1-1-2004

Physiology And Biochemistry Of Sclerotinia Sclerotiorum, A Fungal Plant Pathogen

Erastus Gatebe
Eastern Illinois University

THESIS REPRODUCTION CERTIFICATE

TO: Graduate Degree Candidates (who have written formal theses)

SUBJECT: Permission to Reproduce Theses

The University Library is receiving a number of request from other institutions asking permission to reproduce dissertations for inclusion in their library holdings. Although no copyright laws are involved, we feel that professional courtesy demands that permission be obtained from the author before we allow these to be copied.

PLEASE SIGN ONE OF THE FOLLOWING STATEMENTS:

Booth Library of Eastern Illinois University has my permission to lend my thesis to a reputable college or university for the purpose of copying it for inclusion in that institution's library or research holdings.

12/16/04

Author's Signature Date

I respectfully request Booth Library of Eastern Illinois University **NOT** allow my thesis to be reproduced because:

_____

_____

_____

_____ _____

Author's Signature Date

Physiology and Biochemsitry of Sclerotinia sclerotiorum, a Fungal Plant Pathogen

(TITLE)

BY

Erastus Gatebe

THESIS

SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF

Master of Science

IN THE GRADUATE SCHOOL, EASTERN ILLINOIS UNIVERSITY
CHARLESTON, ILLINOIS

2004

YEAR

I HEREBY RECOMMEND THAT THIS THESIS BE ACCEPTED AS FULFILLING
THIS PART OF THE GRADUATE DEGREE CITED ABOVE

12.9.04
DATE

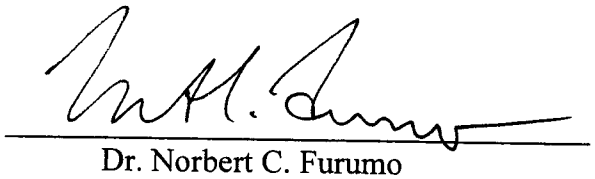
THESIS DIRECTOR

12-13-04
DATE

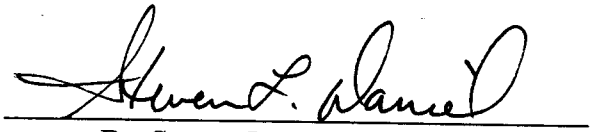
DEPARTMENT/SCHOOL HEAD

Physiology and Biochemistry of *Sclerotinia sclerotiorum*, a Fungal Plant Pathogen

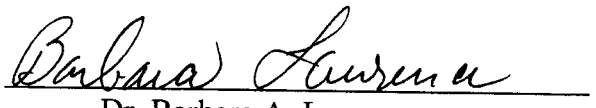
Thesis approved by committee members:



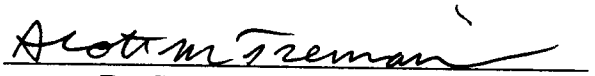
Dr. Norbert C. Furumo — 12.9.04 Date



Dr. Steven L. Daniel — 12/9/04 Date



Dr. Barbara A. Lawrence — 12/9/04 Date



Dr. Scott M. Tremain — 12/9/04 Date

ABSTRACT

This study evaluates the physiology and biochemistry of *Sclerotinia sclerotiorum*. In four isolates, we investigated growth conditions on biomass production, oxalate biosynthesis and protein expression. Oxalate biosynthesis is affected by growth conditions. The production of biomass depends on the isolate and growth conditions investigated. Isolate DE-7 had the highest biomass production in all the eleven conditions compared to other isolates. The choice of method of extraction affects the concentration of protein isolated from the fungus. The study reports the significance of glucose, yeast, soytone and trypton supplements and other co-substrates such as acetate, malate and succinate in oxalate biosynthesis. When glucose concentration was high or low in growth media, oxalate biosynthesis was found to vary proportionally. Protein expression, determined by SDS-PAGE was dependent on isolate investigated as different isolates showed different protein expression. Oxaloacetate acetylhydrolyse (OAH), glyoxylate dehydrogenase (GDH) and malate dehydrogenase (MDH) enzymes activities were determined. OAH and GDH are involved in oxalate biosynthesis while MDH provides oxaloacetate, a precursor of oxalate. This is the first study to report significance of both OAH and GDH in *S. sclerotiorum*. The high MDH specific enzyme activity suggests an anaplerotic role of the enzyme in oxalate biosynthesis.

APPRECIATION

I would like to thank Dr. Norbert Furumo for his guidance, help and patience throughout the project. Also thanks to Dr. Steven Daniel for his guidance on the project and to Dr. Barbara Lawrence and Dr. Scott Tremain for being on the committee. I would also like to thank Bryan Culbertson who was also involved in the project and contributed immensely and Jason DeGroate who joined our research later. Special acknowledgements go to my wife Eunice and son Dennis, my departed parents and the entire Chemistry faculty for their support.

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

APS.....Ammonium persulfate
DTT..... Dithiothreitol
EDTA.....Ethylenediaminetetraaceticacid
GTP.....Guanosine triphosphate
GDH..... Glyoxylate dehydrogenase
HRGPS..... Hydroxyproline-rich glycoprotein genes
ICL..... Isocitrate lyase
KPi.....Potassium phosphate buffer
MDH..... Malate dehydrogenase
MOPS.....4-Morpholinosulphonic acid
NAD^+..... Nicotinamide adenine dinucleotide
OAA.....Oxaloacetate
OAH.....Oxaloacetate acetylhydrolase
PCNB..... Pentachloronitrobenzene
PDA..... Potato dextrose agar
PMSF..... Phenylmethanesulfonylfluoride
SDS..... Sodiumdodecyl sulfate
SDS-PAGE.....Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SSR..... Sclerotinia stem rot
TEMED.....N, N, N', N'-tetramethylethylenediamine

LIST OF FIGURES

LIST OF TABLES

Chapter I

INTRODUCTION

Sclerotinia stem rot (SSR) in soybean is caused by the fungus *Sclerotinia sclerotiorum* (*S. sclerotiorum*). This is considered one of the most destructive diseases affecting this crop. While this pathogen has been implicated in plant disease for over 40 years, it has only become a problem for soybean farmers since the early 1990s with outbreaks becoming more common and widespread.[1] Worldwide, *S. sclerotiorum* reduces soybean yields by more than 1 million metric tons a year. In the United States, SSR reduces soybean yields by nearly half a million metric tons a year, costing farmers ~100 million dollars.[1-3] In a survey conducted by Wrather and co-workers, the disease was ranked second after soybean *cyst* nematode as a leading cause of soybean loss in the North-Central U.S. and Argentina.[1,4]

Sclerotinia is a ubiquitous phytopathogenic Ascomycetes fungus that infects a wide variety of plants including several crop, vegetable, ornamental, fruit, and weed species. Earlier classification of the fungi by Purdy[5] found that the fungus *S. sclerotiorum* is among the most nonspecific and successful of plant pathogens. Plants susceptible to this pathogen encompass 64 families, 225 genera, and 361 species and affects more than 400 plants.[5-6]

During its life cycle, **Figure 1,** *S. sclerotiorum* produces many black fleshy structures called sclerotia, which allows the fungus to survive from one cropping season to the next. The fungus survives primarily as sclerotia on or in the soil, or as mycelium, in infected plant residues. The fungal mycelium has been found to grow inside and outside of the stem and the infected plants die in late growth stages. The sclerotia have been

found to survive, when buried in the soil, for up to seven years. Sclerotia are highly resistant to chemicals, dry heat up to 158 °F, and prolonged freezing and thawing.[6] During wet, cool weather, sclerotia on or just beneath the soil surface germinate by means of tiny, mushroom-like structures (apothecia). Microscopic spores (ascospores) are produced in the apothecia, and are forcibly ejected from the apothecia during wet conditions. Prolonged periods of low soil temperatures (41-59 °F) and high soil moisture have been reported to favor conditions for apothecial development. The most favorable conditions for ascospore production were found to be 54-59 °F and wet soils.[6] However, seed dissemination is not a factor in the epidemiology of white mold.

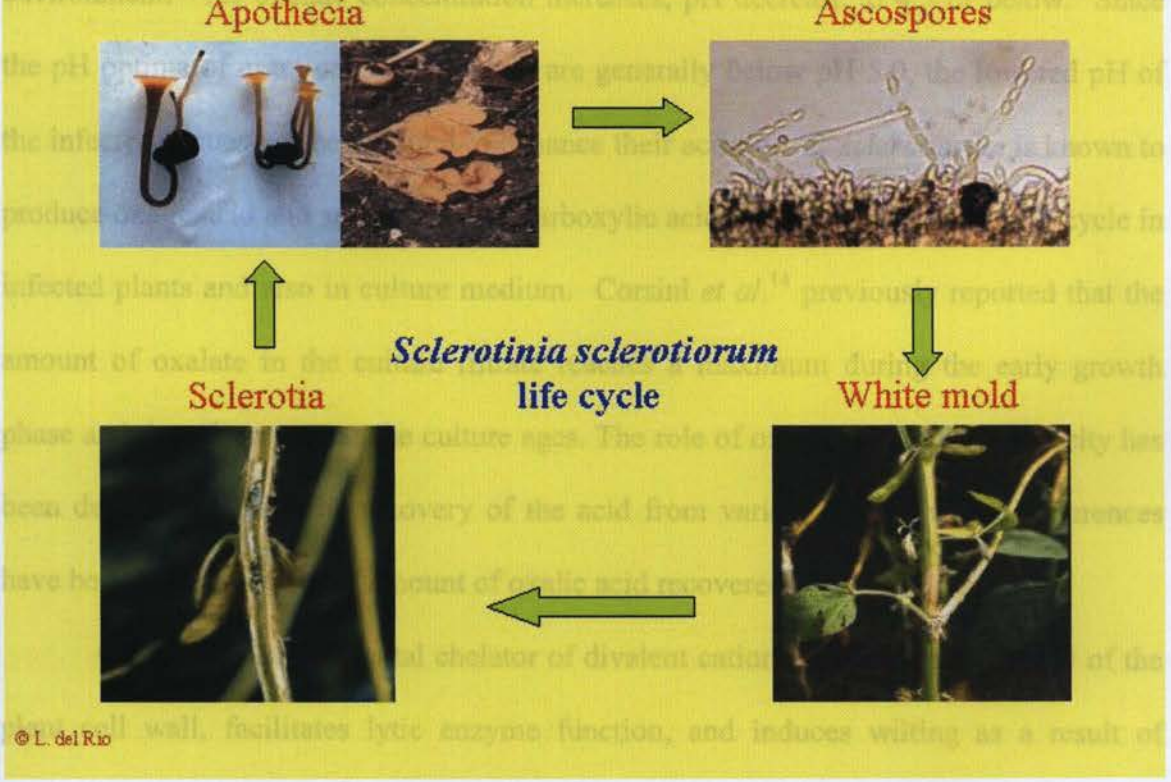


Figure 1: The Life cycle of *Sclerotinia sclerotiorum*.[7]

Oxalic acid plays an important and essential role during the pathogenesis of the host plant by *S. sclerotiorum.* Studies in other laboratories have found that the ability of this fungus to infect soybeans and other crops appears to hinge on its ability to produce oxalic acid. The fungus produces relatively high levels of oxalate, as recovered from diseased tissues.[8-12,39] Oxalic acid is a colorless, crystalline organic acid that melts at 189 °C with sublimation. Oxalic acid and its oxalate salts are poisonous. Oxalic acid is found in many plants, usually as the calcium or potassium salt. Early in pathogenesis, oxalic acid accumulates in infected tissues and increases in concentration as the pathogen colonizes the soybean. The factors affecting oxalate production have been primarily found to be the carbon and nitrogen source in the culture media and the pH of the environment.[13] As oxalate concentration increases, pH decrease to 4.5 or below. Since the pH optima of extra-cellular enzymes are generally below pH 5.0, the lowered pH of the infected tissues has been found to enhance their activity. *S. sclerotiorum* is known to produce oxalic acid and several other dicarboxylic acids of the tricarboxylic acid cycle in infected plants and also in culture medium. Corsini *et al.*[14] previously reported that the amount of oxalate in the culture filtrate reaches a maximum during the early growth phase and then decreases as the culture ages. The role of oxalic acid in pathogenicity has been determined from the recovery of the acid from various strains whose differences have been found only in the amount of oxalic acid recovered.[8,40]

