The University of Southern Mississippi

# The Aquila Digital Community

Dissertations

Spring 5-2008

# *In Vitro* and *In Vivo* Techniques for Screening New Natural Product-Based Fungicides for Control of Strawberry Anthracnose

Maritza Abril
*University of Southern Mississippi*

Follow this and additional works at: https://aquila.usm.edu/dissertations

Part of the Agricultural Science Commons, Agriculture Commons, Agronomy and Crop Sciences Commons, Biology Commons, Food Biotechnology Commons, and the Fruit Science Commons

## Recommended Citation

The University of Southern Mississippi

*IN VITRO* AND *IN VIVO* TECHNIQUES FOR SCREENING

NEW NATURAL PRODUCT-BASED FUNGICIDES

FOR CONTROL OF STRAWBERRY ANTHRACNOSE

by

Maritza Tatiana Abril-Castillo

A Dissertation
Submitted to the Graduate Studies Office
of The University of Southern Mississippi
in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy
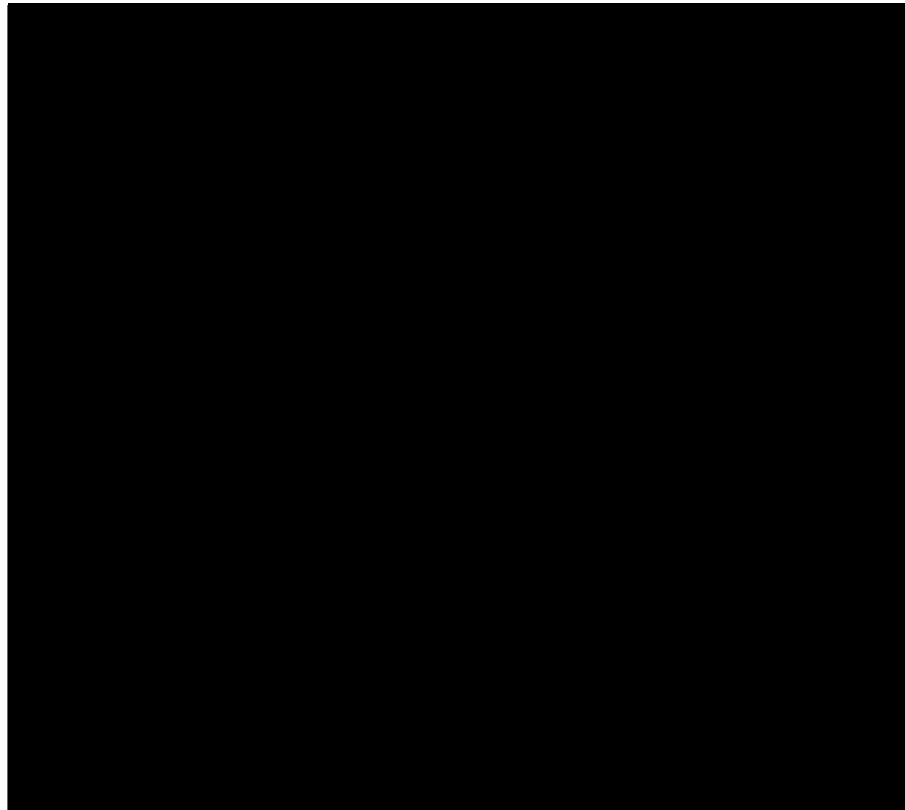
Approved:

May 2008

The University of Southern Mississippi


*IN VITRO* AND *IN VIVO* TECHNIQUES FOR SCREENING

NEW NATURAL PRODUCT-BASED FUNGICIDES

FOR CONTROL OF STRAWBERRY ANTHRACNOSE


by

Maritza Tatiana Abril-Castillo


Abstract of a Dissertation
Submitted to the Graduate Studies Office
of The University of Southern Mississippi
in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy


May 2008

ABSTRACT

*IN VITRO* AND *IN VIVO* TECHNIQUES FOR SCREENING

NEW NATURAL PRODUCT-BASED FUNGICIDES

FOR CONTROL OF STRAWBERRY ANTHRACNOSE

by

Maritza Tatiana Abril-Castillo

May 2008

Seven plant pathogenic fungi (*Botrytis cinerea, Colletotrichum acutatum, C. fragariae, C. gloeosporioides, Fusarium oxysporum, Phomopsis obscurans,* and *P. viticola*) valuable in screening fungicide efficacy were tested. Optimal and reproducible conditions for germination of these selected fungi were established by incorporating Roswell Park Memorial Institute 1640 (RPMI) as a medium of known composition and washing conidia to remove innate germination inhibitors. This step reduced average fungal germination times between 3.5 h and 21.2 h. The natural product-based fungicide, sampangine, seven sampangine analogs (4-bromo-sampangine, 4-methoxysampangine, benzo[4,5]sampangine, liriodenine Mel AMC-XIII-103, onychine, cryptolepine, and liriodenine CDH-II-37), plus seven conventional fungicides (benomyl, captan, cyprodinil, fenbuconazole, fenhexamid, iprodione, and kresoxim-methyl) were tested *in vitro* for their ability to inhibit germination and growth of the targeted fungal species. Microtiter assays demonstrated that sampangine was more efficacious than any of its analogs and than most of the commercial fungicides. In addition, I documented through

microbioassays morphological anomalies in germ tube development caused by 4-bromosampangine. Germ tubes of *B. cinerea* splayed and branched and *C. fragariae* produced germ tubes that branched more frequently than the usual. These anomalies indicate a physical mode of action where appresorial formation and hyphal growth, but not conidial germination, are impaired, suggesting the possibility that 4-bromosampangine may protect hosts after fungi have germinated. The natural product-based fungicides, sampangine and CAY-1, and the commercial fungicide, azoxystrobin, were tested using a detached leaf assay on strawberry (*Fragaria x ananassa* cv. Chandler). All three fungicides reduced anthracnose lesions on the leaves when applied to the host prior to inoculation with the fungal pathogen, but not after inoculation. *In planta* screening procedures exhibited a consistent 10-fold reduction in the number of conida landing on the plant surface. *In vitro* screening procedures demonstrated that conidia adhered to the inner surfaces of spray delivery equipment in high numbers. Practical implications of these findings include incorrectly classifying a fungicide as efficacious or a cultivar as resistant when in reality the amount of inoculum was insufficient to elicit disease.

**TO NICOLAS**

My Sweet Little Angel

You Are the Greatest Miracle

I Have Ever Been Blessed With

# ACKNOWLEDGMENTS

I would like to thank the members of my committee Dr. Kenneth Curry, Dr. Barbara Smith, Dr. David Wedge, Dr. Bryan Kreiser, and Dr. Raymond Scheetz for their advice and support throughout the duration of this project. I especially would like to thank them for providing me with the guidance required never to lose sight of my goals, the inspiration I needed to embrace a whole new level of understanding, the wisdom to perceive the world outside my preconceived conceptions, the strength to endure a painful but rewarding learning experience, and the courage to defy my own fears and reach beyond my own limitations. I thank them for the direction they provided me to strive through new avenues. In particular I want to thank them for properly grooming me with the highest ethical and moral values at the professional environment that I safeguard as my motto in life. I cherish every opportunity they granted me to travel to national and international meetings to get the exposure I so much needed to overcome my shy personality and along the way savor the satisfaction of a well done job in reporting the results of my research. I will always be grateful for the collaborative projects in which they involved me because they have been a rewarding experience and the network of scientists I have joined has allowed me to reach beyond geographical boundaries. I really appreciate their confidence in me. I will always treasure the way they challenged my thought processes, forcing that critical thinking that is at times so evasive. I have greatly benefitted from the opportunities they gave me to excel in penmanship by taking part in the creative process of writing grants and proposals that were fruitful ending in several

# TABLE OF CONTENTS

LIST OF TABLES

Table

LIST OF ILLUSTRATIONS

Figure

CHAPTER I

INTRODUCTION

The focus of my research is the improvement of techniques for reliable

assessment of *in vitro* and *in planta* testing of the efficacy of new fungicides and

identification of potentially resistant strawberry germplasm. Most considerations of the

relationships between fungi and plants focus on the advantages and disadvantages of the

association to the plant while the significance of the role of the fungus is sometimes

overlooked. In addition, disease screening procedures are commonly assessed at the

macroscopic or whole plant level while frequently neglecting the microscopic or (whole)

fungal level.

Accurate characterization of the relationships between plants and fungi is essential

for understanding plant disease. In general terms, fungi that have partial or complete

nutritional dependance on their hosts are referred to as parasites (Bos and Parlevliet,

1995). Organisms that feed on plants from the outside are considered ectoparasites, i.e.,

insects feeding on leaves, while organisms that feed on plants from the inside are

considered endoparasites, i.e., fungi feeding on strawberry plants. Parasites become

pathogens when they incite enough damage to their hosts to produce disease symptoms

(Cooke, 1977). Even though parasitism often leads to disease, not all parasites are

pathogens, and likewise not all pathogens are parasites (Bos and Parlevliet, 1995). The

first scenario is illustrated by fungal latent infections since the fungi can penetrate the

plant and establish themselves within its host, but infection does not necessarily imply the

production of visible disease symptoms (Agrios, 2005). The latter scenario is illustrated

by plants or microorganisms that produce allelopathic substances (Bos and Parlevliet,

1995). Stakman and Harrar (1957) define inoculation as the "process by which spores are brought into contact with plant surfaces." Infection is defined by Agrios (2005) as "the process by which pathogens establish contact with susceptible cells or tissues of the host and procure nutrients from them." Agrios (2005) defined incubation as "the time interval between inoculation and the appearance of disease symptoms." For purposes of this study, I shall address only those plant-pathogenic interactions initiated artificially in the laboratory and greenhouse.

Variation within natural populations in disease levels is common but unpredictable depending on environmental conditions. Therefore, a controlled, experimental approach is needed to measure resistance, where a random sample of individuals from a plant population is experimentally assessed for disease resistance. Difficulties arise in deciding how to measure the resistance of the tested plants. Two general approaches have been used: (1) measuring disease levels after plants have been directly exposed to the pathogen by inoculation and (2) measuring disease levels in the field on naturally randomized experimental plants (Alexander, 1992). Laboratory screening provides pre-inoculation and post-inoculation environments, thereby minimizing variation of the conditions. Inoculum densities are reproducible and different species/isolates of a pathogen can be grown in pure culture. The method by which the pathogen is inoculated onto the plant can be controlled, and ultimately, disease severity can be assessed in laboratory screening procedures (Irwin et al., 1984). Greenhouse studies, of interest here for the identification of efficacious fungicides and disease resistant strawberry germplasm, rely on the artificial inoculation of plants with the pathogen (Irwin et al., 1984). Among the common methods for testing resistance of plants

is the technique of exposing the crop species to an inoculum of the target pathogen species (Niks and Rubiales, 2002). Since variation within treatments is reduced, only a small number of plants are needed to test host resistance for a particular plant/pathogen interaction. Another advantage of greenhouse testing is that single plants or even excised leaves can be used to increase the number of plant populations, isolates, or fungicides that can be tested at once (Alexander, 1992). Studies on variation in resistance have focused on extensive data from inoculation studies of within- and between-population variation in resistance over a broad geographic range or on localized field studies. A complementary approach of greenhouse and field studies allows scientists to establish correlations between resistance patterns measured in the greenhouse and those expressed in the field (Alexander, 1992) since, ultimately, screening under field conditions is the final step in a fungicide testing program.

