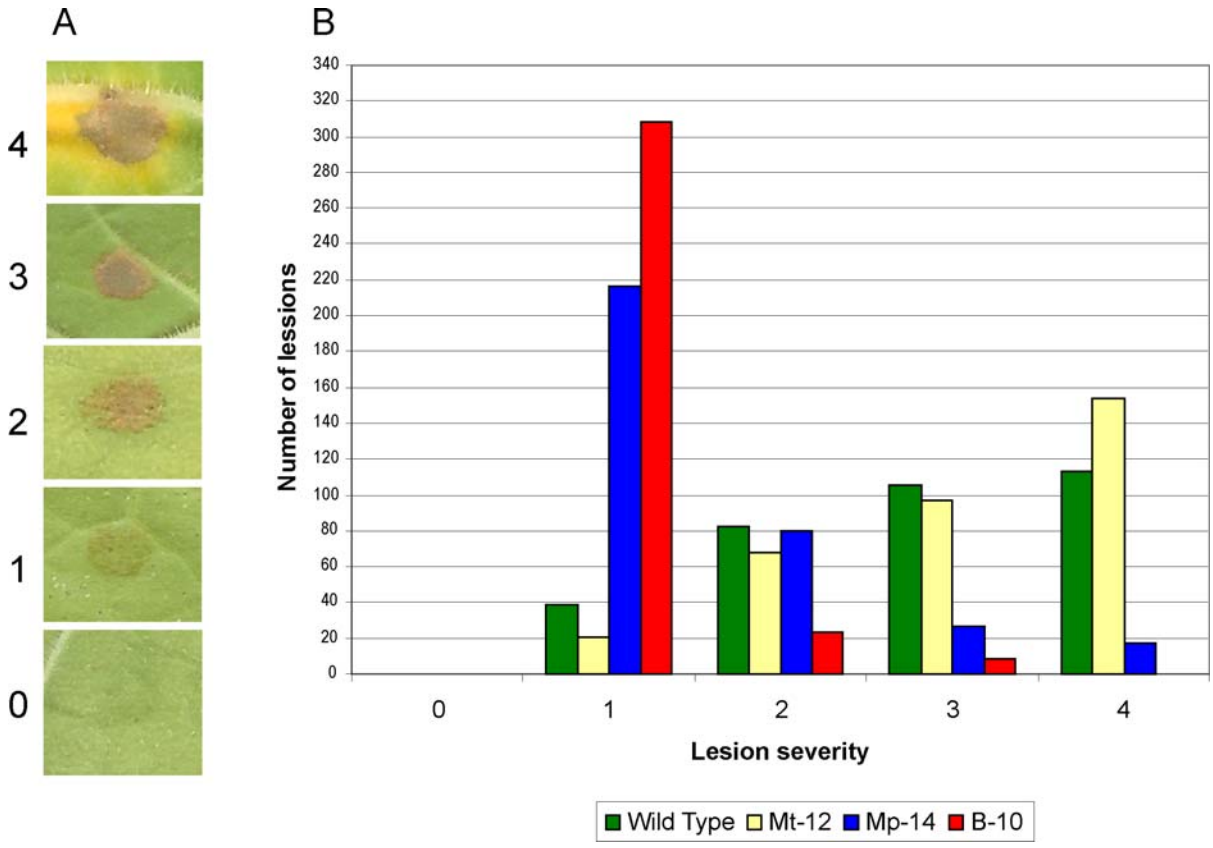

A
4
3
2
1
0
B
Number of lessions
340
320
300
280
260
240
220
200
180
160
140
120
100
80
60
40
20
0
0
1
2
3
4
Lesion severity
Wild Type
Mt-12
Mp-14
B-10