Oxalate is a strong metal chelator of divalent cations, disrupts the integrity of the plant cell wall, facilitates lytic enzyme function, and induces wilting as a result of acidifying the host tissue.[9] Chelation of Ca^{2+} in the cell-wall has also been proposed to compromise the function of Ca^{2+}-dependent defense responses and to weaken the plant

cell wall.[9] However, Mouley and co-workers have also shown that chelation of Ca^{2+} by oxalic acid is unimportant while determining the pathogenicity of *S. sclerotiorum*.[10] Although exposure to increasingly alkaline pH increases the oxalic acid accumulation independent of the carbon source, sclerotial development is reported to be favored by acidic pH conditions and inhibited at neutral pH.[9-10,15] The inhibition of sclerotial development is because several fungal enzymes secreted during invasion such as polygalacturonase have been found to be most active at low pH.[16] These studies suggest that carbon sources may also regulate the growth and oxalate synthesis by *S. sclerotiorum* and that nutritional factors may impact the virulence of this fungal pathogen.[16] Goody and co-workers had earlier reported that prototrophic mutants of *S. sclerotiorum* do not produce oxalate in carbohydrate rich media or in natural substrate like bean blossoms.[9] However, mutants and revertants of wild type *S. sclerotiorum* grown on nutrient media containing sodium succinate supports oxalate production, confirming that oxalate is the pathogenicity determinant.[9-12,15] The role of oxalic acid has been further implicated in pathogenicity by reproducing and correlating various disease symptoms after injecting the acid or extra-cellular filtrates containing the acid into plants. The subsequent recovery of millimolar concentration of oxalate suggests this, although the data is not conclusive since different isolates accumulate different amounts of oxalate *in vitro* and *in vivo* where degradative enzymes are also found.[8-9,16]

The infected plant usually mounts a resistance response against the pathogen through oxidative burst. This is required for all plants species to develop germplasm resistant against the pathogen. Various studies have demonstrated that oxalate suppresses the oxidative burst through inhibiting the activated free radical-generating oxidase

directly or by blocking a signaling step leading to activation of oxidase.[10] Biochemical data available indicate that oxidase activation involves tightly regulated signal transduction pathways including the recognition of pathogen or plant derived elicitor molecules by plasma membrane or cytosolic receptors and the activation of several signaling enzymes including GTP binding proteins, phospholipases and protein kinases.[16] Cessna *et al.* have recently demonstrated that this fungus suppresses the oxidative burst of the host plant through oxalate generated by the virulent strain and the subsequent inhibition of the signaling step positioned upstream of oxidase activation. Thus the revelation of the role of oxalate in suppression of active oxygen generation compromising the defense mechanisms of the host plant has revealed an insight into the mechanism of attack and a new line of investigation.

Since effective pathogenicity by *S. sclerotiorum* requires the secretion of oxalic acid, understanding its biosynthesis is important in determining the enzymes involved in pathogenesis. Recent research focuses on identifying the mechanisms by which oxalate aids in colonization of plant tissues by the fungus and then degrade the cell wall leading to etiolation of the host plant.[8,11,16-19] The reaction of soybean cultivars to *S. sclerotiorum* has been recently described based on field and green-house evaluations. Disease incidences and severity indices have been used to evaluate the reaction of soybean cultivars to *S. sclerotiorum* in the field. Mechanisms of resistance to the pathogen in soybean germplasm are not known. However, factors such as plant architecture, lodging characteristics and relative maturity have been found to influence disease incidence and severity.[10]

Although there is little information on oxalate biosynthesis in *S. sclerotiorum*, several biochemical pathways have been proposed for the potential route by which the pathogen infects the host plant. This has led several workers in our group to investigate proposed mechanisms through the glyoxylate and tri-carboxylic acid cycles, as shown in **Figure 2.**

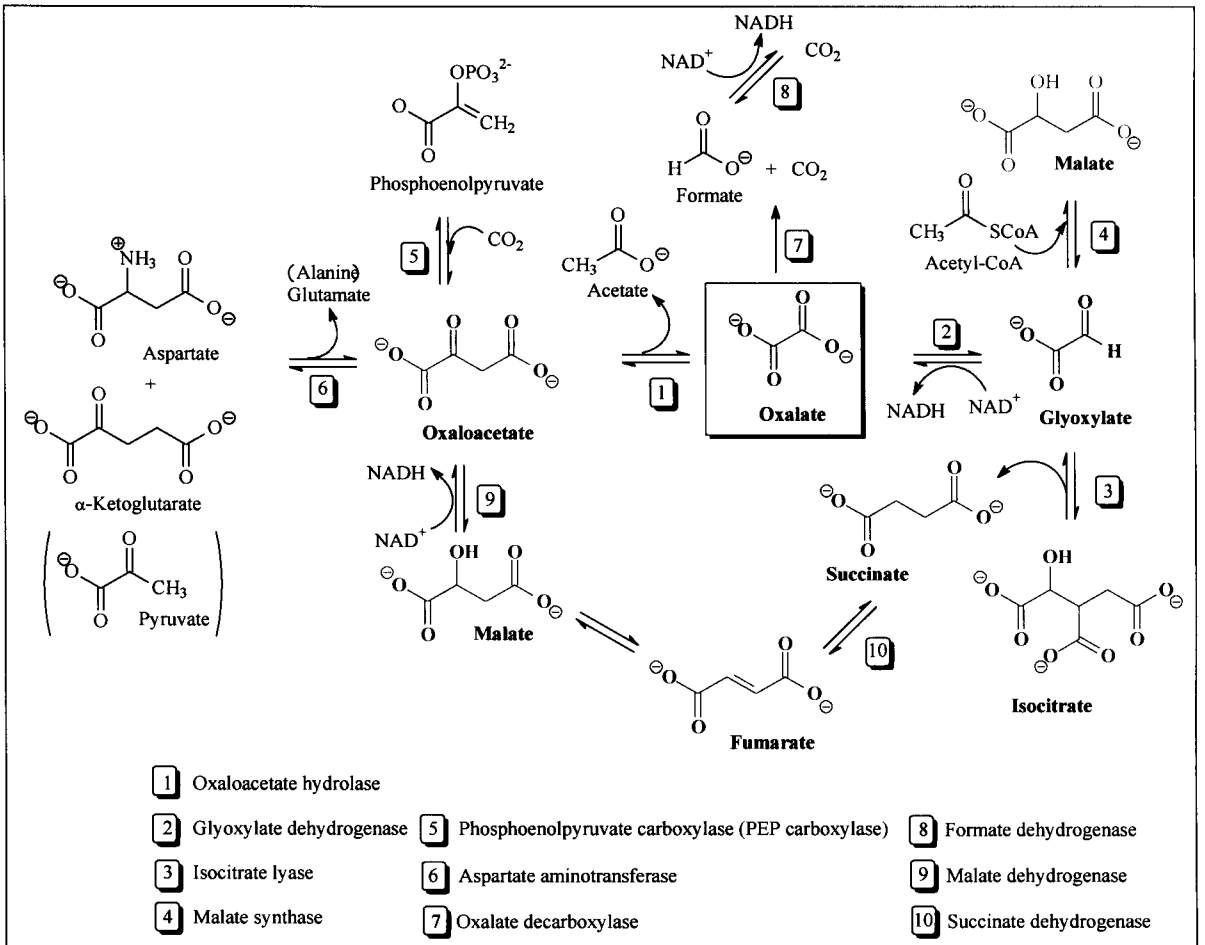


Figure 2: Proposed metabolic pathways of oxalate synthesis

The presence of malate, fumarate and succinate in culture media and from infected plants suggests an operative tricarboxylic cycle as reported for a number of fungi.[14] It is suggested that oxalate biosynthesis in *S. sclerotirum* is a complex

biochemical processes and not a simple TCA cycle, which suggests the reason why it is not understood. It is also suggested that only the glyoxylate cycle, **Figure 3,**[20] can explain the variance in concentration and the presence of high oxalate concentration.[12,14,20]

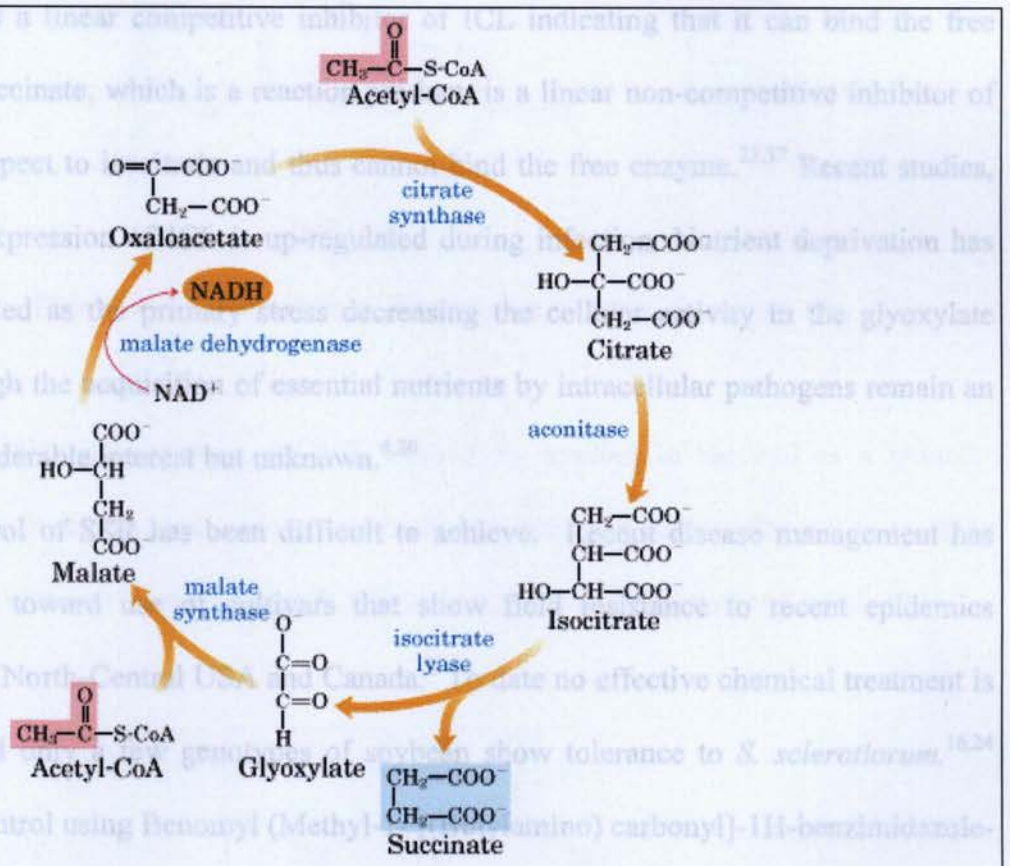


Figure 3: The glyoxylate cycle.[20]

The glyoxylate cycle is an anaplerotic metabolic pathway that allows the biogenesis of carbohydrates from C2-units like acetate or glyoxylate.[20-23] In plants and fungi, the glyoxylate cycle is found in organelles called glyoxysomes. The key enzymes of this cycle are isocitrate lyase (ICL) and malate synthase which catalyze the formation

of L-malate from glyoxylate and acetyl-CoA by an aldol condensation. This reaction is substrate specific and dependent on Mg^{2+}.[21-22,42] Although up-regulated, ICL cannot play any significant role in *S. sclerotiorum* pathogenicity since the organic acid that accumulates in glucose-salts medium opposes the scheme proposed for *Sclerotinia rolfsii* where glyoxylate formed from isocitrate is oxidized to oxalate.[23] Malonate, an analog of glyoxylate is a linear competitive inhibitor of ICL indicating that it can bind the free enzyme. Succinate, which is a reaction product, is a linear non-competitive inhibitor of ICL with respect to isocitrate and thus cannot bind the free enzyme.[23,37] Recent studies, shows the expression of ICL is up-regulated during infection. Nutrient deprivation has been suggested as the primary stress decreasing the cellular activity in the glyoxylate cycle although the acquisition of essential nutrients by intracellular pathogens remain an area of considerable interest but unknown.[4,20]