Pathogenic fungi sprayed on plant surfaces are commonly assumed capable of producing disease (Parbery, 1996). Several studies of *Colletotrichum* spp. have focused on the initial stages of host-pathogen interactions (Perfect et al., 1999; Leandro et al., 2001; Diéguez-Uribeondo et al., 2003). However, early interaction at the microscopic (fungal) level is frequently not considered in screening experiments. Studies addressing aggressiveness and virulence of *Colletotrichum* species may fail to distinguish between the efficacy of the fungicide, the plant being resistant, or the fungi having the appropriate conditions for causing disease.

In studies of the biology, ecology, resistance, biochemistry, and/or epidemiology of *Colletotrichum* species, factors that may affect the physiology of the plant or the physiology and mechanics of the fungal inoculum have not been explored adequately or

may have gone unnoticed since they were not the main focus of the study (Diéguez-Uribeondo et al., 2003; Freeman et al., 2001; Leandro et al., 2003; Pangga et al., 2004; Wharton et al., 2001). The apparent efficacy of a fungicide may depend on the physiological state of the plant, the physiological state of the fungal inoculum, and the mechanics of delivering the inoculum to the plant. While documenting the physiological state of anything this complex in detail may be not possible, some aspects of plant and fungal physiology that may be particularly important in testing fungicides is addressed in the development of a protocol described in my research, thus improving the reliability of fungicide testing in general.

Seemingly minor points, such as not washing the conidial inoculum, can lead to misinterpreted results. The level of conidial germination when spores are not washed is dramatically lower than after washing. But without knowing the level of germination in a screening test, the efficacy of a developmental fungicide might be mistakenly rated high, when in fact, the fungus just failed to germinate at infectious levels for other reasons. It seems that a greater effort in studying the basic biology of the fungi upon landing on the plant surface and monitoring the progress of germination events leading to disease development is desirable.

I have identified several important criteria for reproducible inoculation and germination of *Colletotrichum acutatum* and *C. fragariae* conidia. The next logical step in a screening program for reliable assessment of *in vitro* and *in planta* testing of the efficacy of new natural product-based fungicides and potentially resistant strawberry cultivars would include the application of those parameters.

## Objectives

Two types of *in vitro* assays were conducted. Seven economically important plant pathogens (*Botrytis cinerea, Colletotrichum acutatum, C. fragariae, C. gloeosporioides, Fusarium oxysporum, Phomopsis obscurans,* and *P. viticola*) were tested using these assays to derive complementary information. The new natural product-based fungicide sampangine, seven of its analogs (4-bromosampangine, 4-methoxysampangine, benzo[4,5]sampangine, liriodenine Mel AMC-XIII-103, onychine, cryptolepine, and liriodenine CDH-II-37), and seven commercial fungicides (kresoxim-methyl, azoxystrobin, fenhexamid, iprodione, benomyl, fenbuconazole, and cyprodinil) were evaluated in these assays.

*Objective 1a: Microbioassay.* Determine the effects on the developmental morphology of conidial germination, germ tube elongation, appressorial formation, and hyphal growth of targeted fungi.

*Objective 1b: Microtiter Assay.* Determine the sensitivity of selected plant pathogenic fungi to the natural product-based fungicide, sampangine, its analogs, and seven commercial fungicides.

In this manuscript, microtiter assay will mean assays conducted by challenging fungi with concentrations of natural product-based and commercial fungicides in 96-well cell culture clusters and evaluating fungal growth with a microplate photometer; microbioassay will mean assays challenging fungi with concentrations of natural product-based and commercial fungicides in 24-well cell culture clusters and evaluating growth by direct, microscopic observation.

*In planta* assays were conducted in a greenhouse. The efficacy of natural product-based fungicides sampangine and CAY-1 along with the commercial fungicide azoxystrobin were tested.

*Objective 2a: Detached Leaf Assays for Fungicide Evaluation.* Determine the efficacy and phytotoxicity of the natural product-based and commercial fungicides against *C. fragariae* isolate CF-75 on strawberry leaves from cv. Chandler.

*Objective 2b: Leaf Clearing Technique.* Develop a tool to determine at the microscopic level the efficacy of natural product-based and commercial fungicides as disease control agents in support of routine greenhouse screening procedures to test for resistant strawberry germplasm.

Variables that impact reproducibility of screening techniques were identified. Emphasis was on accountibility of the conidial inoculum to determine levels of both accuracy and precision in artificial inoculations of test plants with pathogenic fungi.

*Objective 3a:* In Planta *Screening Techniques.* To account for the totality of conidia sprayed on the leaf surface of strawberry plants cv. Chandler.

*Objective 3b:* In Vitro *Screening Techniques.* To account for the totality of conidia applied to an artificial glass surface simulating "spot inoculation."

CHAPTER II

LITERATURE REVIEW

Fungal Physico-Chemical Conditions

*Colletotrichum* is a genus of Ascomycete fungi containing many species that cause anthracnose diseases on a wide range of important crops and ornamental plants and represents an ideal model to study fungal-plant interactions in artificial, controlled systems (Perfect et al., 1999). Several *Colletotrichum* species have been used in studies based on traditional artificial inoculation procedures all over the world. These procedures include spraying to run off, spot inoculation, direct application (by rubbing with a swab), or dipping of the specific plant part of interest.

Administration of *Colletotrichum* inocula via spraying until run off has been reported in several studies conducted on anthracnose disease in different pathosystems (Curry et al., 2002; Freeman et al., 2001; Ntahimpera et al., 1999). Spot inoculation was used less frequently (Curry et al., 2002; Vinijsanun et al., 1987). Diéguez-Uribeondo et al. (2003) inoculated almond leaves with 10 µl droplets spaced 5 mm apart while Denoyes-Rothan et al. (1999) inoculated strawberry fruits with 15 µl droplets. Mims and Vaillancourt (2002) took conidia from the cultures by lightly rubbing the colony surface with a sterile cotton swab and applied them directly to the upper surface of maize leaves. Delp and Milholland (1980) injected strawberry crowns with 0.2 ml of the conidial suspension. De Lapeyre de Bellaire et al. (2000) dipped banana fruit in the conidial suspension.

Several factors have proven essential for inducing anthracnose symptoms following inoculation with *Colletotrichum* species in artificial systems. Among those

controlled factors having an influence on disease development are temperature, growth medium, conidial density, culture age, light regime, relative humidity, the use of surfactants (e.g., Tween 20), and washing conidia. Optimum incubation temperature for conidia of *Colletotrichum* species varies from $22 \pm 2\,°C$ to $35 \pm 2\,°C$ (Mould et al., 1991a, 1991b; Simpson et al., 1994; Smith et al., 1999; Wilson et al., 1990).

The media on which different strains of *Colletotrichum* species are grown include potato dextrose agar (PDA), Mathur's medium (MS), strawberry fruit agar (SFA), or modifications of them. PDA is the most common medium (Manaut and Maraite, 1997; McRae and Stevens 1990; Simpson et al., 1994; Wilson et al., 1990; Yang et al., 1990; Zulfiqar et al., 1996). A variation of 1:1 v/v mixture of oatmeal agar and PDA has been included in some studies to increase conidial populations (Smith and Black, 1987; Smith and Gupton, 1993). Modified MS medium was used by Freeman et al. (2001) and Horowitz et al. (2002). SFA was used by Leandro et al. (2001). For long-term conidial storage isolates were grown on PDA amended with streptomycin sulfate and tetracycline, transferred to silica gel, and stored at $4\,°C$ (Khan and Hsiang, 2003; Leandro et al., 2001). McRae and Auld (1988) grew their *C. orbiculare* cultures on water agar sprinkled with irradiated carnation leaf pieces. The size of conidia and appressoria in *Colletotrichum* species may vary depending on the medium on which the fungus is grown (Baxter et al., 1983; Dey, 1933; Gunnell and Gubler, 1992).

Conidial density used to inoculate plant tissues has been consistently reported between $10^4$ and $10^6$ conidia/ml (Freeman et al., 2001; Khan and Hsiang, 2003; Yang et al., 1990; Zulfiqar et al., 1996). Leandro et al. (2001) adjusted their conidial suspensions to a 0.1 ml droplet of $5 \times 10^3$ conidia/ml to inoculate coverslips and to obtain a similar

conidial density to that used on their strawberry leaves. The age of *Colletotrichum*

cultures used to inoculate plant tissues varied from 2 days to 4 weeks (McRae and

Stevens, 1990; Simpson et al., 1994; Smith and Gupton, 1993; Yang et al., 1990; Zulfiqar

et al., 1996).

The light regime to which isolates of *Colletotrichum* species have been exposed

varied from continuous low light (Khan and Hsiang, 2003), continuous fluorescent light

(Mims and Vaillancourt, 2002; Simpson et al., 1994; Smith and Black 1987; Smith and

Gupton, 1993), fluorescent light at 14/10 h photoperiod at 16.7 $\mu mol.m^{-2}. sec^{-1}$ (Curry et

al., 2002), to complete darkness (Wilson et al., 1990). The relative humidity (RH) at

which *Colletotrichum* cultures are kept is rarely reported in the literature. Leandro et al.

(2001) studied conidial germination of *C. acutatum* on plastic coverslips within petri

dishes moist chambers and maintained 100% RH by sealing the petri dishes with

parafilm.

Only a few studies report the addition of any surfactant to the inoculation

procedure of their conidia. Tween 20, the most common surfactant, has been added in

different proportions to increase dispersion of the conidia to the plant surface (Horowitz

et al., 2002). The proportions reported include 0.1% v/v (Delp and Milholland, 1980),

0.02% v/v (Horowitz et al., 2002; Manaut and Maraite, 1997), and two drops of Tween

20 per liter (Smith and Black, 1987; Smith and Gupton, 1993). Other commercial

surfactants including Plus 50 have been added to conidial suspensions (80 $\mu$l per liter)

(McRae and Auld, 1988).

Washing conidia to remove natural germination inhibitors prior to inoculation is a

common part of the inoculation protocol. A conidial suspension is prepared by adding 10

ml of sterile distilled water to the culture plate, gently scraping the agar surface to dislodge conidia, and filtering the resulting conidial suspension through one or two layers of cheesecloth (Curry et al., 2002; Khan and Hsiang, 2003). McRae and Stevens (1990) removed the conidial matrix from the inoculum by centrifuging twice at 11,000 g for 15 min at 15°C, discarding the supernatant, and resuspending the conidial pellet in distilled water each time. Leandro et al. (2001) washed conidia twice by centrifuging at 3,020 g for 2 min, discarding the supernatant, and resuspending the conidial pellet in sterile deionized water each time. De Lapeyre de Bellaire et al. (2000) washed the conidia three times by centrifuging at 4,000 rpm for 10 min, discarding the supernatant, and resuspending the conidial pellet in sterile distilled water each time.