Figure 4

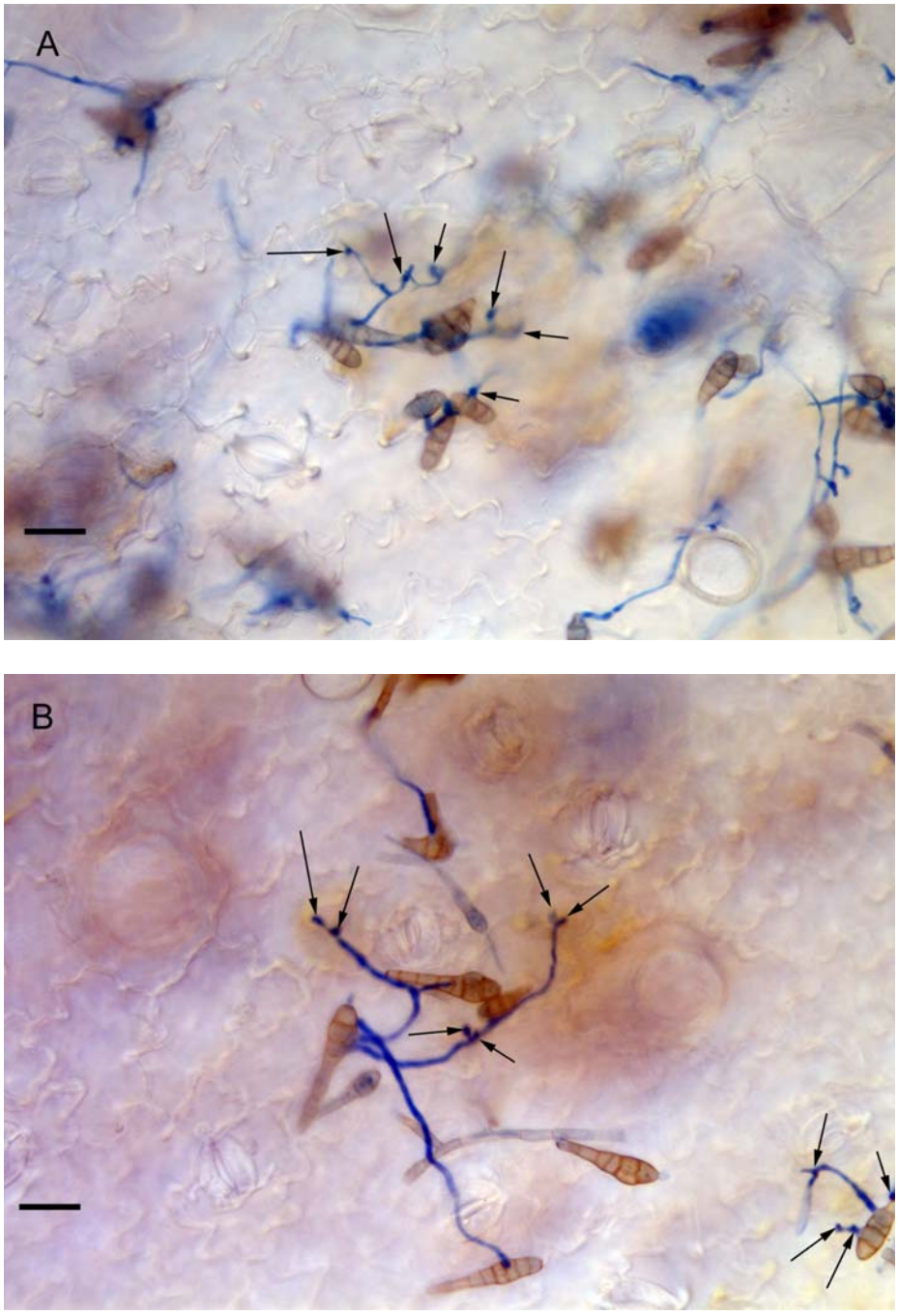