Control of SSR has been difficult to achieve. Recent disease management has been geared toward use of cultivars that show field resistance to recent epidemics especially in North-Central USA and Canada.[2] To date no effective chemical treatment is available and only a few genotypes of soybean show tolerance to *S. sclerotiorum.*[16,24] Chemical control using Benomyl (Methyl-1- [(Butylamino) carbonyl]-1H-benzimidazole-2-ylcarbamate), **Figure 4**, has been reported to be somewhat effective in areas of dry land such as Michigan. However, in irrigated regions, effectiveness is sporadic.[9] The reason for this lack of control is not known but may be related to heavy vine growth of the available indeterminate cultivars, which occurs after application of the fungicide. Benomyl uptake studies have shown that the fungicide cannot be detected in the plant by

penicillium inhibition bioassays more than 2 weeks after the second spray application making its potency suspect and inconsequential.[9]

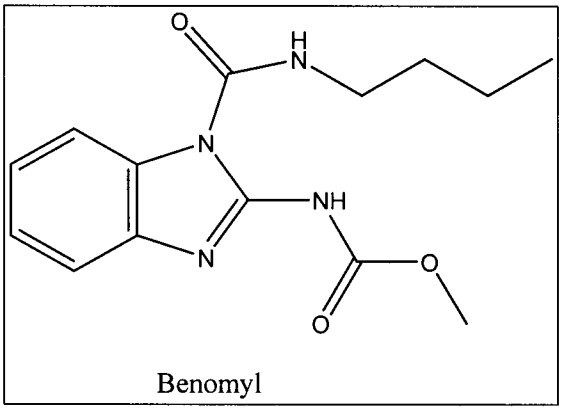


Figure 4: The structure of Benomyl: a systemic fungicide

Pentachloronitrobenzene (PCNB, **Figure 5),** applied to the soil as a granular treatment has been found to reduce apothecia but does not reduce disease or increase yields.[9] Thus chemical control using PCNB is described as ineffective and costly.[25]

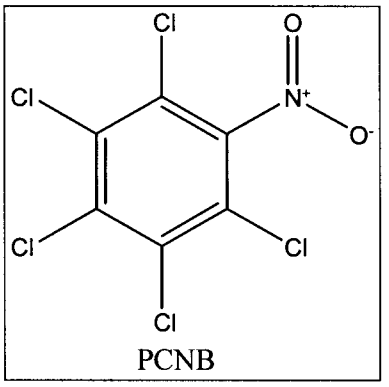
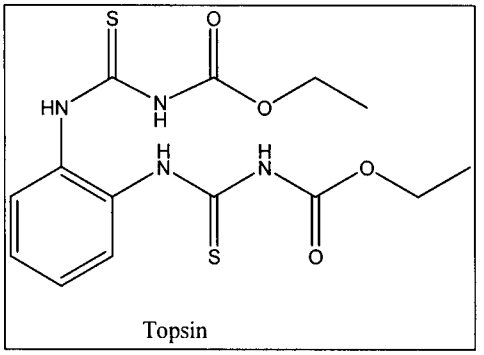


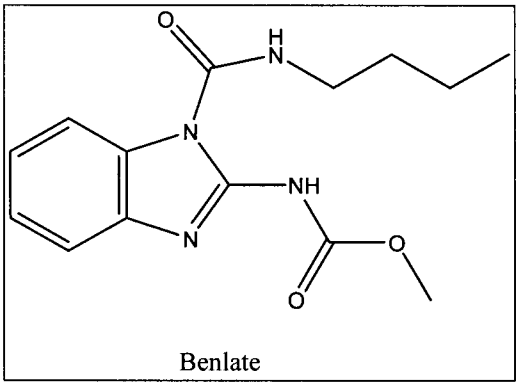
Figure 5: The structure of Pentachloronibenzene (PCNB).

Topsin, **Figure 6,** and Benlate, **Figure 7,** are fungicides that, despite their effective controls of SSR in dry beans and canola have not proven to be economically viable for white mold control in soybeans. Two explanations have been reported for their limited effectiveness in field trials. First, when the fungicides are applied using standard application rates and herbicide application equipment, coverage of possible infection points, namely, the flower petal is poor. Second, flowers that develop after the fungicide application are not protected. In essence, there has not been any significant benefit either in reduced white mold incidence or increased yield from a single R1 (flowering stage) application of Topsin or Benlate making their use in chemical control inefffective.[8]



Topsin

Figure 6: The structure of Topsin.



Benlate

Figure 7: The structure of Benlate.

Use of the herbicide Cobra (lactofen), **Figure 8,** for white mold control has received considerable attention. Cobra application at the beginning of the flowering stage (R1) reduces white mold severity and increases yields. However, the effect of Cobra is dependent both on soybean cultivar and on white mold severity. In addition, Cobra application has been found to decrease yields if white mold does not occur. In a single experiment carried out in Minnesota, Cobra application was found to have no significant effect on either yield or white mold incidence.[26]

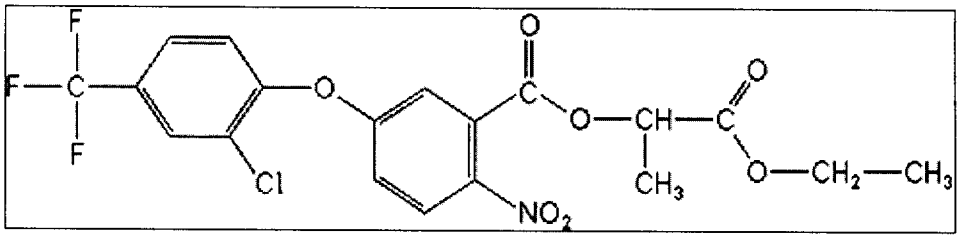


Figure 8: The structure of Cobra (lactofen)

Genetic modification of the cultivars of various plant species affected by the disease in order to improve disease resistance has not been very successful due to the fact that the enzymes involved in the biochemical pathways are not completely understood. At molecular and biochemical levels, most studies concerning the fungus have been geared towards pathogenicity of the fungus where oxalic acid has been identified as the major determinant.[44-46]

Genetic modification of hydrolytic enzymes such as polygalacturonase, pectin and methyl esterase produced by *S. sclerotiorum* has been attempted relative to SSR control.[25,27] These enzymes have been attributed to plant cell wall degradation.[9,11-12,41,43] Based on work by Margo[16] and co-workers showing oxalic acid has elicitor-like activity,

expression of a class of defense-related genes was described on phytoalexin production in soybean by Mouley[25] and co-workers and successfully incorporated transgenic hydroxy proline-rich glycoprotein gene (HRGPS) to sunflower, another *S. sclerotiorum* host. This however, did not improve defense related genes of HRGPS involved in plant cell wall resistance to *S. sclerotiorum* in soybean. Their results showed that the process did not have any significant effects on output and production of soybean although some resistance was improved about 35 %.[25,27] Since natural crop resistance to this disease is inadequate, attempts at breeding resistance genotype cultivars has resulted in poor transfer of results from the laboratory to the field, and resistance arises from disease avoidance rather than physiological resistance. Transgenic mapping is now being used to develop genotype variety resistance to oxalic acid degradation as a result of *S. sclerotiorum* infection.[27] Despite showing improved resistance to *S. sclerotiorum* when a transgenic oxalate oxidase (OXO) gene is incorporated to the plants, transgenic mapping has not resulted in high resistance or yields in soybean. The OXO enzymes from barley and wheat have been found to be responsible for resistance to *S. sclerotiorum.*[27] Giczey and co-workers have utilized gene encoding cell wall degrading enzymes of mycoparasitic fungi.[28] They utilized superior fungal strains that exhibit greater bio-control activities and transgenic plants that exhibit increased resistance to fungal pathogen that has successfully worked well on other plants like canola, barley and sunflower.[27-29] The use of the cloned mycoparasite *Caniothyrium minitians*, a destructive micoparasite that only attacks the *S. sclerotiorum*, did not yield higher resistance against *S. sclerotiorum* as the mechanisms accounting for its degrading activity are rather limited. This has necessitated research in other ways to tackle the pathogen. [27]

In our effort to build on the data available for *S. sclerotiorum*, I propose a different approach to understand the physiology and biochemistry of the pathogen. I intend to investigate:

(i) The growth conditions which supports oxalate and biomass production.

(iii) The expression of proteins relevant to oxalate synthesis.

(iii) The enzymes involved in oxalate biosynthesis.

By identifying and quantifying the enzymes, I intend to use the data to find ways in which to stop the synthesis of oxalate. Since control and management of *S. sclerotiorum* has had little success, a more biochemical approach will be utilized. The long-term goal is to see whether a relationship exists between data obtained in the laboratory and that obtained in field.

In order to understand the pathways involved, I will use **Figure 2** as a model to identify the enzymes involved in the synthesis of oxalate. Specifically, we are interested in the enzymes oxaloacetate hydrolase (OAH), glyoxylate dehydrogenase (GDH) and malate dehydrogenase (MDH) which catalyze biochemical reactions as shown in **Figure 9**.

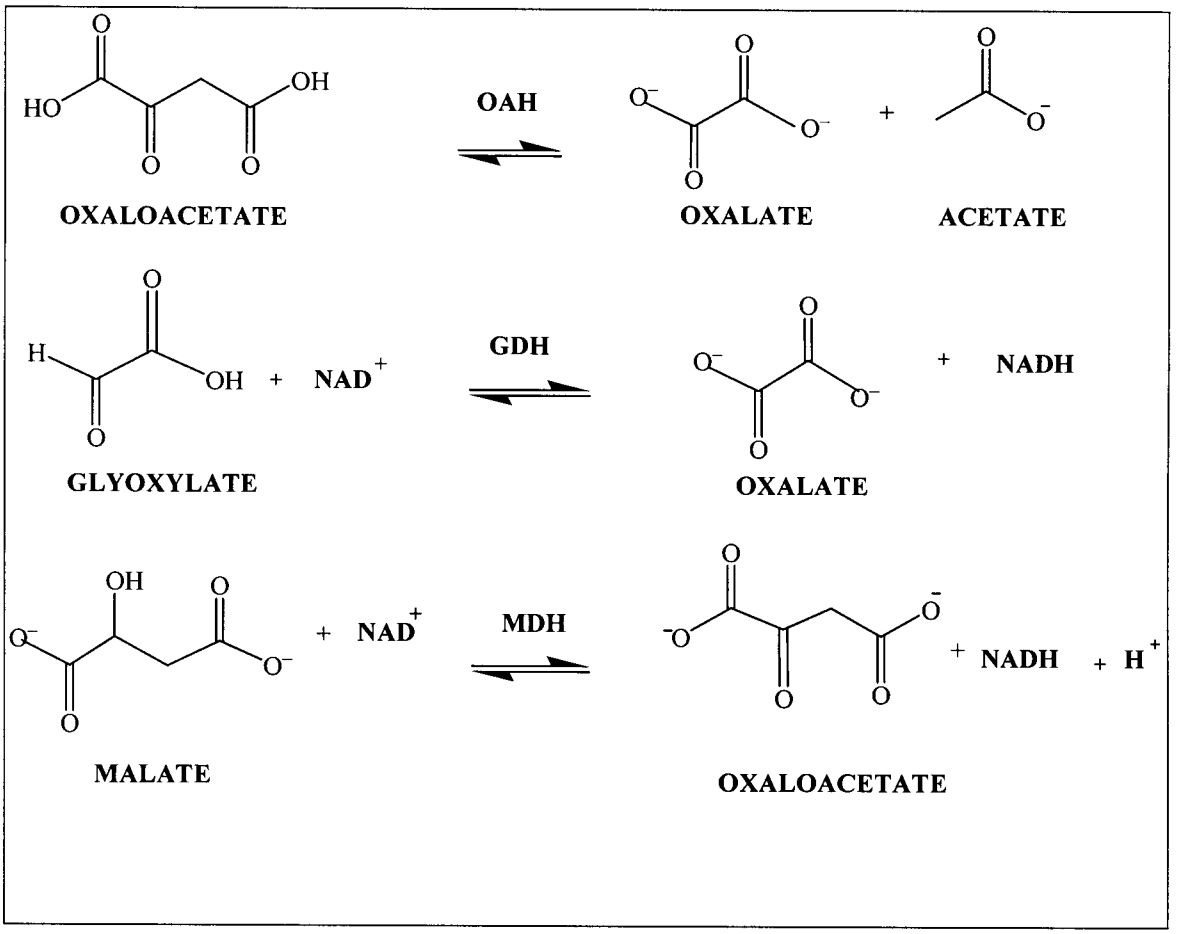


Figure 9: Reaction catalyzed by OAH, GDH and MDH

I plan to study the following virulent isolates of *S. sclerotiorum* commonly found in the environment. Arg-L obtained from Argentina, 105 obtained from Iowa, DE-7 obtained from Dekalb, Illinois, and W-B10 obtained from Watsika, Illinois.