Pathogens may partially or completely loss their pathogenicity when maintained in culture for prolonged periods of time. However some pathogens are capable of recovering their virulence if transferred to their hosts under appropriate conditions (Agrios, 2005). To maintain fungal pathogenicity, strawberry fruit was inoculated with *C. acutatum* every two weeks and the fungus was then reisolated from infected fruits (Ntahimpera et al., 1999; Wilson et al., 1990; Yang et al., 1990). Conidia were scraped from infected fruit and streaked on PDA, and cultures were incubated in the dark for 2–7 days at 25°C. To maintain fungal pathogenicity in their study, McRae and Stevens (1990) inoculated *Xanthium spinosum* with *C. orbiculare*, reisolated it, and maintained it on PDA at 25°C in the dark. To produce conidia for inoculation, the culture was established on water agar sprinkled with irradiated carnation leaf pieces in the dark at 25°C.

Some studies have addressed conidial germination and adhesion to artificial substrates including teflon, mylar, polystyrene petri dishes, plastic coverslips, glass coverslips, glass slides, silicone vacuum grease-coated plastic coverslips, teflon-coated multiwell slides, polystyrene sheets, polystyrene-coated glass slides, dimethyldichlorosilane-coated glass slides, polystyrene microtiter plate wells, 4% agarose, 2% agar, chromatography paper, nylon filter membranes, cellulose acetate filter, cellulose nitrate filter, teflon tape, parafilm, and wood tongue depressors (Chaky et al., 2001; Egley, 1994; Mercure et al., 1994a; Young and Kauss, 1984). Young and Kauss (1984) determined that adhesion of *C. lindemuthianum* conidia to polystyrene surfaces was inhibited by sodium azide (respiratory inhibitor) and antimycin A (transcription inhibitor) and reduced by cytochrome C, hemoglobin, ovalbumin, bovine serum albumin, fetuin, immunoglobulin (proteins), and also by Tween 80. Condial binding was also enhanced by sodium, potassium, calcium, and magnesium chloride (salts). Egley (1994) determined that germination of *C. truncatum* was reduced when conidia were incubated while suspended in water or when incubated on or in partially liquified agar, but increased when conidia were incubated on firm agar. He also noted that more than 50% of the conidia germinated on chromatography paper, cellulose acetate filter, or on plastic cover slips, but very few germinated on cellulose nitrate filter and glass cover slips. Mercure et al. (1994a) obtained the maximum percentage adhesion of ungerminated conidia of *C. graminicola* on polystyrene petri dishes followed by dimethyldichlorosilane (DMS) coated glass slides. They determined that sodium azide and antimycin A had no effect on adhesion of *C. graminicola* conidia to polystyrene surfaces, but brefeldin A (glycoprotein transport inhibitor) and cyclohexamide (protein synthesis inhibitor)

significantly reduced conidial adhesion. However, pronase E prevented adhesion completely, and likewise conidia treated with the lectin Concanavalin A also failed to adhere. Chaky et al. (2001) reported higher values of condial germination of *C. graminicola* on hydrophobic surfaces such as corn leaf, teflon, plastic cover slip, mylar, petri dish, and glass cover slip. Carbon sources including glucose, saccharose, maltose, and yeast extract increased germination in comparison to nitrogen sources such as glutamic acid and ammonium. They also reported that germination remained unaffected by the addition of Concanavalin A, even though appressorial production was reduced.

Other studies have targeted the specific conditions required for optimal development of specialized infection structures. The effects of temperature, carbon dioxide, and metabolic inhibitors on appressoria of several *Colletotrichum* species have been reported in the literature (Dey 1933; Miehle and Lukezic, 1972; Rahe and Kuć, 1970; Skoropad, 1967). Appressoria of *C. lindemuthianum* forms at 27°C (Rahe and Kuć, 1970), appressoria of *C. trifolii* is reduced above 27°C (Miehle and Lukezic, 1972), appressoria of *C. lagenarium* forms between 20–26°C (Ishida and Akai, 1969), but appressoria of *C. graminicola* has a range of 15–35°C (Skoropad, 1967). Cell walls of appressoria in *C. trifolii* produced in an atmosphere low in $CO_2$ exhibited thinner walls and lacked the brown pigment common to "normal" appressoria (Miehle and Lukezic, 1972). The protein synthesis inhibitors cyclohexamine and *p*-fluorophenylalanine blocked germination and appresorium formation in *C. trifolii* (Miehle and Lukezic, 1972).

## Infection Strategies of *Colletotrichum* Species

*Colletotrichum* species use two main strategies for invading host tissues, intracellular hemibiotrophic and subcuticular intramural (Bailey et al., 1992; Perfect et

al., 1999) (Table 1). The initial stages of infection are similar for both strategies.

Conidia adhere to the plant surfaces, germinate, produce germ tubes, and form

appressoria. Upon penetration, subcuticular intramural pathogens develop a network of

hyphae under the cuticle before penetrating deeper into the tissue and obtaining nutrients

from dead host cells which have been killed in advance during a necrotrophic phase

(Bailey et al., 1992; Parbery, 1996). Intracellular hemibiotrophy is the most common

infection strategy among *Colletotrichum* species (Perfect et al., 1999). The length of the

biotrophic phase is variable (Curry et al., 2002; Latunde-Dada et al., 1996; Mould et al.,

1991a, 1991b; Wharton and Julian, 1996). Upon penetration, fungal hyphae grow within

living host cells, obtain nutrients from them and after colonizing one or more host cells,

biotrophic infection hyphae, infection vesicles, and secondary necrotrophic hyphae are

produced (Bailey et al., 1992; Latunde-Dada et al., 1996; O'Connell et al., 1985;

O'Connell 1987; O'Connell et al., 1993; Parbery, 1996; Wharton and Julian, 1996).

Regardless of the initial infection strategy used by *Colletotrichum* species to interact with

their hosts, it is significant that the fungi avoid being recognized by the host plant and that

there are no specific resistance responses, or if there are, that the fungi overcome them

with their specialized infection structures (Green et al., 1995; Mendgen et al., 1996;

Perfect et al., 1999).

<p style="text-align:center">Adhesion</p>

*Hydrophobins in Adhesion and Pathogenesis*

Adhesion of conidia to their hosts is an essential component of the fungal

infection process and therefore considered a requirement for disease development since

adhesion prevents the displacement of the conidia from the plant surface (Hamer et al.,

1988; Mercure et al., 1994b; Nicholson and Epstein, 1991). Conidia of some *Colletotrichum* species can adhere to both host (Curry et al., 2002; Smith et al., 1999; Wharton et al., 2001; ) and artificial substrates (Chaky et al., 2001; Egley, 1994; ; Mercure et al., 1994a; Young and Kauss, 1984), but the chemical and/or physical bases for adhesion remain to be ascertained. Microscopic studies on conidial adhesion of *Colletotrichum* species to plant surfaces are documented by Swinburne (1976) who detected scars left on the host surface after conidia were removed, and O'Connell et al. (1992) reported the presence of a mucilaginous material around germinating conidia. The only direct evidence of conidial adhesion is provided by Young and Kauss (1984) who demonstrated that conidia of *C. lindemuthianum* adhered to bean hypocotyls within one hour after being suspended in water, and the fact that these conidia also adhered to polystyrene surfaces suggests that adhesion is a non-specific event. Mercure et al. (1994a) found that conidia of *Colletotrichum graminicola* adhere to artificial, hydrophobic surfaces immediately after contact with the surface and hours before the onset of germination.

Hydrophobins are small secreted proteins (~100 amino acids) that carry out a broad spectrum of functions in fungal growth and development (Wessels, 1994). They are involved in the formation of hydrophobic structures such as aerial hyphae, conidia, and fruiting bodies (e.g., mushrooms) (Wessels, 1997). In addition they mediate attachment of hyphae to hydrophobic surfaces (Wösten et al., 1994) resulting in morphogenetic changes which are relevant in the initial steps of fungal pathogenesis where the fungus must attach to the hydrophobic surface of the host before penetration takes place (Talbot et al., 1996). Spores of *Magnaporthe grisea* excrete a hydrophobin

that allows them to adhere tightly to hydrophobic surfaces at the apical region (Hamer et al., 1988). Even though surface hydrophobicity has been proven important for adhesion of the conidia of several plant pathogenic fungi ( Sela-Buurlage et al., 1991; Young and Kauss, 1984), adhesion may not always require a hydrophobic surface. That is the case of conidia of *Magnaporthe grisea* that adhere to hydrophobic surfaces and also adhere to hydrophilic surfaces although less efficiently (Hamer et al., 1988).

A remarkable characteristic of hydrophobins is the capacity for self-assembly at any hydrophilic-hydrophobic interface (water-air, water-oil, and water-hydrophobic plant surface) (Wessels, 1994; Wessels, 1997; Wösten et al., 1994; Wösten and Wessels, 1997). It seems that the confrontation of the monomer with a hydrophilic/hydrophobic interface causes a conformational change in the protein and results in the formation of a stable two-dimensional assemblage (amphipathic membrane) in which polar groups face the hydrophilic phase and apolar groups face the hydrophobic phase (Wessels, 1997). Once the hydrophobin is excreted by the fungus, self-assembly is expected to occur in both the side of the spore facing the air and the side facing the hydrophobic plant surface. The hydrophilic fungal wall becomes firmly glued to the hydrophobic plant surface by an insoluble amphipathic membrane even when wet. The hydrophobicity of the surface is sensed by a molecule at the outer wall surface that itself subsequently serves as an adhesive to glue the two incompatible surfaces together (Wösten et al., 1994).

Several investigations support the fact that adhesion by means of hydrophobins generate signals, e.g., via mechanosensitive ion channels in the fungal plasmalemma, that trigger morphogenesis of infection structures (Nicholson, 1996). In addition, abundant transcription of hydrophobin genes is concomitant with early stages of fungal infection

and during disease symptom development (Wessels, 1997; Wösten and Wessels, 1997).

Through gene disruption Talbot et al. (1993) demonstrated that mutants of the

hydrophobin gene exhibited reduced ability to cause disease symptoms that appeared to

result from an impaired ability to undergo appressorial formation caused by the absence

of a hydrophobin-mediated contact between the fungal wall and the hydrophobic surface

leading to the expression of a morphogenetic signal for appressorial formation.

Chaky et al. (2001), Egley (1994), and Young and Kauss (1984) focused their

studies on whether or not the hydrophobicity of the substratum influenced conidial

adhesion.  Studies on the initial attachment of several *Colletotrichum* conidia to natural

and artificial substrates indicate hydrophobic interactions (Mercure et al., 1994a;

Nicholson and Epstein, 1991).  These investigations also showed that, in addition to

surface hydrophobicity, adhesion might be affected by chemical substances and physical

parameters.  Specifically cycloheximide (protein synthesis inhibitor), brefeldin A

(glycoprotein synthesis and transport inhibitor), and sodium azide and antimycin

(respiration inhibitors), significantly reduced conidial adhesion of *C. lindemuthianum, C.*

*musae*, and *C. graminicola* while pronase E prevented conidial adhesion of *C.*

*graminicola* completely (Mercure et al., 1994a; Sela-Buurlage et al., 1991;Young and

Kauss, 1984).  The fact that conidial adhesion in *C. musae* and *C. graminicola* was

inhibited after treatment with a proteolytic enzyme suggests that, for these fungi,

preformed proteins on the surface of the spores are required for their initial attachment

(Mercure et al., 1994b; Sela-Buurlage et al., 1991).  Exposure of conidia to different

temperatures delays their ability to adhere to hydrophobic surfaces (Mercure et al., 1994a;

Sela-Buurlage et al., 1991).  Adhesion of spores to hydrophobic surfaces decreased

significantly as spores aged (Mercure et al., 1994a). These facts provide evidence that in

addition to hydrophobic interactions, conidial attachment in *C. lindemuthianum*, *C.*

*musae*, and *C. graminicola* requires an active metabolic process, including protein

synthesis (Mercure at al., 1994b; Sela-Buurlage et al., 1991;Young and Kauss, 1984).