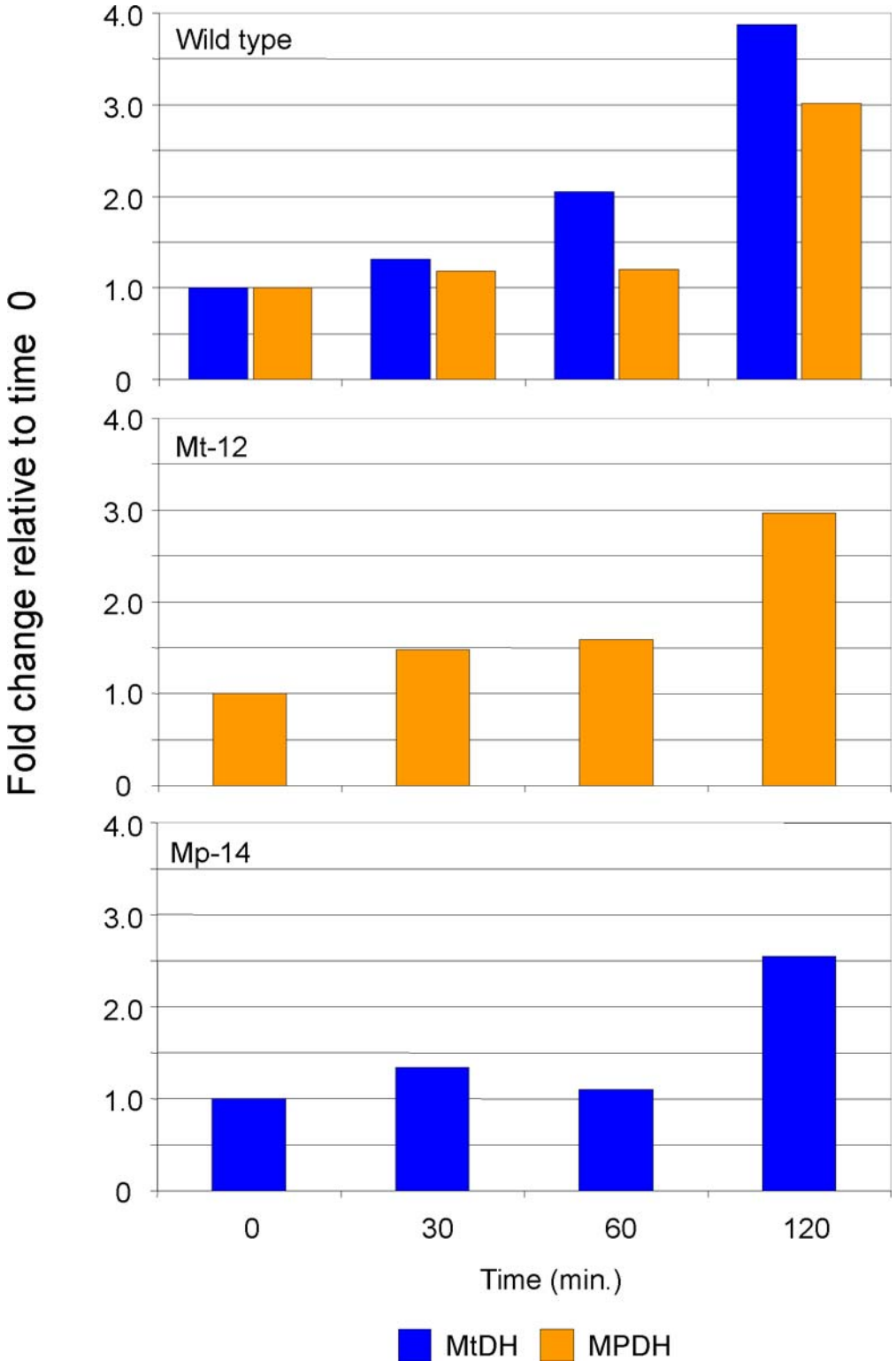