Chapter II

MATERIALS AND METHODS

In my attempt to investigate the physiology and biochemistry of S. *sclerotiorum,* eleven growth conditions were investigated. These were used to optimize growth conditions that would produce higher fungal mycelia (biomass) and result in greater oxalate production.

Cultivation of Fungal Isolates

Fungal isolates Arg-L, 105, DE-7 and W-B10 were first grown with shaking (200 rpm) in culture flasks containing the following undefined culture medium: basal medium (BM), BM + Glucose (BMG), final concentration 25 mM sterile glucose in culture flasks and in Fernbach flasks at room temperature. Glucose was prepared as 500 mM stock solution and filter sterilized. The basal media consisted of 50 mL of each of the following stock solutions with the final concentrations indicated. **STOCK 1**: 57.4 mM, potassium phosphate monobasic, **STOCK 2**: 151.4 mM $(NH_4)_2SO_4$, 73.5 mM KH_2PO_4 (dibasic), 154.1 mM NaCl, 20.3 mM $MgSO_4$ and **TRACE METALS SOLUTION**: 389 μ M Na_3 nitrilotriacetate, 180 μ M $FeSO_4 \cdot 7H_2O$, 40 μ M $CuSO_4 \cdot 5H_2O$, 73 μ M $ZnCl_2$, 59 μ M $MnSO_4 \cdot H_2O$, 41 μ M $Na_2MoO_4 \cdot 2H_2O$. The final volume was 1000 mL. The media was then autoclaved for 25 minutes at 121 °C and 15 PSI. The pH of the medium after cooling was 4.2-4.4. A five mm biscuit plug from the edge of a colony grown on PDA in a Petri plate and maintained at 4 °C was aseptically seeded into 100 mL of autoclaved growth medium in 250 mL culture flasks, stoppered and put on a shaker for five days at room temperature.

Growth conditions were changed by the addition of different co-substrates to the BMG. Co-substrates used were malate, succinate and acetate prepared as 500 mM filter-sterilized stock solutions, final concentration 25 mM. These were added to the culture medium after autoclaving and prior to inoculation. Other growth conditions included BMG + 0.1% or 1% soytone (SE), BMG + 0.1% or 1% yeast extract (YE) and potato dextrose broth (PDB). After five days of growth, contents of the flasks were used to inoculate one-liter Fernbach flasks containing identical media.

Biomass Collection

After five days of growth, mycelia from the Fernbach flasks were collected by using a Buchner funnel and vacuum filtration. Biomass was washed with dH_2O or KPi three times in a centrifuge bottle followed by centrifugation at 7000 x *g* for 15 min. Before centrifugation, the rotor was allowed to cool to 4°C for 30 minutes at 16,000 rpm using a Beckman Avanti-T301 HS Centrifuge. After centrifugation, the biomass (fungal mycelium) was collected from culture flasks on membrane filters by vacuum filtration and weighed to determine the grams (wet weight) of mycelium formed during incubation. The mycelia was used immediately or stored at –20°C after freezing in liquid nitrogen.

Extraction of Biomass

For extraction of protein from the mycelia, I attempted the following methods to maximize fungal protein extraction.

(a) Bead-Beater Method

Lysis of mycelia from *S. sclerotiorum* was accomplished by combining one part of the cell paste and two parts of KPi (homogenization buffer) in a Bead-Beater and homogenizing at 0 °C in an ice bath. The Bead-Beater chamber was half filled with glass

beads (acid washed) and then filled with 5 g of mycelia (small chamber) or 10 g (big chamber) plus buffer i.e., PGSK or KPi. It was then cooled in a freezer for 15 minutes before homogenization. The mixture was homogenized for one minute and then allowed to cool for two minutes on ice. The cycle was repeated six times for a total blending time of six minutes. Centrifugation was then carried out at 16,000 rpm for 30 minutes at 4 °C. Based on Bradford protein assays, six minutes yielded the most protein. Microcon concentrators were used unsuccessfully to concentrate proteins from the extracts. The extract was then decanted into clean, plastic conical centrifuge tubes and capped tightly.

(b) Sonication

Sonication was carried out using a sonicator (Biosonik III) and a magnetic stirrer bar in a beaker. The intensity was set to 30% of full power for the small probe. The treated crude extract was subjected to sonication for 30 seconds on and one minute off to cool for a total sonication time of six minutes. However, based on a Bradford protein assay, **Figure 10**, this method was found to yield low protein concentrations.

(c) French Press

A French Press cell method was used (courtesy of University of Illinois, Urbana-Champaign, Department of Microbiology). The mycelia cells were fractionated by two passages through the cell at 1,300 PSI. Ultra centrifugation at 18,000 rpm (64,285 x g) was done using Beckman SW 50.1 rotor at 4 °C after French press extraction to remove mycelia debris.

Treatment of Extracts

All the protein extracts were brought to 1 mM DTT, dispensed in approximately 1.5 mL aliquots using micro centrifuge tubes, frozen in liquid nitrogen (77K) and then stored at –20 °C. During our initial studies we treated the crude extracts that had co-substrate with 1 mM DTT, 1 mM PMSF and 1 mM EDTA. For the extracts from the French press method, the extracts were brought to 1mM PMSF, 1mM DTT, and 0.05μg /mL DNAse1 and the KPi buffer for extraction adjusted to pH 7. PMSF is used to inhibit serine protease enzymes by sulfonation while EDTA is a metal chelator that inhibits metallo proteases. DTT is used to prevent interchain disulfide bond formation upon lysis. Thus, it is a reducing agent that protects cysteines. DNAse 1 reduces amino acids viscosity, thus helps in protein solubolization.

Method of Protein Analysis

Different analytical techniques were used to determine the biological properties of the fungus after growth and protein extraction. These include: HPLC analysis for concentration of oxalate, substrates and co-substrates, Bradford for protein concentration, SDS-PAGE for protein expression, and UV-Vis spectrophotometry for enzyme activity.

Determination of Oxalate Concentration

Concentrations of oxalate and co-substrates in culture filtrates were determined using a Beckman HPLC and a 300-mm BioRad Aminex HPX-87H column. HPLC conditions were: column temperature, 55 °C; flow rate, 0.6 mL/min of 0.01 N H_2SO_4; injection volume, 10 μL; detector, 210 nm and refractive index. The extra-cellular filtrate was prepared for analysis by filtering 1 mL using 0.2 μ m filters into HPLC vials

and then running the samples for twenty minutes. The HPLC was calibrated using standard stock solutions of glucose, acetate, malate, oxalate and succinate.

Bradford Method of Protein Determination

Bradford solution (Bio-Rad) was diluted in the ratio 1: 4. The reagent (4 mL of Bradford + 16 mL dH_2O) was mixed to make the diluted Bradford solution. Test samples contained 2.5 mL dilute Bradford and 1 mL of extract diluted in KPi or dH_2O. A blank contained 2.5 mL dilute Bradford /1 mL Buffer (KPi) or dH_2O. Bovine serum albumen (BSA) was used as a standard. Absorbance was measured at 595 nm after mixing the reagents and allowing five minutes for color development. **Figure 10** shows a representative BSA standard curve for determination of protein concentration in extracts.

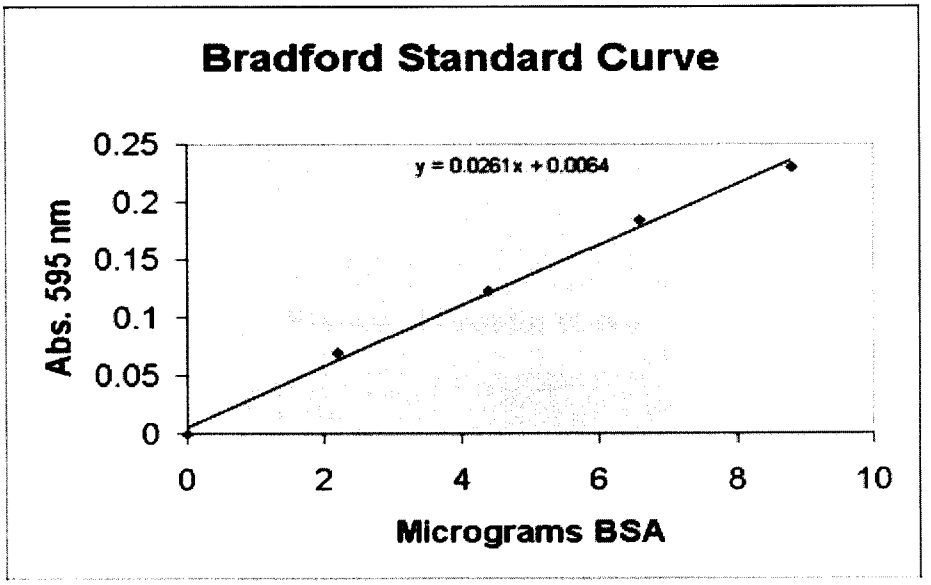


Figure 10: Protein standard curve, Bradford method.

Gel Electrophoresis.

SDS-PAGE was performed according to Laemmli.[47] We initially used Bio-Rad 12% polyacrylamide precast running gels and later we used freshly prepared gels as described later. Protein samples for SDS gel electrophoresis were mixed with sample loading buffer and heated in a boiling water bath for five minutes. After cooling, the protein extracts were subsequently loaded into the wells and run at 120V or 200V using a Bio-Rad Mini Protean III set up. The number of micrograms of cell free extract loaded into each well was between 15 μg and 60 μg. Bands were visualized by staining with Commassie Blue R-250 in methanol/acetic acid for 24 hours or by using Bio-Rad Commassie G250 stain for one hour. The gel was then distained using a solution of 40% methanol/10% acetic acid. R_f distances were measured and the molecular weights of proteins were determined by comparing with a standard curve produced from standard molecular weight markers, **Figure 11**.

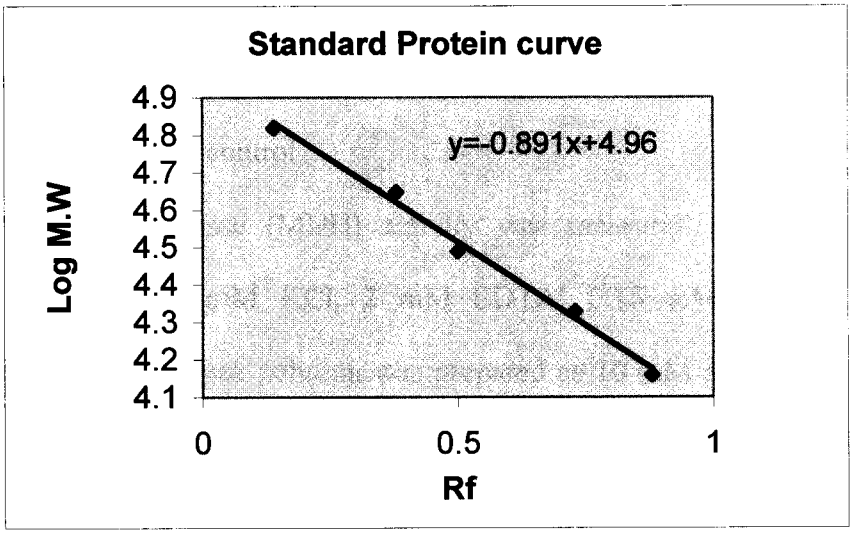


Figure 11: Protein standard curve, log molecular weight vs. R_f.

Fungal Enzyme Assays

All enzyme activities were measured at 25 °C in a total volume of 1.0 mL. Oxaloacetate acetylhydrolase (OAH) activity was determined in 100 mM MOPS (4-morpholinopropanesulphonic acid), 2 mM $MnCl_2$, and 1 mM OAA (0.1 mL of 10 mM stock solution). The activity was calculated from the decrease in absorbance at 255 nm using an extinction coefficient of 1.1 $mM^{-1}cm^{-1}$ for OAA. A stock solution of OAA was prepared as 10 mM in Millipore water. $MnCl_2$ was prepared in 100 mM MOPS buffer, pH 7.5. The assay was initiated by adding 10-100 μL of extract (diluted if necessary). A blank (no extract added) was run as a control.