Furthermore, Mercure et al. (1994a) determined that in *C. graminicola* neither the

extracellular mucilage (essential for conidial survival) nor a mucilage component affected

the ability of conidia to adhere to artificial surfaces.

*Acervulus*

Several species of *Colletotrichum* commonly produce conidia in acervuli

(Nicholson, 1992). This reproductive structure is formed from a hyphal stroma

developing within an intact epidermal and subepidermal layer despite the massive

destruction of host tissue underneath these layers (Curry et al., 2002; Sutton, 1966). Two

types of acervuli have been described. The most frequent type in *Colletotrichum* species

is the pulvinate type and the less common is the hypostromatic type (Bailey et al., 1992;

Sutton, 1966). Pulvinate acervuli are established in epidermal cells and seem to depend

on the mechanical force of the conidiophores, setae, and stromatic tissue to rupture the

epidermal host wall and to perforate the cuticle simultaneously. Development of

pulvinate acervuli in *C. acutatum* and *C. fragariae* was described by Curry et al. (2002).

Hypostromatic acervuli form when the epidermal host wall is ruptured by hyphae from

the stromatic tissue and the cuticle is perforated separately by setae and conidiophores

(Sutton, 1966). This is the case of *C. sublinoleum* where the production of acervuli

involves localized enzymatic penetration of the epidermal cell wall by narrow hyphae

while the cuticle appears to be ruptured mechanically, following the expansion of conidia,

conidiophores, and setae between the cell wall and the cuticle (Wharton et al., 2001).

Hypostromatic acervulus has been reported in all *Colletotrichum* species infecting grasses

(Sutton, 1966).

Lenné et al. (1984) reported observations made on conidial production by setae in

isolates of *Colletotrichum* species from Australia, U.S.A, and Colombia. In culture,

conidia produced from setae are of identical size as those produced in conidiophores,

while on the host, conidia produced from setae may be identical or even smaller than

those produced on conidiophores. Lenné et al. (1984) also pointed out the existence of

two types of setae produced in an acervulus: fertile setae that produce conidia and sterile

setae that do not produce conidia. Fertile setae are truncate bearing hyaline apices while

sterile setae exhibit darker pointed apices. Conidia produced by conidiophores embedded

in a matrix in the acervulus are dispersed by rain drops and insects while conidia

produced on setae are readily dislodge by air (Lenné et al., 1984).

*Conidial Matrix*

Whether produced in acervuli on infected plant tissue or in culture, conidia of

many *Colletotrichum* species are encased in a hydrophilic mucilaginous material, referred

to as a spore matrix (Curry et al., 2002; Kuo, 1999; McRae and Stevens, 1990; Mims et

al., 1995; Mould et al., 1991a; Nicholson, 1992). This water soluble mucilage may

completely surround conidia, or it may be confined to the spore apices (O'Connell et al.,

2000). This matrix is composed mostly of polysaccharides, glycoproteins, and enzymes

including invertase, polygalacturonases, cellulase, pectin lyase, protease, cutinase,

laccase, β-glucosidase, non-specific esterase, non-specific hydrolase, DNase, RNase, and

alkaline phosphatase (Bergstrom and Nicholson, 1981; Bonnen and Hammerschmidt,

1989a, 1989b; Louis and Cooke, 1985b; Louis et al., 1988; McRae and Stevens, 1990; Nicholson and Moraes, 1980; Porter, 1969; Ramadoss et al., 1985). However, the contribution of these enzymes to supply nutrients during germination, penetration of plant surfaces, or subsequent growth within tissues is not supported by direct experimental evidence. The principal component of the mucilage of *C. graminicola* is a mixture of high molecular weight glycoproteins including mannose, rhamnose, galactose, and glucose and relatively high levels of hydrophobic (aspartic acid, glutamic acid, proline, glycine, alanine, valine, methionine, ilsoleucine, leucine, tyrosine, phenylalanine, histidine, lysine, and arginine) and hydroxylic amino acids (threonine and serine) (Ramadoss et al., 1985). The glycoprotein composition of this mucilage also includes an unusually high concentration of proline (Ramadoss et al., 1985). These proline-rich proteins are responsible for selective binding to toxic phenolic compounds and they protect conidia from toxic secondary plant metabolites such as tannins (Nicholson et al., 1986). By detoxifying phenolic compounds these proteins ensure that conidia are not inhibited or killed as they are dispersed from the acervulus to new potential infection sites (Nicholson et al., 1989).

The matrix has several roles. It is responsible for the antidesiccant properties of mucilage and assists in the survival of conidia during periods of drought (Nicholson and Moraes, 1980). The fact that spores lacking the matrix and exposed to high relative humidity exhibited extensive disruption of their cytoplasm, such as lack of vacuolation, suggests that the matrix is associated at least in part with preventing spore dessication (Nicholson and Moraes, 1980; Louis et al., 1988). In addition, since spore dessication does not occur at 100% relative humidity in spores devoid of matrix, the matrix may

suppress spore metabolism and prevent the use of nutritional reserves in an environment not conducive to germination (Nicholson and Moraes, 1980). These findings question the assumption that species of *Colletotrichum* only require wet conditions for germination and dispersal and lead to the realization that as conidial masses become dry they may be dispersed by wind as particulate matter (Nicholson, 1992). Therefore, as spore viability is retained within the dry conidial masses by the matrix that binds spores together in the acervulus, it seems that those conidial masses can act as a secondary inoculum for spreading the pathogen and subsequent disease development even under field conditions where relative humidity is variable and the availability of water is of periodic duration. Gemination of these plant pathogens requires the release of conidia from conidial masses and the dissemination of spores occurs readily in the presence of water, while dry spore masses are distributed by the wind (Nicholson and Moraes, 1980). There is also evidence that the matrix maintains viability of spores under conditions of low humidity, e.g., within dry acervuli or within dispersed dry spore masses by reducing spore metabolism to the extent that intracellular nutrients essential for germination are not depleted (Nicholson and Moraes, 1980). It may also protect spores from extremes of temperature and ultra-violet light (Nicholson et al., 1986).

Several studies have shown that the spores remain within acervuli, however, self-inhibitory substances in the matrix prevent premature germination of conidia and ensure that ungerminated, viable inoculum can be dispersed along the plant surface where secondary infections can occur (Leite and Nicholson, 1992; Louis and Cooke, 1985a). The nature of some inhibitors has been established (Arpin and Bouillant, 1981; Seebach et al., 1989). A gloeosporone has been identified in *C. gloeosporioides* (Seebach et al.,

1989) and two inhibitors have been identified in the mucilage of *C. graminicola* (Arpin and Bouillant, 1981; Leite and Nicholson, 1992). One of the inhibitors of *C. graminicola* is a water soluble inhibitor of low molecular weight classified as a mycosporin-alanine (Arpin and Bouillant, 1981; Leite and Nicholson, 1992). The principal effect of this inhibitor appears to be to prevent germination within the acervulus and to inhibit spore germination and appressorium formation until conditions that ensure dispersal of the pathogen are favorable (Leite and Nicholson, 1992). The second inhibitor present in the matrix of *C. graminicola* has a volatile nature and appears to influence the basal metabolism of conidia by reducing the rate of lipid reserve depletion (Leite and Nicholson, 1992). In the presence of this inhibitor, conidia of *C. graminicola* survive up to 70 days at 25°C after which they lose viability, but in the absence of such inhibitor, their survival is limited to a few days, presumably because of the depletion of lipid reserves (Leite and Nicholson, 1992). This fact is relevant when considering the long term survival of plant pathogens that are poor competitors in the field (Vizvary and Warren, 1982; Lipps, 1983).

Evidence that the conidial matrix directly influences the infection process is less well documented, although McRae and Stevens (1990) showed that the addition of a matrix to a conidial inoculum will expedite the onset of anthracnose symptoms in maize. Invertase and hydrolase are two enzymes identified among the components of the mucilage that may affect the ability of *C. graminicola* to survive and to function as a sorghum and maize pathogen (Bergstrom and Nicholson, 1981). Even though the functional significance of both enzymes for survival of conidia and their role in disease development have yet to be ascertained, it has been suggested that invertase is involved in

the acquisition of carbon by the germinating spores and that hydrolase activity accounts for the partial degradation of the maize leaf cuticle and therefore leads to more efficient penetration (Bergstrom and Nicholson, 1981). Invertase and hydrolase enzymatic activity in the spore matrix is not eliminated by desiccation of the matrix at relative low humidity indicating that under drought conditions in the field these enzymes would be present but they would be inactive (Nicholson and Moraes, 1980). The importance of cutinase enzymes to fungal pathogenicity has been associated with the penetration of the host cuticle (Kolattukudy, 1985). The assumption that cutinases are required by plant pathogens remains controversial (Bonnen and Hammerschmidt, 1989a, 1989b). However, the function of cutinases may be involved in surface recognition or adhesion rather than in pathogenesis (Pascholati et al., 1993).

## Germination

Even though *Colletotrichum* conidia respond to physical and chemical signals from the plant surface to undergo germination and differentiation into appressoria (Perfect et al., 1999), they also react to environmental signals such as light and temperature (Emmett and Parbery, 1975). Thigmotrophic responses direct germ tubes on host surfaces and enable plant pathogens to recognize an array of anticlinal walls or the stomatal openings (Hoch and Staples, 1991). Germination and appressorium formation are selectively triggered by chemical inducers such as ethylene produced by the host which may occur at ripening as has been reported for *C. gloeosporioides* infecting avocado and *C. musae* infecting banana (Podila et al., 1993; Kolattukudy et al., 1995). Once conidia of *C. gloeosporioides* land on developing fruit they germinate and penetrate

the host cuticle after being triggered by the surface wax, but then remain latent until the fruit ripens (Kolattukudy et al., 1995).

Ultrastructural studies have shown that conidia of diverse *Colletotrichum* species have several features in common. Ungerminated, young conidium harvested from 5–7 day old cultures of *C. gloeosporioides*, *C. graminicola*, and *C. truncatum* are characterized by lipid bodies that constitute the main endogenous nutritional reserve and occupy most of the cytoplasm (Kerrigan and Mims, 1993; Mims et al., 1995; Schadeck et al., 1998; Van Dyke and Mims, 1991). Mitochondria, microbodies, microtubules, multi-vesicular bodies, ribosomes, elements of smooth or tubular endoplasmatic reticulum (ER), short strands of rough ER, simple cisternal elements, Woronin bodies, and numerous small vacuoles with electrodense contents are also present in young conidia of *C. graminicola* and *C. truncatum* (Van Dyke and Mims, 1991; Mims et al., 1995; Schadeck et al., 1998). Most conidia are uninucleate with a centered nucleus and most of the structures are oriented roughly parallel to the long axis of the conidium.