Figure 5


Wild type
Mt-12
Mp-14
4.0
3.0
2.0
1.0
0
Fold change relative to time 0
0
30
60
120
Time (min.)
MtDH
MPDH

APPENDIX 1: Transformation of *Alternaria* species

Objective

In order to study the possible acquisition and transmission of pathogenicity factors between members of the genus *Alternaria*, several species were transformed to express antibiotic resistance markers, hygromycin B and phleomycin.

Methods

Protoplast isolation

Transformation of wild type *Alternaria alternata* A5, *Alternaria mali* AT328, *Alternaria arborescens*, and *Alternaria brassicicola*, with the constructs carrying the hygromycin B and phleomycin markers was carried out as previously described (Akamatsu et al., 1997) with some modifications. For protoplast isolation, fungal mycelial plugs harvested from a V-8 culture plate were inoculated into 50 mL GYB medium and grown in the dark for two days with shaking at 200 rpm at 25°C. The mycelia were harvested by centrifugation at 2706 × g and washed three times with a protoplast salt solution (0.7 M KCl, 0.2 M CaCl2). The mycelium mass was incubated in 20 mL of 10 mg/mL kitalase (Wako Chemicals USA Inc., Richmond, VA) dissolved in the protoplast salt solution for 3-4 hours at 25°C with constant shaking at 80 rpm. The digested protoplasts were filtered through cheesecloth and a 60 micron mesh-screen (TWP Inc. Berkeley, CA) and spun at 1730 × g for 10 min at 4°C. The supernatant was decanted and the protoplasts were washed with 10 mL of protoplast salt solution followed by centrifugation at 1730 × g for 10 min at 4°C. This last step was

repeated twice. The protoplasts were washed with 10 mL of STC buffer (1 M sorbitol, 50 mM Tris pH 8.0, 50 mM CaCl) and spun at 1730 × g for 10 min at 4°C. The supernatant was decanted and the protoplasts were suspended in fresh STC buffer at 10^8 protoplasts per mL.

Hygromycin and phleomycin markers

PCR with primers 5'-CGGCGTAGAGGATCCTCT-3' and 5'-GGGATCCTCTAGAGTCGACA-3' were used to amplify the hygromycin marker form plasmid pUCATPH (Lu et al., 1994), which contained the gene encoding hygromycin resistance (*hph*) flanked by a fungal promoter (*A. nidulans trpC* promoter) and terminator (*A. nidulans trpC* terminator). In the same manner, PCR with primers 5'-AATTCCCTTGTATCTCTACACA-3' and 5'-ACATCGAACTGGATCTCAA-3' were used to amplify the phleomycin markers from the plasmid pBC-Phleo (McCluskey 2003), which contained the gene encoding phleomycin flanked by a fungal promoter (*A nidulans gpdA* promoter) and terminator (*S. cerevisiae* CYC1 terminator).

Fungal transformation

For transformation, 80 µL of protoplasts were gently mixed with 10-20 µL of either hygromycin or phleomycin PCR mix The protoplasts were incubated with the PCR mix on ice for 30 min, followed by the addition of 500 µL of 40 % PEG 4000 (in STC) and a further 30 min incubation at room temperature. Five mL of liquid RM (0.5 M sucrose, 0.1 % yeast extract, 0.1 % casein amino acids, and 0.1 % mannitol)was added to the transformation mix, and the protoplasts were allowed to regenerate in the dark overnight at 28°C. The

regenerated protoplasts were plated on solid RM with either 150 µg/mL hygromycin or 10 µg/mL phleomycin. Transformants that appeared after 2-3 days on selective media were transferred onto solid V-8 medium containing 250 µg/mL hygromycin or 20 µg/mL phleomycin.

Results

Transformants for *A. mali* (phleo), *A. arborescens* (Hyg and Phleo), *A. alternata* (Hyg and Phleo) and *A. brassicicola* (Phleo) were obtained that could grow on either hygromycin or phleomycin. These transformants were given to Dr. Kelly Craven (The Center for Integrated Fungal Research, NC State University, Raleigh, NC) to further study the possible acquisition and transmission of pathogenicity factors by pairing different isolates expressing different makers.

References

Akamatsu, H., Y. Itoh, M. Kodama, H. Otani, and K. Kohmoto. 1997. AAL-toxin-deficient mutants of *Alternaria alternata* tomato pathotype by restriction enzyme-mediated integration. Phytopathology. 87:967-972.

Lu, S., L. Lyngholm, G. Yang, C. Bronson, O. C. Yoder, and B. G. Turgeon. 1994. Tagged mutations at the Tox1 locus of *Cochliobolus heterostrophus* by restriction enzyme-mediated integration. Proc. Natl. Acad. Sci. USA 91:12649-12653.

McCluskey, K. 2003. The Fungal Genetics Stock Center: from molds to molecules. Adv. Appl. Microbiol. 52:245-262.