Glyoxylate dehydrogenase (GDH) enzyme activity was determined in 50 mM CHES buffer, (pH 9.2), 3 mM NAD^+ (0.300 mL of 10 mM stock solution), and 2 mM glyoxylate (0.050 mL of 40 mM stock solution). NAD^+ stock solution was prepared as 10 mM in dH_2O and the glyoxylate solution was prepared as 40 mM stock solution in CHES buffer, pH 9.2. The enzyme activity was calculated from the decrease in absorbance at 340 nm using an extinction coefficient of 6.22 mM^{-1} cm^{-1} for NADH. Assay was initiated by adding 10-100 μL of extract (diluted if necessary). A blank (no extract added) was run as a control.

Malate dehydrogenase (MDH) activity was measured in 50 mM potassium phosphate, pH 7.2, 100 mM KCl, 2 mM EDTA, 0.15 mM NADH and 1 mM oxaloacetate. The OAA stock solution was prepared as 10 mM in potassium phosphate buffer containing 100 mM KCl, and 2 mM EDTA, pH 7.2. NADH stock solution was prepared as 0.300 mM in potassium phosphate buffer. The enzyme activity was calculated from the decrease in absorbance at 340 nm using an extinction coefficient of

6.22 mM^{-1} cm^{-1} for NADH. Assay was initiated by adding 10-100 µL of extract (diluted if necessary). A blank (no extract added) was also run as a control.

Enzyme activities were calculated in terms of specific enzyme activity (µmol min^{-1} g^{-1} protein) and in terms of total (µmol $min^{-1}gram^{-1}$ of biomass). One unit of enzyme activity can be defined as the amount of enzyme that catalyzes the formation of one µmol product per minute or the consumption of one µmol substrate per minute under the experimental conditions described.

Preparation of Reagents

Reagents and solvents were of commercial quality unless otherwise noted. The water used throughout the investigation was Millipore Q (dH_2O). The following solutions were prepared.

•*100 mM Potassium phosphate buffer, pH 7.5 (KPi)*

The buffer solution was prepared by dissolving 17.418 g K_2HPO_4 /L in dH_2O and adjusting the pH to 7.5. This was used for washing and extraction of the mycelia and for Bradford protein analysis.

• *10x Electrode (Running) Buffer, pH 8.3*

This was prepared by dissolving 30.3 g Tris base, 144.0 g glycine, 10.0 g SDS in 1.0 L dH_2O. The running buffer was stored at 4 °C and used for SDS-PAGE.

• *Acrylamide/Bis (30%/2.67%)*

This was prepared by dissolving 29.2 g acrylamide, 0.8 g N'N'-bis-ethylene-acrylamide. The volume was brought up to 100 mL with dH_2O. This was used in casting gels for SDS-PAGE.

• *10% (w/v) SDS*

This was prepared by dissolving 1.0 g SDS in 100 mL dH_2O and was used for SDS-PAGE.

• *1.5 M Tris-HCl, Resolving gel buffer, pH 8.8*

The buffer was prepared by dissolving 18.2 g Tris base in 80 mL dH_2O. The pH was adjusted to 8.8 with 6 M HCl, and the volume was brought up to 150 mL with dH_2O.

• *0.5 M Tris-HCl, Stacking gel buffer, pH 6.8*

The buffer was prepared by dissolving 6 g Tris base in 60 mL dH_2O and the pH was adjusted to 6.8 with 6 M HCl. The volume was brought up to 100 mL with dH_2O.

• *Sample Loading Buffer (SDS Reducing Buffer)*

This was prepared by combining 3.55 mL dH_2O, 1.25 mL 0.5 M Tris-HCl, pH 6.8, 2.5 mL glycerol, 1.0 mL 10% (w/v) SDS and 0.2 mL 0.5% (w/v) bromophenol blue for a total volume of 9.5 mL. Fifty microliters of β-mercaptoethanol was added to 950 μL of sample loading buffer before use.

• *PGSK Extraction Buffer*

The buffer was prepared by dissolving 0.52 g sodium phosphate monobasic; monohydrate, 8.8 g sodium phosphate dibasic, 2.83 g sodium chloride, 0.372 g potassium chloride and 11 g glucose. The volume was brought up to 1.0 L with dH_2O.

• *SDS-PAGE Resolving gel (12% Acrylamide)*

This was prepared by combining 3.4 mL dH_2O, 4 mL 30% Acrylamide/Bis, 2.5 mL resolving gel buffer, 0.1 mL 10% (w/v) SDS, and immediately before pouring, 50 μL of 10% APS (prepared by dissolving 100 mg ammonium persulfate in 1 mL dH_2O). Finally, 5 μL TEMED was added and the components were mixed thoroughly before casting gels.

• *SDS-PAGE Stacking gel (4% Acrylamide)*

This was prepared by combining 6.1 mL dH_2O, 1.3 mL 30% Acrylamide/Bis, 2.5mL, stacking gel buffer, 0.1 mL 10% (w/v) SDS, and immediately before pouring, 50 μL 10% APS and 10 μL TEMED were added. The components were mixed thoroughly before casting gels.

Chapter III

RESULTS

During our studies, various isolates of *S. sclerotiorum* were grown under different growth conditions in order to determine optimal fungal growth and oxalate production. Basal media containing glucose as a substrate and acetate, malate and succinate co-substrates were initially used. The study was repeated with 0.1 % and 1% yeast extract and 0.1 % and 1% soytone as supplements. The pH of the filtrate was measured after five days of growth. Potato dextrose broth was used as a control.

The data in **Table 1** shows the growth media, biomass, protein expression and oxalate produced for isolate Arg-L. The pH of the media was adjusted to pH 7 before inoculation. The results show that PDB supports maximum mycelia growth of the isolate with 90 g of dry biomass harvested while basal media with glucose had the lowest with 2.6 g of biomass. The results of protein concentrations are an average of triplicate data.

Table 1: Growth media, biomass, protein and oxalate concentrations for isolate Arg-L.

Growth condition [pH 7]	Biomass (g)	Protein (mg/mL)	Concentration (mM) of oxalate
BMG	2.6	1.4	0.1
BMG + Acetate	7.92	2.2	16.1
BMG + Malate	4.9	2.0	24.0
BMG + Succinate	3.0	0.9	19.4
PDB	90.0	2.2	6.3

SDS-PAGE of the above extracts showed different protein expression (see **Figure 12).** Different growth conditions showed different protein expression. For example, PDB had only one heavy protein band and relatively few others compared to other growth conditions. BMGA had several distinct heavy protein bands while BMGM and BMGS had almost similar protein expression. BMG had only three protein expressions with one heavy band compared to other growth conditions.

Lanes 1 and 2: 30 and 45 µg Protein; BMG
Lanes 3 and 4: 44 and 66 µg protein; BMGA
Lane 5: 25µL of low M.W. Marker diluted 1:20
Lanes 6 and 7: 17 and 26 µg; BMGS
Lanes 8 and 9: 36 and 54 µg; 1% BMGM
Lane 10: 60 µg ; PDB extract

Figure 12: SDS-PAGE gel of isolate Arg-L.

After obtaining the above results, we investigated other isolates under different growth conditions. Results are shown in **Table 2.**

Table 2: Growth media and biomass of four isolates.

	Isolate biomass, (g)			
Growth condition [pH 5]	**DE-7**	**105**	**W-B10**	**Arg-L**
PDB	23.0	30.1	35.3	34.0
BMG	103.0	37.9	3.1	30.0
BMG + SE	7.4	52.0	7.9	31.4
BMG + YE	22.9	33.4	20.7	74.4

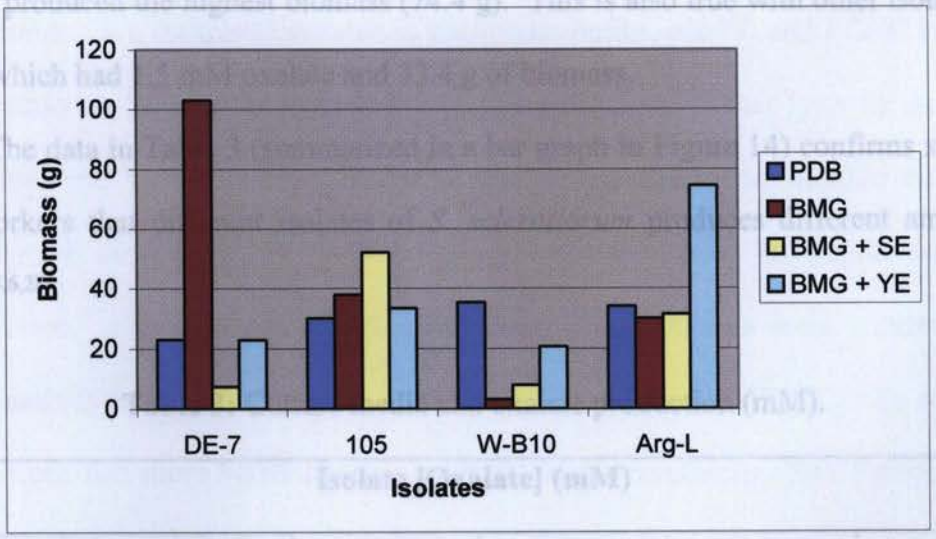


Figure 13: Comparison of Fungal biomass (g).

From data in **Table 2** and **Figure 13** (a bar graph of data from **Table 2),** it appears that different isolates produced different amounts of biomass under similar conditions. The pH of the media was maintained at pH 5 through out the investigation after our initial

study (**Table 1**). As the data illustrate, DE-7 produced more biomass in glucose media and comparatively less biomass in all other supplements. After the change in pH from 5 to 7, Arg-L produced less biomass i.e., from 90 g to 34 g in PDB. Isolate 105 had almost similar biomass production apart from growth in soytone supplement. This was the reason why we settled on the two isolates 105 and DE-7 for further studies since they grew consistently in all growth media.

The amount of oxalate produced by the four isolates was also measured. Since oxalate production has been implicated in pathogenesis [4-8,26,29], we wanted to investigate if a relationship exists between biomass production and oxalate concentration. For example, isolate Arg-L had the highest oxalate production (1.65 mM) with yeast extract and also produced the highest biomass (74.4 g). This is also true with other isolates such as 105, which had 1.5 mM oxalate and 33.4 g of biomass.

The data in Table 3 (summarized in a bar graph in Figure 14) confirms studies by other workers that different isolates of *S. sclerotiorum* produces different amounts of oxalate.[5,6,28]

Table 3: Culture media and oxalate production (mM).

Isolate [Oxalate] (mM)				
Growth condition [pH 5]	**DE-7**	**105**	**W-B10**	**Arg-L**
PDB	0.2	4.3	0.2	0.2
BMG	0.2	0.2	0.9	0.9
BMG +SE	1.1	2.6	0.5	1.4
BMG + YE	0.6	1.5	0.5	1.7

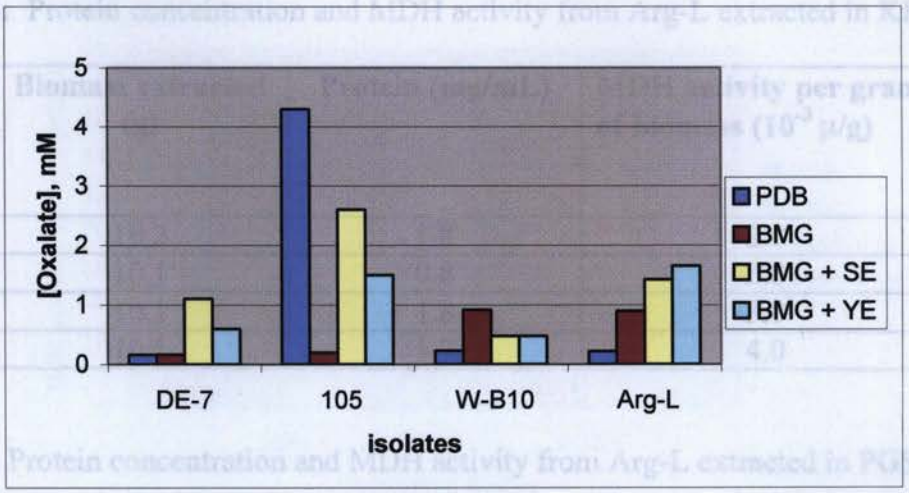


Figure 14: Quantitative comparison of oxalate production (mM).