Older conidia of *Colletotrichum* species harvested from 30–35 day old cultures exhibit significantly different changes in the ultrastructure. These changes are indicators of physiological changes that affect the ability of the conidia to survive as they age. They include a decrease in the ability of conidia to germinate in old cultures indicating the onset of senescence in the population (Leite and Nicholson, 1992). This decreased viability of older conidia seems to be related to the depletion of their primary energy reserves, glycogen and lipids, as they contain fewer and smaller lipid bodies and lack the large glycogen deposits. In addition, evidence of autophagic vacuolar activity indicates that these conidia undergo nutrient stress (Mims et al., 1995; Schadeck et al., 1998,

2003). Older conidia also are highly vacuolated and contain numerous, long strands of rough ER and extensive arrays of tubular ER. Mitochondria are conspicuous in old conidia along with numerous microbodies arranged in clusters (Van Dyke and Mims, 1991; Mims et al., 1995). Woronin bodies, multi-vesicular bodies, and microtubules are present in old conidia, as well as simple cisternal elements even though fewer vesicles are associated with these structures than in young conidia. The conidial wall is similar in young and old conidia of *C. graminicola*, *C. truncatum*, and *C. fragariae* and consists of a thicker inner electron transparent layer and a very thin outer layer composed of fine electron dense fibers (Curry et al., 2002; Mims et al., 1995; Van Dyke and Mims, 1991). The inner wall layer labels with wheat germ agglutinin-gold (WGA) complexes indicating the presence of chitin (Curry et al., 2002; Mims et al., 1995; Van Dyke and Mims, 1991). The outer wall layer of the conidium of *C. graminicola* labels with $\propto$-amylase-gold complexes specific for $\propto$-1,4 glucans (Mims et al., 1995).

*Germ Tube Formation*

The events leading to germ tube formation have been described for *C. truncatum* by Van Dyke and Mims (1991). Most conidia of *Colletotrichum* species contain a single nucleus that undergoes mitosis (Kuo, 1999; Skoropad, 1967; Staples et al., 1976; Van Dyke and Mims, 1991). A septum develops near the middle of the conidium, resulting in two uninucleate cells. Just prior to or during septum development, a germ tube emerges laterally, usually near one end of the conidium. Upon septum formation, a central pore and Woronin bodies are associated with it. Woronin bodies are also present in the tips of developing germ tubes (Van Dyke and Mims, 1991). The inner wall of the septum laid down by *C. fragariae* labels with WGA (Curry et al., 2002). After a couple of hours the

nucleus of the germinating cell moves into the germ tube and divides mitotically. One of the resulting daughter nuclei moves back into the conidium and positions itself near the base of the germ tube while the other moves into the germ tube tip which by this time has curved sharply (Van Dyke and Mims, 1991).

During germination organelles move from the germinating conidial cell into the developing germ tube. However, the germinating cell of *C. truncatum* never becomes highly vacuolated or conspicuously devoid of organelles, even though some germ tubes are larger than the parent conidium (Van Dyke and Mims, 1991). The cytoplasm of germ tubes is highly organized. The cytoplasm of the germ tube of *C. truncatum* is very dense and exhibits the same organelles present in germinating conidia. A cluster of vesicles is located in the apical area and behind it several mitochondria are present. Endoplasmic reticulum and the Golgi bodies are distributed throughout the cytoplasm except within the apex. The shape of the hyphal tip wall is determined by a continuous apical secretion of wall glycoproteins (processed in the Golgi apparatus) and polysaccharides, such as chitin and glucans, that are assembled into microfibrils at the apex of the hyphae as a result of hydrogen bonding and cross linking of adjacent polysaccharide chains that become rigid as they move outward (Wessels, 1993, 1994). This process seems to be mediated by the discharge of cytoplasmic vesicles along the walls that migrate from the Golgi apparatus to the hyphal tip and interact in the middle of the apex with the Spitzenkörper (Hardham, 1992; Heath, 1990). A correlation between vesicle production and morphogenesis in hyphae has been established since the accumulation of actin in the hyphal tip supports the assumption that the movement of vesicles appear to be directed or mediated by the cytoskeleton. At the hyphal tip, actin forms a net that appears to reach into the

Spitzenkörper (Bourett and Howard, 1991). Wessels (1990) suggested that this actin net contributes to the firmness of the hyphal apex in the extension zone where vesicle fusion, synthesis of wall polymers, and protein secretion occur. The Spitzenkörper seems to function as a center of microtubule nucleation and organization (Roberson and Vargas, 1994), it provides directional mass transport of vesicles toward the hyphal apex (López-Franco et al., 1995), and it may also control vesicle secretion of enzymes required for host cell wall degradation. The Spitzenkörper has in general a central position in the apical dome of the hyphal tip, but in the germ tubes of *Colletotrichum* it is eccentric and is located near the substrate where it might help recognize topographical features of the plant surface (Roberson and Vargas, 1994).

Developing germ tubes and appressoria produce large amounts of additional extracellular, material consisting of a distinct coating of electron dense, fibrillar material (Kuo, 1999; Van Dyke and Mims, 1991). This material blends into the remnants of the matrix initially surrounding the conidia to the point that they cannot be differentiated. During germination some of the matrix persists around the conidia, although it changes its appearance from finely granular to a loose network of interconnected strands (Van Dyke and Mims, 1991).

Germ tubes grow to various lengths before forming appressoria (Van Dyke and Mims, 1991). The length of the germ tube of *C. acutatum* is variable and it appears to relate directly to moisture conditions, with wetter conditions favoring longer germ tubes (Van Dyke and Mims, 1991). Apical growth cessation coincides with the sharp curving of the tip of the germ tube and seems to be triggered by contact with an artificial or

natural surface. The tip of the germ tube begins to swell and subsequently differentiates into an appressorium (Van Dyke and Mims, 1991).

During the germ tube and appressorial formation concomitantly with aging of the conidia of *C. graminicola*, and *C. gloeosporioides*, lipid deposits are mobilized and digested by vacuoles until they eventually disappear and the vacuoles become enlarged (Kerrigan and Mims, 1993; Schadeck et al., 1998). The vacuolar system plays an important role during the germination process of *C. graminicola* where the initial stages of lipid metabolization take place. Large vacuoles have been also reported in the appressoria of *C. graminicola* and *C. lindemuthianum* (O'Connell et al., 1985; Politis, 1976; Politis and Wheeler, 1973).

*Appressorium Differentiation*

Some *Colletotrichum* species form differentiated appressoria. Appressoria have been described as swollen structures attached to conidia or at the ends of distinct germ tubes or at the tips of mycelial branches that acquire different forms such as globose (*C. lindemuthianum* and *C. sublinoleum*), and with or without lobes (Cox and Irwin, 1988; Dey, 1933; Sutton, 1966; Wharton et al., 2001). Appressoria of different species differ in size (O'Connell et al., 1992, 1996).

Several experiments have been carried out to characterize stimuli and mechanisms acting during appressorial formation. In *Colletotrichum* differentiation of appressoria requires a specific sequence of nuclear events. During germination, a septum is produced by conidia of *C. graminicola* (Skoropad, 1967) and *C. truncatum* (Staples et al., 1976; Van Dyke and Mims, 1991) following mitosis. The septum delimits the tip that eventually differentiates into a swollen appressoria (Van Dyke and Mims, 1991). One

nucleus migrates into the germ tube where a second mitosis occurs and a second septum is formed. The result is the presence of a single nucleus in the cell that subsequently develops into an appressorium. During maturation of appressoria, another mitosis takes place generating a mature binucleate appressorium. Upon infection one nucleus moves with the infection peg into the infected tissue, while the other remains in the appressorium.

Mature appressoria of *C. sublinoleum* contain abundant mitochondria, lipid globules, glycogen granules, polyribosomes, multi-vesicular bodies, and vacuoles. Appressoria are surrounded by extracellular matrix material. Commonly, the area of appressorial cell wall in contact with the host cuticle is small. The appressorial wall consists of two distinct layers: an outer thin highly electron opaque and an inner thicker moderately electron opaque. A third electron lucent layer is deposited at the base of the appressorium, between the inner wall layer and the plasma membrane, forming a thickened ring around the penetration pore that is continuous with the cell wall of the infection peg which emerges through the pore and penetrates the host cuticle and epidermal cell wall directly (Wharton et al., 2001).

During penetration of plant surfaces, the basal portion of the appressorial wall of *C. lindemuthianum* (O'Connell et al., 1985; Xuei et al., 1988), *C. gloeosporioides* (Brown, 1977), and *C. trifolii* (Mould et al., 1991a) in contact with the host cuticle forms a pore. The pore wall is very thin but frequently has reinforced borders and the structure becomes the appressorial cone (Landes and Hoffman, 1979a). This cone seems to be an extension of the infection peg wall and probably directs hydrostatic pressure into the penetration site (Landes and Hoffman, 1979b; Wolkow et al., 1983). This pore provides

an opening for the emerging penetration hyphae. The appressorial wall surrounding the pore is prolonged inside the appressorium forming a funnel-shaped structure called a collar. The cone is formed upon the collar and extends beyond the collar edge within the appressorium to the pore where it is continuous with the wall of the emerging hypha (Brown 1977; Landes and Hoffman, 1979b; Mould et al., 1991a; O'Connell and Bailey, 1991; Xuei et al., 1988). *Colletotrichum graminicola* and *C. truncatum* produce only a pore from which the penetration hypha develops (Politis and Wheeler, 1973; Van Dyke and Mims, 1991). The absence of an appressorial cone in several *Colletotrichum* species including *C. lindemuthianum* I57 and *C. graminicola* (Politis and Wheeler, 1973) suggests that these morphological variations could be related to the range of plants these pathogens attack and with the different mechanisms used for successful pathogenesis. The penetration pore of *C. lagenarium*, *C. trifolii*, and *C. lindemuthianum* is surrounded by a funnel shaped elaboration of the appressorial wall called the appressorial cone (Landes and Hoffman, 1979a, 1979b; Mercer et al., 1971; Mould et al., 1991a; Xuei et al., 1988). The appressorial cone is absent in *C. sublinoleum*, *C. graminicola*, *C. destructivum*, and *C. truncatum* and the penetration pore is surrounded by an annular wall thickening called the pore wall overlay (Latunde-Dada et al., 1996; Mims, 1991; Politis, 1976; Politis and Wheeler, 1973; Wharton et al., 2001). Deformation of the cuticle and localized changes in staining properties of the host cell wall caused by *C. sublinoleum* around the infection peg suggests that penetration involves both mechanical force and enzymatic dissolution (Wharton et al., 2001).