The use of different extraction buffers confirms that they do not affect protein concentration. We compared potassium phosphate buffer, pH 7.0 and PGSK buffer, pH 7.0 as extraction buffers for isolate Arg-L, using the Bead-Beater lysis method. Both extractions were carried out at 0 °C. Our aim was to determine whether there is the possibility of denaturing the protein during extraction and its effects on protein concentration. However, we found that protein concentrations were similar. MDH enzyme activity was determined for each extract from KPi and PGSK. We found that PGSK extract had more MDH activity on PDB growth conditions. See **Tables 4**, **5** and **Figure 15**. Since results seemed consistent in KPi buffer, we used KPi in subsequent extractions. **Tables 4** and **5** compare protein concentration and MDH activity from Arg-L extracted with KPi and PGSK buffers.

Table 4: Protein concentration and MDH activity from Arg-L extracted in KPi buffer.

Growth condition [pH 5]	Biomass extracted (g)	Protein (mg/mL)	MDH activity per gram of biomass (10^{-3} μ/g)	MDH activity/mg protein (10^{-2} μ/mg)
PDB	10.2	1.8	2.0	1.1
BMG	10.1	0.8	2.0	2.5
BMG +SE	10.1	1.8	3.0	1.7
BMG + YE	10.1	1.7	4.0	2.3

Table 5: Protein concentration and MDH activity from Arg-L extracted in PGSK buffer.

Growth condition [pH 5]	Biomass extracted (g)	Protein (mg/mL)	MDH activity per gram of biomass (10^{-3} μ/g)	MDH activity/mg protein (10^{-3} μ/mg)
PDB	10.1	1.6	30.0	18.5
BMG	10.1	0.8	3.0	3.8
BMG +SE	10.1	1.4	2.0	1.4
BMG + YE	10.2	2.8	12.0	4.2

Figure 15 is a bar graph comparing MDH specific enzyme activity and extraction buffer. We investigated MDH enzyme activity due to its greater activity in our extracts as compared to either OAH or GDH which were not always detected depending on the extraction method.

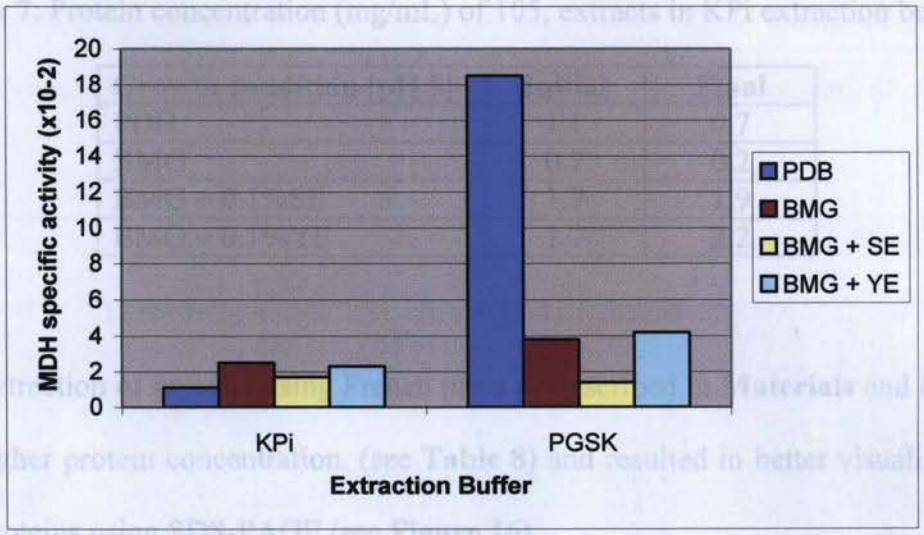


Figure 15: Comparison of MDH specific enzyme activity in different extraction buffers.

The above results suggest that the buffer used for extraction affects MDH enzyme activity. Some enzyme activity may be lost in the course of fracturing especially in KPi and this may explain our data since we used the Bead-Beater method for both extractions.

In our attempts to increase protein concentration we used Centricon concentrators (Millipore Corp.) and compared the initial concentration of the extract to the final concentration. Data shows that we managed to increase the concentration in some extracts but not all (see **Tables 6** and **7**).

Table 6: Protein concentration (mg/mL) of DE-7, extracts in KPi extraction buffer.

Growth condition [pH 5]	Initial	Final
PDB	0.2	0.3
BMG	0.7	0.5
BMG + 0.1%SE	1.5	5.1
BMG + 0.1%YE	1.6	8.1

Table 7: Protein concentration (mg/mL) of 105, extracts in KPi extraction buffer.

Growth condition [pH 5]	**Initial**	**Final**
PDB	1.1	0.7
BMG	0.7	0.2
BMG + 0.1%SE	1.7	1.9
BMG + 0.1%YE	1.7	2.2

Extraction of mycelia using French press as described in **Materials** and **Methods** gave a higher protein concentration, (see **Table 8)** and resulted in better visualization of soluble proteins using SDS-PAGE (see **Figure 16)**.

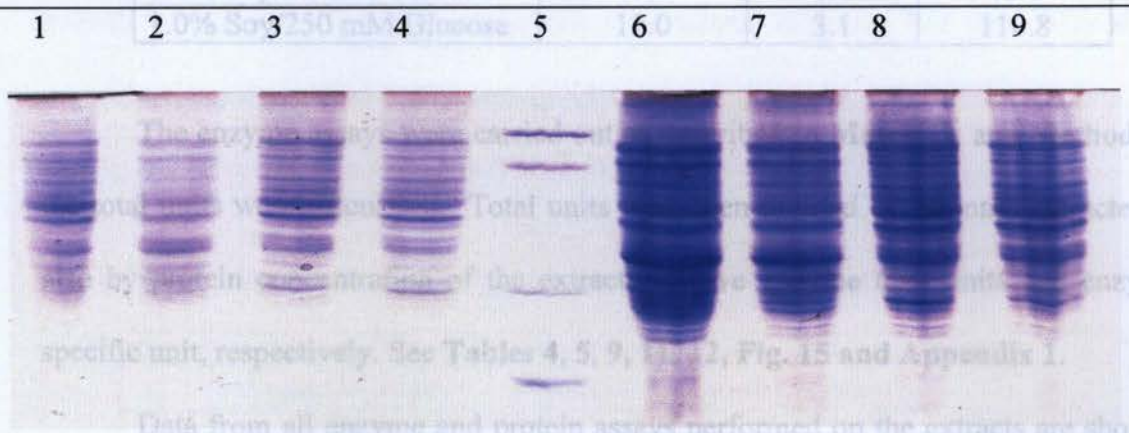
Table 8: Biomass, protein concentration, and oxalate concentration, DE-7.

Growth condition [pH 5]	**Biomass (g)**	**Protein concentration (mg/mL)**	**Oxalate (mM)**
0.1% Soy/250 mM Glucose.	11.5	7.9	0.02
1.0% Soy/-	15.0	14.8	21.0
1.0% Soy/25 mM Glucose.	18.8	15.3	21.0
1.0% Soy/250 mM Glucose.	18.3	12.7	7.0

Growth conditions were varied using isolate DE-7 to investigate the effect of higher glucose concentration (see **Table 8)**. This resulted in higher oxalate production with low glucose concentration. As the glucose concentration increased, oxalate concentration decreased. High soy concentration supported oxalate production. For example, when the isolate was grown on 1.0%, oxalate concentration was found to be 21 mM.

The SDS-PAGE results (**Figure 16)** suggest that we should have used through out our investigation the French press method of extraction. **Figure 16** also reveals that the fungi might posses complex soluble proteins that are affected by the extraction method.

Since we treated our extracts with DNase 1, this might have helped purify the protein by reducing DNA viscosity in the protein extract and thus caused less smearing of gels.



Lanes 1 and 7: 15 and 30 µg: 0.1% Soy + 250 mM glucose
Lanes 2 and 8: 15 and 30 µg: 1 % Soy + Water
Lane 5: 20µL of low M.W. Marker diluted 1:20
Lanes 3 and 8: 15 and 30 µg: 1 % Soy + 25 mM glucose
Lanes 4 and 9: 15 and 30 µg: 1% Soy + 250 mM glucose

Figure 16: SDS-PAGE gel of DE-7.

We investigated OAH, GDH and MDH enzyme activity on the above extracts. The data reveals encouraging results as all enzymes were detected. The enzyme activities are shown in **Table 9**. OAH, GDH and MDH activities of isolate DE-7 were obtained immediately after extraction. The assays were carried out before freezing the extracts.

Table 9: Enzyme specific activity (units per mg protein, μ/mg)

Growth conditions	OAH	GDH	MDH
0.1% Soy/250 mM Glucose	1.0	3.8	24.9
1.0% Soy/-	11.6	0.2	22.1
1.0% Soy/25 mM Glucose	13.4	1.4	46.9
1.0% Soy/250 mM Glucose	12.0	3.1	110.8

The enzyme assays were carried out as described in **Materials** and **Methods** and the total units were calculated. Total units were then divided by biomass extracted and also by protein concentration of the extracts to give enzyme total units and enzyme's specific unit, respectively. See **Tables 4, 5, 9, 11, 12, Fig. 15 and Appendix 1**.

Data from all enzyme and protein assays performed on the extracts are shown in **Appendix 1.** After three days, the extracts represented by data in **Table 9** were thawed and the above enzyme assays were repeated. However, we were unable to detect any enzyme activity for OAH, GDH, and MDH. Thus, enzyme activities are affected by freeze/thaw of protein in *S. sclerotiorum*. Protein concentration was also re-determined. The results revealed decreased protein concentration by almost a third of the initial one, (see **Table 10**) apart from the one grown under 0.1% soy and 250 mM glucose.

Table 10: Comparison of protein concentration, DE-7

Growth condition [pH 5]	Initial	Final
0.1% Soy/250 mM Glucose	7.9	7.4
1.0% Soy/-	14.8	10.2
1.0% Soy/25 mM Glucose	15.3	11.2
1.0% Soy/250 mM Glucose	12.7	10.0

The above data shows that *S. sclerotiorum* has soluble proteins that are affected by the freeze/thawing process. This can be explained by protein precipitation on thawing as was observed in all extracts.

We also repeated our earlier studies (see **Tables 4** and **5**) to determine the significance of extraction methods. As the results show, the extracts had higher protein concentration and also higher enzyme activity than our previous extracts under similar growth conditions. Oxalate concentration remained similar. We were also able to quantify the presence of OAH and GDH enzymes that was not possible in our initial extracts. The results are shown in **Table 11.**

Table 11: Growth condition, biomass, oxalate concentration, and specific activity.

Isolate	Growth cond.	Biomass (g)	Oxalate (mM)	Protein (mg/mL)	Enzyme activity (μ/mg)		
					OAH	GDH	MDH
Arg-L	PDB	10.0	0.04	1.3	0.6	0.1	0.7
W-B10	PDB	10.0	11.8	1.5	1.2	0.1	0.5
DE-7	PDB	10.0	4.5	0.6	6.6	0.1	0.6
Arg-L	BMGA	6.2	1.9	4.9	5.6	0.1	5.2

We carried out further studies using isolates DE-7 and WB-10 grown in the absence of glucose to determine how significantly it would impact enzyme activity (see **Table 12**). As the results show, higher activity of OAH, GDH, and MDH enzymes were detected. Higher enzyme activity was detected from DE-7 than from WB-10. The results also demonstrate that OAH, GDH, and MDH activities are not significantly affected by absence of glucose.

Growth Condition	DE-7			W-B10 (x 10^{-3})		
	OAH	GDH	MDH	OAH	GDH	MDH
BM	0.07	0.02	0.26	0.7	7	200
BMS	0.4	0.08	0.85	300	1.5	800
BMT	0.5	0.03	0.23	2500	50	600
BMYE	0.5	1.03	11.5	5500	60	1100

Table 13 shows the data obtained for the molecular weight markers.

Table 13: Protein, R_f, and log M.W. of Molecular Weight Markers.

Protein	R_f	Log M.W.	M.W.
Serum albumin	0.1	4.8	66,200
Ovalbumin	0.4	4.7	45,000
Carb. Anhydrase	0.5	4.5	31,000
Trypsin inhibitor	0.7	4.3	21,500
Lysosome	0.9	4.2	14,400

Table 14 contains results of SDS-PAGE analysis of Arg-L extracts (**Figure 12**). Only major bands were measured. Bands with overlapping R_{fs} were recorded once.

Table 14: R_f, log M.W. and M.W. of proteins extracted from Arg-L

R_f	Log M.W.	M.W.
0.3	4.8	59,500
0.3	4.6	42,700
0.3	4.6	38,200
0.4	4.5	30,600
0.5	4.4	23,200
0.6	4.2	14,900
0.7	4.0	9,000

The R_f distance was then fitted to an equation calculated from the molecular weight standard curve (**Figure 11**) and molecular weights of unknown proteins were calculated. Similar treatment of data was repeated for isolate DE-7. Results are shown in **Table 15**.

Table 15: R_f, log M.W. and M.W. of proteins extracted from DE-7

R_f	Log M.W.	M.W.
0.3	4.7	45,800
0.5	4.4	25,200
0.7	4.1	13,100
0.8	4.0	9,900
0.9	3.9	9,000

Chapter IV

DISCUSSION

The general inability of agronomically important plants to develop a mechanism of defense against the pathogen *S. sclerotiorum* and lack of any scientific advances in controlling the spread of the disease among crops has increased the effort to understand and contain the disease caused by this pathogen. Since many studies have demonstrated the importance of oxalate in fungal pathogenesis, considerable efforts have been focused on unraveling the mechanisms by which oxalate is involved in colonization and spread of disease in plant tissues.[8-12,30-31] Although the pathogen has been somewhat contained in wheat, barley and sunflower through genetic modification of oxalate oxidase enzymes that are involved in the primary line of defense against *S. sclerotiorum* infection, this has not been replicated in other important crops such as soybean.[21,24-25,27-28,32-34] Our research has been focusing on the effects of growth conditions on oxalate synthesis and on biomass production, protein expression and enzyme activity involved in oxalate biosynthesis.

In our study of effects of growth conditions on biomass production, *S. sclerotiorum* shows differences across all isolates investigated of Arg-L, WB-10, DE-7 and 105. In all isolates, biomass production was relatively high in PDB growth media where we harvested between 23 g to 90 g and lowest in soytone supplement when 7.4 g to 31.4 g was obtained depending on isolate, except for isolate 105 where the biomass was 52 g (see **Tables 1** and **2).** Thus, growth conditions affect biomass production.

Protein concentration in *S. sclerotiorum* is affected by which isolate was investigated, extraction method, extraction buffer, and growth conditions (**Tables 1, 5-8** and **Figure 12**). Protein concentrations are highly dependent on extraction method. The French press extraction method yielded higher protein concentration compared to Bead-Beater or sonication. For example isolate DE-7 grown on PDB had 0.23 mg/mL on average after Bead-Beater but had 1.54 mg/mL after French press. Use of co-substrate or supplements also affected the protein concentration (see **Tables 4-8**). In all isolates, yeast supplement yielded higher protein concentration of the extracts under similar growth conditions.

The high oxalate concentration in the growth medium has also confirmed earlier studies by other workers that oxalate is produced in high millimolar concentration by this fungal pathogen under different growth conditions.[7-8,11,16] We have also found that oxalate concentration as determined by HPLC (**Tables 3, 8, 11 Figure 13,** and **Appendix 1)** is affected by growth conditions. It was found to be highest in isolate Arg-L, 24 mM, in undefined basal media with 25 mM glucose plus 25 mM malate and lowest, 0.02 mM, in undefined basal media supplemented with 0.1% soytone and 250 mM glucose. We also report the effect of high and low concentrations of glucose (25 mM and 250 mM) and soytone (1% and 0.1%) on oxalate production (**Table 8**). This was investigated using isolate DE-7 extracted by the French press method. As the glucose concentration was increased, there is decreased oxalate production. An opposite trend was observed with soy. For example, when the fungus was cultured in basal media plus 1.0% soytone, oxalate production was found to be 21 mM compared to 0.02 mM when the same culture had 1% soy and 250 mM glucose. We thus report oxalate biosynthesis is inhibited by

high glucose concentration and promoted by high soy concentration (see **Tables 1, 2, 8 and Figure 12**). This is the first research to report this effect of glucose on oxalate biosynthesis in *S. sclerotiorum.*

The pH dependent activity of this fungal pathogen as reported by other workers has also been investigated.[9-10] In their studies, Cessna and co-workers found that *S. sclerotiorum* has maximum fungal activity at pH of 4-5 and very low activity at a pH higher than 5. We found that when the pH of the growth media was adjusted from 7 to 5, biomass production decreased drastically. For example, isolate Arg-L grown on PDB decreased from 90 g of biomass to 34 g (**Tables 1 and 2**). *S. sclerotiorum* growth is reportedly favored at pH 5 or lower.[8-12] Our data contrasts the earlier reported findings since lowered pH resulted in a decrease in biomass production under similar growth conditions. Repetition of the same experiment under similar growth conditions (BMG) with isolate Arg-L yielded consistent results and a lower amount of biomass, 1.8 g after 5 days of incubation. A similar trend was observed in isolate 105 grown on PDB where we harvested 6.7 g of biomass.

Protein expression was analyzed by SDS-PAGE. Treatment of the extracts with DNAse 1 by French press extraction helped in protein visualization by SDS-PAGE (see **Fig. 15**). When we froze the protein extracts, we observed precipitation upon thawing. This corroborates studies by other workers that the proteins in the fungus easily precipitate.[35] We were unable to reproduce the initial protein concentration due to protein precipitation. This explains the drop in protein concentration (see **Tables 6, 7** and **10**). This research has thus shown that freezing and thawing the extracts affects protein concentration since in all cases studied, we were unable to recover the initial

concentration after precipitation. The SDS-PAGE results improved after changing our extraction method from Bead-Beater to French press, which achieved higher protein concentration (see **Figure 15**). In order to obtain the molecular weight of proteins that are found in *S. sclerotiorum*, we measured the R_f distances on the gels. This was then fitted into a standard curve (log molecular weight of standard vs R_f) as shown on **Figure 11**. Visible protein expressions whose log molecular weights were determined (see **Table 13, 14, 15** and **Figure 11**) are shown. The SDS-PAGE data shows the presence of several proteins in different growth conditions. Specific proteins were however not identified. From the data represented, protein expression seems to be influenced by different growth conditions.

We investigated the presence of three enzymes that are found in glyoxylate and tricarboxylic acid cycles and which may play a significant role in oxalate biosynthesis. We report the presence of OAH, GDH and MDH activity in *S. sclerotiorum.* Our data demonstrate that despite the lack of a direct relationship between MDH and oxalate biosynthesis (see **Figure 2**), it is one of the enzymes that are found in *S. sclerotiorum.* We were also able to quantify high specific activity of GDH (3.1 μ/mg) and OAH (13.4 μ/mg) in *S. sclerotiorum* enzymes that are involved in oxalate biosynthesis. This study has revealed that the MDH enzyme may play a significant role in oxalate biosynthesis. This is attributed to its relatively high activity in fractured mycelia under different growth conditions. For example, it had the highest specific activity; 110.8 μ/mg under BM + 1.0% Soy and 250 mM glucose and lowest in BM + 0.1% soy and 25 mM glucose; 1.4 x 10^{-3} μ/mg. Our results also demonstrate that MDH activity is higher in all isolates grown on BMG + YE and minimal in growth media of undefined basal medium plus glucose

alone (see **Appedix 1**). OAH enzyme was also detected but had lower specific activity (13.4 μ/mg) compared to MDH (110.8μ/mg). Also see **Table 9**.

The failure to detect considerable OAH enzyme activity in extracts of the fungal mycelia was probably because it was inactivated during extraction or other interfering enzymes exist. This is apart from the fact that OAH catalyzes the hydrolytic cleavage of oxaloacetate to oxalate and acetate as reported in other pathogenic *Sclerotinia* species that produces oxalate.[31,36,38] We suspect that the low OAH activity as compared to MDH may be due to other factors. This has lead us to hypothesize that there is a possible inhibitor of OAH enzyme as we were unable to determine any activity in our initial investigation and also reported low OAH quantity compared to MDH in subsequent investigations. We also conclude that the extraction method affects the activity as we have found that after changing the extraction method from Bead-Beater to French press, we were able to detect OAH in all extracts. This was not possible after extraction with Bead- Beater.

The presence of the GDH enzyme also validates our earlier assumption that this enzyme plays an active role in this fungal pathogen. Although the GDH enzyme specific activity (3.1 μ/mg) was lower than OAH (13.4 μ/mg), its presence points to an active role played by this enzyme in oxalate biosynthesis. We are also able to infer that oxalate synthesis in *S. sclerotiorum* is more significant due to OAH enzyme activity and to a less extent GDH activity. For example, isolate DE-7, WB-10, and Arg-L grown in PDB had high OAH and similar GDH activity, implying that OAH is more active than GDH (see **Table 11**). The inability to detect high GDH activity (see **Tables 9, 11 and 12**) in extracts of *S. sclerotiorum* may be attributed to growth conditions. We have been able to

conclude that the method of extraction also affects the specific enzyme activity as demonstrated in **Tables 4, 5** and **9**. Our data is the first to report the presence of two active enzymes involved in oxalate biosynthesis in *S. sclerotiorum*. Other workers have not reported the presence of both MDH and GDH working concomitantly to produce oxalate, but only one enzyme as directly responsible for oxalate biosynthesis.

FUTURE STUDIES

Our future project will be to investigate the role of MDH in *S. sclerotiorum,* and also establish whether we can correlate our data with field studies. We will also characterize the proteins expressed in this fungus.

ACKNOWLEDGEMENTS

This research was supported by a grant from Illinois Soybean Program Operating Board and was a joint project between the Departments of Chemistry and Biological Sciences at Eastern Illinois University.

Appedix I

Summary of Data

DE-7		Wet	Biomass				Centricon Concentration of 1 mL							
	[Oxalate], mM	Biomass (g)	Extracted	mg/mL	Vol. of extract (mL)	Total protein (mg)	mg/mL	Vol. of concentrate (mL)	Total protein (mg)	Abs./min	Units (MDH)	Dilution	Total Units	pH of media
PDB	0.16	23	10	0.23	49	11.27	0.32	0.5	0.16	0.364	0.56	10	27.44	
BMG	0.17	103	10	0.66	44	29.04	0.52	0.5	0.26	0.042	0.0067	1	0.29	
BMG + YE	1.1	110	10	1.54	48	73.92	5.1	0.5	2.55	0.017	0.03	10	1.31	
BMG + SE	0.6	7.4	7.4	1.63	48	78.24	8.1	0.5	4.05	0.134	0.2	10	9.60	
PGSK														
BMG + YE (a)			10.03	2.2	48	105.6				0.392	0.63	10	30.24	
BMG + YE (b)			10.03	0.138	55	7.59				0.517	0.083	1	4.57	

105		Wet	Biomass				Centricon Concentration of 1 mL							
	[Oxalate], mM	Biomass (g)	Extracted	mg/mL	Vol. of extract (mL)	Total protein (mg)	mg/mL	Vol. of concentrate (mL)	Total protein (mg)	Abs./min	Units (MDH)	Dilution	Total Units	pH of media
PDB	4.3	30	10	1.07	48	51.36	0.67	0.5	0.335	0.093	0.015	1	0.72	
BMG	0.2	38	10	0.67	50	33.5	0.16	0.5	0.08	0.0085	0.014	10	0.70	
BMG + YE	2.6	52	10	1.68	50	84	1.9	0.5	0.95	0.4	0.64	10	32.00	
BMG + SE	1.5	33	10	1.7	48	81.6	2.2	0.5	1.1	1.9	31	100	1488.00	

WB-10		Wet	Biomass				Centricon Concentration of 1 mL							
	[Oxalate], mM	Biomass (g)	Extracted	mg/mL	Vol. of extract (mL)	Total protein (mg)	mg/mL	Vol. of concentrate (mL)	Total protein (mg)	Abs./min	Units (MDH)	Dilution	Total Units	pH of media
PDB	0.23	7.9	7.9	1.1	50	55	nd	nd	#VALUE!	0.492	0.791	10	39.55	
BMG	0.92	3.1	nd	ND	nd	#VALUE!	nd	nd	#VALUE!	nd	nd	na		
BMG + YE	0.48	10	10	1.4	50	70	nd	nd	#VALUE!	0.104	0.64	10	32.00	
BMG + SE	0.48	10	10	1.9	50	95	nd	nd	#VALUE!	0.066	31	10	1550.00	