Fungicides

*Studies of Mode of Action of Fungicides*

Sisler (1996) points out several reasons for the importance of modes of action studies of fungicides. They allow identification of pathways and target sites that support the development of new and more effective fungicides. This is the case of the sterol biosynthetic pathway where mode of action studies revealed the existence of several potential target sites for various fungicides. Mode of action studies also identify systemic fungicides useful for elucidation of biological and biochemical processess since such fungicides are highly effective inhibitors of specific cellular processes. Benomyl represents a great example since this fungicide selectively binds to fungal tubulin (Davidse, 1986). In addition, mode of action studies provide useful information on potential hazards of fungicides to nontarget organisms including plants, mammals, birds, and insects (Lyr, 1995). The knowledge of the risks involved in the use of synthetic compounds with toxic effects that are environmentally undesirable represents a subject of particular concern among all the parties involved including growers, research laboratories, and manufacturers. Mode of action studies provide the basis for understanding fungal resistance. Mode of action studies allow identification of target sites which enhance fungicide resistance management programs by guiding the selection, combination, and/or alternation of fungicides with different target sites to minimized cross-resistance (Dekker, 1995). These types of studies provide an understanding of resistance mechanisms leading to appropriate measures such as the use of fungicide analogs (Kato et al., 1984). In summary, mode of action studies provide the knowledge required for the development of more effective fungicides and their improvement and

maintenance. Furthermore, potential target sites that remain unidentified can only be detected by mode of action studies and screening techniques.

A distinction between a physical and a biochemical mode of action of a fungicide has been addressed in the literature. The term physical mode of action is used to describe "the observable effects of a fungicide on the host-pathogen interaction with respect to the timing and/or placement of the fungicide application (e.g., protectant, post-infection (curative), post-symptom, and vapor activities)" (Wong and Wilcox, 2001). On the other hand, the term biochemical mode of action refers to the fungicide activity targeted at a specific site in the pathogen. Genetic changes by the fungus or a naturally insensitive fungus have a much greater chance to overcome the fungitoxic effect resulting in the development of resistance since they only affect one biochemical function or pathway (Rimelspach and Boehm, 2005).

*Types of Fungicides by Function*

*Multi-site inhibitors.* Multi-site inhibitors are non-systemic and are not susceptible to the development of resistance (Köller, 1994). These organic fungicides were introduced in the market between 1940 and 1950. Dithiocarbamates (ferbam and thiram) were the first organic fungicides manufactured followed by the ethylene *bis*-dithiocarbamates (zineb, maneb, and propineb). Organic fungicides containing *N*-trichloromethylene thio groups included captan, captafol, folpet, tolyfluanid, dichlofuaniol, and dithianon. All these fungicides are still in use although restrictions have been applied to reduce toxicological effects on nontarget organisms and the environment (Köller, 1994). Captan is one of the oldest R-S-CCl$_3$ phthalimide fungicides

that inhibits spore germination by inhibiting several enzymes involved in the energy supply process (Leroux, 1996).

All these organic fungicides have a common biochemical mode of action (Buchenauer, 1990). Numerous enzymes in many organisms contain a SH group as an essential component of their biocatalytic activity. SH groups are highly reactive and are chemically modified by the fungicide itself, or by a highly active conversion product originated from the fungicide. The chemical modification leads to the deactivation of the respective enzyme and the network of interconnected enzymatic reactions within a fungal organism is disrupted at crucial points. As a result, all of these fungicides have a biochemical mode of action where damage to the fungal cell is imminent and the fungitoxic action of the fungicide leads to the death of the fungal cell (Buchenauer, 1990).

Unfortunately, enzymes with SH groups are abundant in nature and not restricted to fungal organisms. Therefore, a multi-site biochemical mode of action implies that not only fungal but also plant enzymes are susceptible to inhibition and plant tissue would be severely damaged in the case when multi-site fungicides penetrate the plant (Buchenauer, 1990). Since phytotoxic effects are undesirable in crop production, multi-site fungicides must be restricted to the cuticle (Köller, 1991). Consequently, multi-site inhibitors exhibit protective activity only interfering with the pathogen before it becomes established underneath the cuticle (Köller, 1994). Such protective activity is limited since fungicides cannot stop disease development after the cuticle has been penetrated and subcuticular hyphae are established (Köller, 1991). For this reason, the stages of fungal

development accessible to the inhibitory action of multi-site fungicides are spore germination, germ tube elongation, and appressorial formation (Köller, 1994).

Measures of disease control promote the avoidance of unnecessary fungicide applications and/or applications as required (Köller, 1994). At this point addressing how long after infection has taken place treatment with protective fungicides should be initiated becomes relevant. To address this issue the classification of fungicide activities according their physical mode of action provided by Szkolnic (1981) and refined by Köller (1994) becomes rather useful:

(a) Protectant activity: refers to the ability of a fungicide residue to prevent conidia from penetrating the cuticle. Consequently, the residue must be on the plant before infection is initiated.

(b) Post-infection or kick-back activity: refers to the ability of a fungicide to completely prevent growth and development of the pathogen, if applied within a given period after infection has occurred.

(c) Pre-symptom or curative activity: is defined as an extension of the post-infection activity. A fungicide with curative properties will allow small chlorotic lesions to develop when applied following an infection period. However, it will reduce the production of conidia from those lesions.

(d) Post-symptom or eradicant activity: refers to the ability of a fungicide to prevent the continued production of spores when applied to an actively sporulating lesion. Pre-symptom and post-symptom activity reduce the spread of secondary inoculum. However, since the secondary inoculum

Under selective pressure by the fungicide such populations build-up and become more competitive and their frequency increases until control of the disease is no longer possible (Dekker, 1995).

*Types of Fungicides by Chemical Class*

Kresoxim-methyl, azoxystrobin, benomyl, cyprodinil, fenbuconazole, fenhexamid, and iprodione are highly fungal-specific and have various degrees of post-infection, pre-symptom, and/or post-symptom activities. The physical modes of action of these fungicides have been previously documented ( Duben et al., 2002; Hänßler and Pontzen, 1999; Latorre et al., 2002; Leroux and Gredt, 1995; Leroux et al., 1998; Richmond and Pring, 1971; Rosslenbroich and Stuebler, 2000; Soto-Estrada et al., 2003; Ypema and Gold, 1999).

*Strobilurins*. Strobilurins include all natural and synthetic compounds based on the lead molecule, strobilurin A, derived from the basidiomycete *Strobilurus tenacellus* (Ypema and Gold, 1999). Kresoxim-methyl and azoxystrobin are members of the strobilurin class which includes broad spectrum fungicides that provide control against oomycetes, ascomycetes, and basidiomycetes (Wong and Wilcox, 2001).

The biochemical mode of action of the strobilurins includes their ability to inhibit mitochondrial respiration. Strobilurins bind to the ubiquinonone oxidation center of cytochrome b and consequently hinder the transfer of electrons from ubiquinone to cytochrome $c_1$ in fungi (Gold et al., 1996; Ypema and Gold, 1999). This reduces drastically aerobic energy production, thereby inhibiting growth of the fungus (Leinhos et al., 1997).

Azoxystrobin. Azoxystrobin was first registered in 1997 as a systemic fungicide for control of black rot, powdery mildew, downey mildew, Phomopsis cane, leaf spot, Botrytis bunch rot, rice blast, apple scab, and Septoria (Baldwin et al., 1996; Wong and Wilcox, 2000, 2001). It has been used worldwide to control disease on cereals, rice, potatoes, tomatoes, cucurbits, vegetables, peanuts, turf, ornamentals, stone fruit, and other fruit crops such as citrus, grapes, bananas, and apples (Baldwin et al., 1996).

The physical mode of action of azoxystrobin has been determined in greenhouse, field trials, and growth chamber conditions (Bertelsen et al., 2001). Azoxystrobin inhibits spore germination and hyphal growth of *Alternaria alternata* and *Cladosporium macrocarpum* on wheat (Bertelsen et al., 2001). Wong and Wilcox (2001) reported the physical mode of action for azoxystrobin against *Plasmopara viticola* on grapevine downey mildew as that of a protectant fungicide with strong eradicant activity and also with effective translaminar activity. In addition, azoxystrobin exhibits protectant and curative activity against *Mycosphaerella* spp. (Bertelsen et al., 2001) and protectant, curative, and eradicant activity against *Cercospora beticola* and *Erysiphe betae* on sugar beet (Anesiadis et al., 2003).

Although azoxystrobin is one of the first fungicides from the strobilurin group, certain fungi that attack wheat developed resistance to it in Germany around 1998. Azoxystrobin resistance spread throughout wheat crops in Europe the following year (Ishii et al., 2001). In Japan resistance to azoxystrobin has spread to fungi on cucumber crops (Ishii et al., 2001). Reduced disease control of potato blight caused by *Alternaria solani* was reported in the U.S.A in 2000 (Pasche et al., 2004). In order to minimize the risk of resistance development, azoxystrobin is used in limited number of times during a

growing season and in addition is rotated with fungicides that have different modes of action (Wong and Wilcox, 2002).

Kresoxim-methyl. Ypema and Gold (1999) compiled extensive studies conducted under laboratory, greenhouse, and field conditions that document the physical mode of action of kresoxim-methyl. These studies identify the developmental stages inhibited by this fungicide against phytopathogenic fungi. On detached leaf assays kresoxim-methyl exhibits protectant effects on germination and sporulation of the rust fungi *Puccinia recondita* and *Uromyces appendiculatus*, and the powdery mildews *Podosphaera leucotricha* on apple, *U. necator* on grape, *Sphaerotheca fuliginea* and *Erysiphe cichoracearum* on cucumber, and *Erysiphe graminis* f. sp. *tritici* and *hordei* on wheat and barley (Ypema and Gold, 1999). In addition, greenhouse and field studies have confirmed the efficacy of kresoxim-methyl against germination and sporulation of *Venturia inaequalis, E. graminis* f. sp. *tritici, P. recondita, U. necator, U. appendiculatus, P. leucotricha, E. cichoracearum, E. graminis* f. sp. *tritici* and *hordei,* and *S. fuliginea* (Ypema and Gold, 1999).

Greenhouse studies reporting the inhibitory effect of kresoxim-methyl on mycelial growth have been documented for many powdery mildew fungi since their hyphae develop exclusively on the leaf surface rendering all developmental stages accessible to this fungicide (Ypema and Gold, 1999). Kresoxim-methyl provides protectant, post-infection, and/or eradicant activity against scab, powdery mildew, and downy mildew of apple, grape, cucurbits, pear, rose, lettuce, broccoli, cherry, cereals, and pecan (Gold et al., 1996; Leroux, 1996; Olaya and Köller, 1999; Ypema and Gold, 1999).

The translaminar activity of kresoxim-methyl has been observed in several host-pathogen systems including *U. appendiculatus* on bean, *E. graminis* f. sp. *tritici* and *Puccinia recondita tritici* on wheat, *E. graminis* f. sp. *hordei* on barley, *V. inaequalis* on apple, *U. necator* and *Plasmophora viticola* on grape, and *Eryphise chicoracearum* on cucumber (Gold et al., 1996). The vapor phase activity of kresoxim-methyl was observed by its inhibitory effect on germination of *V. inaequalis* on apple (Gold et al., 1996).

The first report of field resistance to strobilurin fungicides was confirmed in wheat powdery mildew in Germany in 1998 and a few years later in Japan (Ishii et al., 2001). It has been determined that a single mutation in the cytochrome b gene, resulting in the substitution of glycine by alanine, caused resistance in isolates of the downy mildew *Podosphaera fusca* (Ishii et al., 2001).

*Benzimidazoles*. The group of benzimidazole fungicides includes benomyl which was introduced as a systemic fungicide in the market in the early 1970s (Delp, 1987). The physical mode of action of benomyl includes inhibition of several developmental stages of important plant pathogenic fungi (Köller, 1994). Benomyl exhibits post-infection activity for the control of Botrytis blossom blight and Botrytis fruit rot in blueberries (Smith, 1998), protectant activity for the control of rust in stone crops (Soto-Estrada et al., 2003) and activity for the control of blue mold in apples (Rosenberg et al., 1979).

The biochemical mode of action of benzimidazole fungicides is the specific binding to fungal β-tubulin and consequent disruption of microtubule function (Hippe, 1991). Microtubules constitute the cytoskeleton of the cell and maintain the integrity of the Spitzenkörper which contains the material necessary for cell wall synthesis and

indicates the direction of cell expansion (Howard and Aist, 1980). Benzimidazoles cause a gradual disappearance and redistribution of the Spitzenkörper and as a consequence microtubules no longer remain as ubiquitous components in the apical cytoplasm (Richmond and Pring, 1971). Presumably, the reduction of cell expansion is a result of the disruption of microtubule mediated transport of cell wall material (Howard and Aist, 1980). In addition, inhibition of microtubule assembly disturbs cellular processes that depend on microtubules such as mitosis, meiosis, the intracellular transport of molecules, and the movement of organelles such as chromosomes (Davidse, 1986).

Resistance to β-tubulin occurs by a mutational change of a single nucleotide that results in a single amino acid substitution (Davidse, 1987). Development of field resistance to benomyl has been reported in Australia and U.S.A for the blue mold in apples and ornamental crops (Rosenberg et al., 1979; Wicks, 1977; Yourman and Jeffers, 1999). In addition numerous cases of benomyl resistance have been reported for *Colletotrichum* spp., *Mycosphaerella citri*, and *Elsinoe fawcetti* on various crops (Goes and Kimati, 1994; Hewitt, 1998; Peres et al., 2004; Whiteside, 1980a, 1980b).

*Anilinopyrimidines*. Cyprodinil is an anilinopyrimidine fungicide introduced in the market by Ciba-Geigy and currently developed as a systemic fungicide for cereals, grapes, vegetable crops, and deciduous fruits (Heye et al., 1994). On cereals cyprodinil exhibits a broad spectrum of activity against *Pseudocercosporella herpotrichoides*, *Erysiphe graminis*, *Pyrenophora teres*, *Rhynchosporium secalis*, and *Septoria nodorum* (Heye et al., 1994; Knauf-Beiter et al., 1995). On grapes, vegetable crops, and strawberries, cyprodinil exhibits excellent activity against *Botrytis* spp. On deciduous fruit the target pathogens include *Venturia*, *Alternaria*, and *Monilinia* spp. (Heye et al.,

1994; Knauf-Beiter et al., 1995). In addition, cyprodinil is a promising product for integrated disease control practices because its toxicological properties make it fully compatible with modern human and environmental safety requirements (Heye et al., 1994).

The toxicity of anilinopyrimidines depends on their ability to inhibit the secretion of extracellular proteins (i.e., cutinases, pectinases, and cellulases) associated with pathogenesis (Milling and Richardson, 1995; Miura et al., 1994; Rosslenbroich and Stuebler, 2000). Studies carried out with *Botrytis cinerea* also indicate that anilinopyrimidines interfere with the biosynthesis of methionine (Masner et al., 1994). In greenhouse studies, the protectant and curative activity of cyprodinil was determined against *Erysiphe graminis* f.sp. *tritici* on wheat, *Drechslera teres* on barley, *Venturia inaequalis* on apples, and *B. cinerea* on grapes (Knauf-Beiter et al., 1995; Rosslenbroich and Stuebler, 2000). Even though curative applications did not completely prevent disease symptoms caused by *E. graminis*, sporulation was reduced 100%.

Histological studies have shown that secondary haustoria are the stage in the infection cycle of *E. graminis* most sensitive to cyprodinil. Symptom development of *V. inaequalis* is effectively minimized by cyprodinil through reduction in the growth of the subcuticular stroma, and the inhibition of intra- and intercellular mycelial growth (Knauf-Beiter et al., 1995).

Even though no cross-resistance of cyprodinil to any current market product has been reported for North America or Europe (Foster and Staub, 1996; Heye et al., 1994), one case of resistance in populations of *B. cinerea* in Chile (South America) to this

fungicide has been reported in an attempt to control gray mold of the grapevine *Vitis vinifera* (Latorre et al., 2002).

*Triazoles*. Sterol-biosynthesis inhibiting fungicides contain functionally related compounds that belong to different chemical groups including triazoles and pyrimidines among others (Hippe, 1991). Sterols are required for growth and reproduction by eukaryotic organisms and they play a crucial role in the structure and function of the membranes of many fungi (Hickey and Yoder, 1981). Sterol biosynthesis is one aspect of general lipid metabolism in which acetate is converted into mevalonate and takes place in the smooth portion of the endoplasm reticulum (Siegel, 1981). The biochemical mode of action of sterol inhibitors involves the inhibition of ergosterol biosynthesis (Siegel, 1981). Even though ergosterol biosynthesis is inhibited by these compounds, mycelial growth and various aspects of metabolism including respiration and protein and nucleic acid synthesis are only moderately affected for a period of time after the reduction of the synthesis of the sterol. Upon depletion of ergosterol biosynthesis signs of reduction of the synthesis of fungal membrane are evident. Growth inhibition and changes in morphology and metabolism and accumulation of sterol intermediates also ensue. In addition, free fatty acids accumulate after cessation of membrane synthesis. This accumulation results from continued *de novo* synthesis of fatty acids, a decline in the utilization in the triglycerides and polar lipids, and from degradation of the existing phospholipids in membranes (Siegel, 1981). Treatment with some sterol biosynthesis inhibiting fungicides affects the morphogenesis and development of certain fungi. Some of these modifications include disruption of the Spitzenkörper and cytoskeletal integrity in germ tube tips, undulations of the plasmalemma, enlargement of the endoplasmic

reticulum, disturbances of the mitochondria, accumulation of lipids, vacuolization and exocytosis, thickening of cell walls, and incomplete septa formation (Hippe, 1991). It has been found that other sterol biosynthesis inhibiting fungicides prevent haustorial formation in powdery mildew rather than germ tube development and/or formation of appressoria. Degeneration of haustoria involved swelling of the sheath membrane, damage and vacuolization of the cytoplasm, accumulation of electrodense material, and thickening of the cell walls (Hippe, 1991). Two independent biochemical modes of action for some sterol inhibitors have been described by Dahmen et al. (1988). It appears that at low concentrations the physical strength of the germ tube wall is weakened, even though permeability and turgor pressure remain unaltered. This leads to the bursting of the hyphal tip. In addition, some sterol inhibitor fungicides at low dosage inhibit the germ tube elongation on *Puccinia graminis* f. sp. *tritici* which constitutes the major fungistatic effect caused by these compounds (Siegel, 1981). On the other hand, at higher concentrations, rapid damage to the cell membranes caused by this fungicides also induces cessation of germ tube elongation, but results in cytoplasmatic disarrangement at the ultrastructural level and leakage of electrolytes, thus reducing turgor pressure and preventing bursting of the germ tube tips (Dahmen et al., 1988).

Although sterol inhibitor fungicides differ in their effective dosage rate and ability to control scab or powdery mildew, these fungicides are active in the control of a wide range of diseases caused by Ascomycetes, Basidiomycetes, and Deuteromycetes (Hickey and Yoder, 1981; Shabi et al., 1981; Siegel, 1981). Sterol inhibitor fungicides with agricultural use have varying degrees of systemicity and have protectant, post-infection, curative, and eradicative activity against apple diseases in the U.S.A and Israel. They

have been used for control of apple scab caused by *Venturia inaequalis*, powdery mildew

caused by *Podosphaera leucotrida*, and cedar-apple rust caused by *Gymnosporangium*

*juniperi-virginianae* (Hickey and Yoder, 1981; Jones, 1981; Shabi et al., 1981; Zuck and

MacHardy, 1981).

Resistance to sterol inhibitors appears to be mutagenic because the level of

fungicide required to inhibit growth of mutant strains varies considerably. Mutagenic

resistance could imply multiple sites of actions within the ergosterol biosynthetic pathway

and/or more than one mechanism of resistance (Siegel, 1981).

*Hydroxyanilides*. Fenhexamid is the only representative of this new chemical

class of hydroxyanilide fungicides (Hänßler and Pontzen, 1999; Rosslenbroich and

Stuebler, 2000). It was introduced worldwide in the market in 1999 as Elevate®,

Teldor®, and Password® (Duben et al., 2002; Rosslenbroich and Stuebler, 2000).

The biochemical mode of action of fenhexamid has not been established, but since

it does not exhibit cross-resistance with benzimidazoles, dicarboximides, triazoles, or

anilinopyrimidines it is considered an option to prevent infection of *Botrytis* spp.,

*Monilinia* spp., and *Sclerotinia* spp. (Hänßler and Pontzen, 1999; Rosslenbroich and

Stuebler, 2000).

The physical mode of action of fenhexamid has been determined under laboratory

and greenhouse conditions (Hänßler and Pontzen, 1999). The effect of fenhexamid *in*

*vitro* was determined on various developmental stages of *Botrytis cinerea* (Hänßler and

Pontzen, 1999). Fenhexamid does not inhibit conidial germination of *B. cinerea* but it is

a strong inhibitor of subsequent developmental stages such as germ tube elongation and

mycelial growth (Hänßler and Pontzen, 1999). Upon germination, germ tubes stop

growing and develop abnormalities that include bulges and excessive branching (Hänßler and Pontzen, 1999). *In vivo* fenhexamid exhibits protectant activity against *B. cinerea* avoiding penetration of the pathogen into the host (Hänßler and Pontzen, 1999). After germination the cytoplasm coagulates, detaches from the walls, and retreats from the tip of the germ tube causing conidia and germ tubes to collapse thus preventing infection (Hänßler and Pontzen, 1999). Fenhexamid also has an inhibitory effect when applied to growing hyphae of *B. cinerea*. Hyphal tips show abnormal excretion of cytoplasm or cell wall associated material suggesting that the integrity and function of the cytoplasmic membrane and/or the cell wall are affected by fenhexamid (Hänßler and Pontzen, 1999). It is assumed that fenhexamid disrupts cell wall synthesis in *B. cinerea* allowing weak points to develop in the tips of mycelia through which cytoplasm is discharged causing them to collapse preventing infection (Hänßler and Pontzen, 1999; Rosslenbroich and Stuebler, 2000).

*Dicarboximides*. The biochemical mode of action of dicarboximides has been described as a lipid peroxidation mechanism based on the generation of activated oxygen products such as superoxide, free hydroxyl radicals, and hydrogen peroxide. Free radicals are thought to be produced as a result of a blockage of the mitochondrial or microsomal electron flow. A cascade process is subsequently induced leading to the peroxidation of unsaturated fatty acids in the cellular membranes of sensitive fungi. Inhibition of fungal growth in *Mucor mucedo* and *Botrytis cinerea* is correlated with the level of intracellular lipid peroxidation responsible for the lysis of the cristae in mitochondria, damage to the membranes of the endoplasmic reticulum and the nuclear envelope, and increased coagulation of the cytoplasm (Edlich and Lyr, 1995).