Arg-L (Kpi extract)		Wet	Biomass				Concentration of 1 mL							
	[Oxalate], mM	Biomass (g)	Extracted	mg/mL	Vol. of extract (mL)	Total protein (mg)	mg/mL	Vol. of concentrate (mL)	Total protein (mg)	Abs./min	Units (MDH)	Dilution	Total Units	pH of media
PDB	0.22	34.2	10.21	1.77	50	88.5	nd	nd	#VALUE!	0.278	0.045	1	2.25	4.48
BMG	0.9	29.9	10.1	0.807	50	40.4	nd	nd	#VALUE!	0.217	0.034	1	1.70	3.29
BMG + YE	1.42	74.4	10.1	1.79	50	89.5	nd	nd	#VALUE!	0.356	0.057	1	2.85	2.9
BMG + SE	1.65	31.4	10.1	1.72	50	86.0	nd	nd	#VALUE!	0.442	0.071	1	3.55	3.31

Arg-L (PGSK extract)		Wet	Biomass				Concentration of 1 mL						
	[Oxalate], mM	Biomass (g)	Extracted	mg/mL	Vol. of extract (mL)	Total protein (mg)	mg/mL	Vol. of concentrate (mL)	Total protein (mg)	Abs./min	Units (MDH)	Dilution	Total Units
PDB	0.22	34.2	10.13	1.62	50	81.0	nd	nd	#VALUE!	0.418	0.672	10	33.60
BMG	0.9	29.9	10.12	0.8	47.5	38.0	nd	nd	#VALUE!	0.09	0.072	5	3.42
BMG + YE	1.42	74.4	10.16	1.43	50	71.5	nd	nd	#VALUE!	0.213	0.0343	1	1.72
BMG + SE	1.65	31.4	10.22	2.85	47.5	135.4	nd	nd	#VALUE!	0.155	0.25	10	11.88

References

1. Wrather, J.A; Anderson, T.R.; Arsyad, D.M.; Gai, J.; Ploper, L.D.; Porta-Puglia, A.; Ram, H.H.; Yorimori, J.T. Soybean Disease Loss Estimates for the Top 10 Soybean Producing Countries in 1994. *Plant Disease* **.1997,** *81,* 107-110.
2. Yang, X.B.; Lundeen, P.; Uphoff, M.D. Soybean Varietal Response and Yield Loss Caused by *Sclerotinia sclerotiorum. Plant Disease.* **1999,** *83*, 456-461.
3. Pratt, P.W.; Wrather, J.A. Soybean Disease Loss Estimates for the Southern United States, 1994 to 1996. *Plant disease,* **1999**, *95*, 114-116.
4. Hoffmann, D.D.; Hartman, G.L.; Mueller, D.S.; Leitz, R.A.; Nickel, C.D.; Pederson, W.L. Yield and Seed Quality of Soybean Cultivars Infected with *Sclerotinia sclerotiorum, Plant disease,* **1998,** *82*, 826-829.
5. Purdy, L.H. *Sclerotinia sclerotiorum*: History, disease and symptomatology, host range, geography distribution, and impact. *Phytopathology.* **1979**, *69*, 875-880.
6. Scott, D. H.; Shaner, G.E.; Abney, T.S. Sclerotinia Stem Rot (White Mold) of Soybeans *Plant Pathology.* **1998**, *43,* 34-39.
7. **http://www.plantpath.wisc.edu/soyhealth/biocont1.**
8. Marciano, P.; Margro P.; Favaron, F. *Sclerotinia sclerotiorum* growth and oxalic production on selected culture media. *FEMS Microbiology Letters.* **1989,** *61*, 57-60.
9. Godoy, G.; Steadman, J.R.; Dickman, M.B.; Dam, R. Use of mutants to demonstrate the role of oxalic acid in pathogenicity of *Sclerotinia sclerotiorum* on Phaseolus vulgaris. *Physiology and Molecular Plant Pathology.* **1990**, *37,* 179-191.

10. Cessna, G.C.; Sears, V.E.; Dickman, M .B. Oxalic Acid, A Pathogenicity factor for Sclerotinia sclerotiorum, Suppresses the Oxidative Burst of the Host Plant. *The Plant Cell.* **2000**, *12*, 2191-2199.

11. Marciano, P.; Margro, P.; Di Lena, P. Cell wall-degrading enzyme and pH pathogenesis and their significance in the virulence of two *Sclerotinia sclerotirum* isolates on sunflower. *Physiological Plant Pathology.* **1983**, *22*, 339-345.

12. Rowe, D.E. Oxalic Acid Effects in Exudates of *Sclerotinia trifoliorum* and *S. sclerotiorum* and Potential Use in Selection. *Crop Science.* **1993**, *33*, 1146-1149.

13. Dutton, M.V.; Evans, C.S. Oxalate production by the fungi: its role in pathogenicity and ecology in the soil environment. *Canadian Journal of Microbiology.* **1996**, *42*, 881-890.

14. Corsini, D.L.; Tourneau, D.L. Organic Acid Metabolism in *Sclerotinia sclerotiorum. Arch. Mikrobiol.* **1973**, *90*, 59-64.

15. Noyes, R.D.; Hancock, J.G. Role of oxalic acid in the *Sclerotinia* wilt of sunflower *Physiological Plant Pathology.* **1981**, *18,* 123-132.

16. Margro, P.; Marciano, P.; Di Lena, P. Enzymatic oxalate decarboxylation in isolates of *Sclerotinia sclerotiorum. FEMS Microbiology Letters.* **1988**, *49*, 49-52.

17. Huttner, S.; Mecke, D.; Frohlich, K.U. Gene cloning and sequencing, and enzyme purification of the Malate synthase of Streptomyces arenae. *Gene.* **1997,** *188,* 239-246.

18. Loewus, F.A.; Saito, K.; Suto, K.R.; Maring, E. Conversion of D-Arabinose to D-Erythroascorbic acid and oxalic acid in *Sclerotinia sclerotiorum.* Biochemical and *Biophysical Research Communication.* **1995**, *212*, 196-203.

19. Keates, S.E.; Loewus, A.F.; Helms, G.L.; Zink, D.L. 5-O- (α-D-Galactopyranosyl)-D-Glycero-Pent-2 Enono-1, 4-Lactone: characterization in the oxalate –producing fungus, *Sclerotinia sclerotiorum*. *Photochemistry.* **1998**, *49*, 2397-2401.

20. Lorenz, M.C.; Fink, G.R. The glyoxalate cycle is required for fungal virulence. *Letters to the Nature.* **2001**,*412*, 83-86.

21. Huttner, S.; Mecke, D.; Frohlich, K.U. Gene cloning and sequencing, and enzyme purification of the Malate synthase of Streptomyces arenae. *Gene.* **1997,** *188,* 239-246.

22. Eastmond, P.J.; Graham, I.A. Re-examining the role of the glyoxalate cycle in oil seeds. *Trends in Plant Science.* **2001**, *2*, 72-77.

23. Hoyt, J.C.; Robertson, E.F.; Berlyn, K.A.; Reeves, H.C. *Escherichia coli* isocitrate lyase: properties and comparisons. *Biochimica et Biophysica Acta.* **1988**, *966*, 30-35.

24. Burke, J.M.; Rieseberg, L.H. Fitness Effects of Transgenic Disease Resistance in Sunflowers. *Science.* **2003,** *300,* 1250-1256.

25. Mouley, A.; Rumeau D.; Tugaye, M.T.E. Differential accumulation of hydroxyproline-rich glycoprotein transcripts in sunflower plants infected with *Sclerotinia sclerotiorum* or treated with oxalic acid. *Plant Science.* **1992**, *85*, 51-59.

26. **http://www.soybeans.umn.edu/home.htm**

27. Donaldson, P.A.; Anderson, T.; Lane, B.G. Soybean plants expressing an active oligomeric oxalate oxidase from the wheat gf-2.8 (germin) gene resistant to the oxalate-secreting pathogen *Sclerotinia sclerotiorum*. *Physiological and Molecular Plant Pathology.* **2001**, *59*, 297-307.

28. Giczey, G.; Kerenyiz, Z.; Fulop, I.; Hornok, L. Expression of cbl an Exo-β-1-3-Glucanase Gene from Coniothyrium minitans, increasing during Sclerotial Parasitism. *Applied and Environmental Microbiology.* **2001**, 67, 865-871.

29. Favaron, F.; Destro, T.; D'Ovidio, R. Transcript Accumulation of Polygalacturonase Inhibiting protein (PGIP) Pathogen infections in soybean. *Plant Pathology.* **2000,** *82*,103-109.

30. Ferrar, P.H.; Walker, J.R.L. o-Diphenol oxidase inhibition – an additional role for oxalic acid in phytopathogenic arsenal of *Sclerotinia sclerotiorum* and *Sclerotinia rolfsii. Physiology and Molecular Plant Pathology.* **1993,** *43*, 415-422.

31. Maxwell, D.P. Oxalate formation in *Whetzelinia sclerotiorum* by oxaloacetate acetylhydrolase. *Physiological and Plant Pathology.* **1973**, *3*, 279-288.

32. Zhou, T.; Boland, G.J. Mycelial growth and production of oxalic acid by virulent and hypovirulent isolates of *Sclerotinia sclerotiorum. Canadian journal of Plant Pathology.* **1999**, *21, 93*-99.

33. Lane, G.B. Oxalate, Germins, and Higher-Plant Pathogens. *IUBMB life (Review Article).* **2002**, *53*, 67-75.

34. Raymond, P.; Deleage, G.; Rascle, C.; Fevre, M. Cloning and Sequence analysis of a polygalacturonase-encoding gene from the Phytopathogenic fungus *Sclerotinia sclerotiorum. Gene.* **1994**, *146*, 233-237.

35. Akamatsu, Y.; Ma D.B.; Higuchi, T.; Shimada, M. A novel enzymatic decarboxylation of oxalic acid by the lignin peroxidase system of white-rot fungus Phanerochaete chrysosporium. Wood Research Institute. **1990**, *269*, 261-263.

36. Albert, T.; Roberts, D.J. Enzymatic formation of oxalate and acetate from oxalo-acetate. *National Science foundation.* **1956**, *87*, 5126-5127.

37. Gradin, C.H.; Hederstedt, L.; Baltschefsky, H. Soluble Succinate Dehydrogenase from the Halophilic Archaebacterium, Halobacterium halobium. *Archive of Biochemistry and Biophysics.* **1985**, *25*, 200-205

38. Houck, D.R.; Inamine, E. Oxalic Acid Biosythesis and Oxaloacetate Acetylhydrolase Actvity in Cattleya. *Archives of Biochemistry and Biophysics*. **1987**, *259*, 58-65.

39. Rai, J.N.; Dhawan, S. Studies on Purification and identification of Toxic metabolites produced by *Sclerotinia sclerotiorum* causing white rot disease of crucifers. *Indian Phytopathology.* **1976**, *29*, 407-411.

40. Colatelo, Nicholas. Fungal Exudate. *Canadian Journal of Microbiology*. **1978**, *24*, 1178-1181.

41. Sridhar, J.; Eitman, M.A.; Wiegel, W. Elucidation of Enzymes in Fermentation Pathways Used by Clostridium thermosuccinogenes Growing on Inulin. *Applied and Environmental Microbiology*. **2000**, *66*, 246-251.

42. Li, Q.H.; Matsuda, I.; Fujise, Y.; Ichyama, A. Short-chain Acyl-CoA-Dependent Production of Oxalate from Oxaloacetate by Burkholderia glumae, a Plant Pathogen Which Causes Grain Rot and Seedling Rot of Rice via the Oxalate Production. *Journal of Biochemistry*. **1999**, *126*, 243-253.

43. Georgiou, C.D.; Petropoulou, K.P. Role of erythroascorbate and ascorbate in sclerotial differentiation in *Sclerotinia sclerotiorum. Mycol. Res*. **2001**, *105*, 1364-1370.

44. Muller, H.M. Utilization of Gluconate by *Aspergillus niger*. Enzymes of Degradation Pathways and Main End Products. *Zentralbl. Mikrobiol.* **1998**, 141, 461-469.

45. Gloer, J.B. Antiinsectan Natural Products from Fungal Sclerotia. *Acc. Chem. Res.* **1995**, *28*, 343-350.

46. Jung, C.H.; Wells, W.W. Spontaneous Conversion of L- Dehydroascorbic Acid to L-Ascorbic Acid and L- Erythroascorbic Acid. *Archives of Biochemistry and Biophysics.* **1998**, *355*, 9-14.

47. Laemmli, U.K., *Nature,* **1970**, *227*, 680.