Dicarboximide fungicides exhibit protectant, and some systemic and curative systemic, activities against a variety of phytopathogenic fungi, particularly on grapes and strawberry (Hippe, 1991; Legard et al., 2001; Pommer and Lorenz, 1982). The physical mode of action of dicarboximides cause the inhibition of spore germination but more efficiently inhibit growth of hyphae in *Botrytis cinerea* (Hisada et al., 1977; Pappas and Fisher, 1979) and cause swelling and bursting of germ tubes and hyphal cells, followed by extrusion of their cytoplasm (Hisada et al., 1977). These abnormalities suggest that dicarboximides have a direct effect on cell wall synthesis.

The first resistant isolates to dicarboximide fungicides were found in vineyards in Germany around 1978 and later in Switzerland and France (Pommer and Lorenz, 1982). Pommer and Lorenz (1982) concluded that there are strong indications that resistant isolates to dicarboximide fungicides were frequently less vigorous and/or less pathogenic than sensitive ones and they also found that dicarboximide resistance was not stable. They attributed to those elements the slow increase in resistant isolates in the field and the limited dispersion of resistant strains to untreated plots.

*Sampangine*. The naturally occurring compound, sampangine, was first patented by the University of Mississippi in 1990 as a therapeutic treatment for human fungal infections. Sampangine is an alkaloid isolated from the root bark of *Cleistopholis patens* (Benth.) Engl. and Diels (Annonacea), a tree endemic to West Africa. The stem bark of *C. patens* is used against hepatitis and stomach disorders, while the root is used as a vermifuge, and the leaves are employed to treat fever (Seidel et al., 1999). Phytochemical studies conducted on the stem and root bark of this tree have resulted in the isolation of monoterpenes, sesquiterpenes, and alkaloids (Atti et al., 1982; Waterman and

Muhammad, 1985). Among the alkaloids isolated and characterized are the oxoaporphine alkaloids, isomoschatoline and liriodenine (Atti et al., 1982) and onychine (Hufford et al., 1987; Waterman and Muhammad, 1985). Partially acetylated tri- and tetrarhamnoside dodecanyl ether derivatives have been extracted from leaves of *C. patens* (Seidel et al., 1999). Root bark extracts containing the alkaloids eupolauridine and onychine have active compounds against *Candida albicans* (Hufford et al., 1987).

Most recently, D.E. Wedge from the USDA-ARS, Natural Products Utilization Research Unit, located in the National Center for the Development of Natural Products, and D.G. Nagle from the University of Mississippi have been issued a patent for sampangine and seven of its analogs (4-bromosampangine, 4-methoxysampangine, benzo[4,5]sampangine, liriodenine Mel AMC-XIII-103, onychine, cryptolepine, and liriodenine CDH-II-37) as a group of new, promising chemicals with broad-spectrum and low-toxicity on the agrochemical horizon for fungal control (Patent # 6,844,353B2; Wedge and Nagle, 2005). Sampangine showed promising antifungal activity against several economically important phytopathogenic fungi including *B. cinerea*, *C. acutatum*, *C. fragariae*, *C. gloeosporioides*, and *F. oxysporum* (Patent # 6,844,353B2; Wedge and Nagle, 2005). Data obtained from *in vitro* bioautography and 96-well microbioassay indicated that sampangine and its analogs are active against benzimidazole and dicarboximide resistant strains of *B. cinerea* ($IC_{50} < 3.0$ µM), *C. acutatum* ($IC_{50} < 3.0$ µM), *C. fragariae* ($IC_{50} < 3.0$ µM), *C. gloeosporioides* ($IC_{50} < 3.0$ µM), and *F. oxysporum* ($IC_{50} < 3.0$ µM) (Patent # 6,844,353B2; Wedge and Nagle, 2005).

*CAY-1*. CAY-1 is a steroidal saponin with a molecular weight of 1243.35 Da that was first isolated by A.J. De Lucca from the fruit of cayenne pepper, *Capsicum frutescens*

(De Lucca et al., 2002). CAY-1 has fungicidal activity *in vitro* against germinating

conidia of the filamentous fungi *Aspergillus flavus*, *A. fumigatus*, *A. parasiticus*, and *A.*

*niger*. In addition, CAY-1 is also an effective fungicide against the opportunistic yeasts

*Candida albicans*, *Pneumocystis carinii*, and *Cryptococcus neoformans* (De Lucca et al.,

2002; Renault et al., 2003).

The biochemical mode of action of CAY-1 has been primarily associated to the

detergent properties of the saponins. It has been suggested that the antimycotic activity of

some saponins is a result of their ability to interact with membrane sterols leading to

changes in the membrane ultrastructure, followed by a leakage of cell components, and

ultimately leading to cell death (Polacheck et al., 1991; Segal and Schlösser, 1975). The

alteration of the cell wall permeability and the disruption of the integrity of the membrane

has been documented for a number of saponins and damaging effects have been reported

against a variety of fungi including *Neurospora crassa*, *Candida albicans*, *Cryptococcus*

*neoformans*, *Trichoderma viride*, *Saccharomyces cerevisiae*, *Penicillum expansum*,

*Botrytis cinerea*, *Rhizoctonia solani*, *Bipolaris oryzae*, and *Acremonium* spp. (Lalitha and

Venkataraman, 1991; Polacheck et al., 1991; Renault et al., 2003; Segal and Schlösser,

1975). In addition, Renault et al. (2003) also documented the inhibition of (1,3)ß-glucan

synthase activity in *A. fumigatus*.

CAY-1 did not induce significant inhibition of cell growth when tested in

concentrations up to 100 µg/ml at the National Cancer Institute against 55 panels of

human tissue culture lines including leukemia, melanoma, lung cancer, colon cancer,

CNS cancer, ovarian cancer, renal cancer, prostate cancer, and breast cancer (Renault et

al., 2003). De Lucca et al. (2002) also found that CAY-1 is not cytotoxic to A 549 lung carcinoma cells or HeLa cells at effective fungicidal concentrations.

In addition to the antimicrobial properties, saponins have ample biological properties. Commercial saponins from the bark tree of *Quillaja saponaria* are natural larvicidal agents against *Aedes aegypt* (vector of dengue fever) and *Culex pipiens* (vector for Western Nile virus) (Pelah et al., 2002). Saponins extracted from various plants, fruits, and seeds have molluscicidal effects against several snails including the vectors of schistosome, *Biomphalaria glabrata* and *Oncomelania quadrasi* (Abdel-Gawad et al., 2000; Li et al., 1998; Mahfouz et al., 2000; Okunji et al., 1996). Saponins also exhibit antimalarial and antileishmanial activity (Okunji et al., 1996).

Need for Discovery, Synthesis, and Screening of New Fungicides

One hundred and three fungicides are currently being used in agriculture worldwide (Ehr et al., 2005) with an acceptable margin of safety to humans, wildlife, crops, and the environment. However, in spite of the range of available fungicides, there are several reasons for the agrochemical industry to continue fungicide discovery programs. Often two or three different types of fungicides are required to control a disease on a crop because of limitations with respect to their activity, spectrum, selectivity, and persistence of action among other properties (Lyr, 1995). Furthermore, some fungal crop diseases cannot be controlled by chemicals (such as the take-all disease of wheat) or have only been partially controlled (such as the eyespot disease of cereals) (Brent, 1996). In some instances, pathogens have acquired resistance to fungicides (i.e., acylalanines, benzimidazoles, thiophanates, carboxianilides, hydroxypyrimidines, and organophosphates) making it impossible to control diseases (Dekker, 1995). This was the

case of benomyl (Benlate®) which on December 31, 2001 after 30 years on the market as a fungicide used by growers worldwide was discontinued by DuPont (Hicks, 2001) due to its lack of efficacy as many fungi developed resistance to it. Resistance to fungicides is one of the major problems faced by disease management programs. It causes unexpected crop loses for the grower, especially if no adequate substitutes are available. It also affects the profits of the manufacturer who developed the fungicide at a high cost. In the long run the development of resistance also has consequences for the extension officers, the regulatory authorities, and eventually the consumer and the economy (Dekker, 1995).

Many fungicides including captafol, binapacryl, organomercurials, and ethylene *bis*-dithiocarbamates have been banned from the market because they represent environmental risks (Dekker, 1995; Leroux, 1996). In 1996 the Food Quality Protection Act increased the requirements for pesticide safety to reduce infant and child exposure to fungicides such as iprodione. In the past this fungicide could be applied to strawberries in the field up until the day of harvest. These strawberries were then harvested directly into consumer packs without washing, and often consumed without washing. In order to comply with mitigation measures determined by the Environmental Protection Agency to lessen human health risk and ecological effects, the iprodione's manufacturer, Rhône-Poulenc, has restricted the use of iprodione on strawberries by not allowing it to be applied after the first flowers are on the plant (Legard et al., 2001). The use of this fungicide on crops such as strawberry is subject to re-registration of their product to abide by today's standards (Smith, 1999). In the face of such adversity, there is no question that the agrochemical industry is interested in investing in the development of natural product-

based fungicides as an alternative to the traditional arsenal of available disease control
agents.

For the reasons mentioned above, all the parties involved in fungicide discovery
will benefit from a greater diversity of available fungicides. The identification of a
promising lead molecule constitutes the direct path to the synthesis of any number of
potentially active analogs of chemical compounds (Brent, 1996). Moreover, the
discovery of new fungicides primarily isolated from natural sources is advantageous since
natural products represent a vast untapped reservoir of chemical compounds (Sisler,
1996). The principal advantage of this approach over chemical synthesis or modification
of existing compounds is the probability of discovering fungicides with different modes
of action and different chemical structures and, therefore, dissimilar toxicities and cross-
resistance to present fungicides (Dekker, 1995). Novel active molecules present in
natural product-based fungicides represent potential new groups of fungicides with broad
spectra for plant disease control employing different modes of action that make them
prime candidates to be included in plant disease management programs. Research
centers, universities and/or the industry can set up large scale, high-throughput test
programs that synthesize and screen between ten and twenty thousand new compounds
per year (Brent, 1996). However, less than 0.01% of the randomly synthesized chemicals
reach the market (Brent, 1996), and less than 1% of the natural product-based fungicides
and their chemical analogs synthesized at the USDA–ARS Natural Products Utilization
Research Unit leads to the identification of effective compounds (Wedge, unpublished
data).

CHAPTER III

MATERIALS AND METHODS

*In Vitro* Testing

*Fungal Isolates*

Seven fungal isolates were used in various aspects of this study. *Colletotrichum acutatum* Simmonds isolate Goff, *C. fragariae* Brooks isolate CF-63, and *C. gloeosporioides* (Penz.) Penz. & Sacc. in Penz. isolate CG-162 were provided by B.J. Smith (Smith et al., 1990; Smith and Black, 1993a, 1993b), USDA-ARS, Small Fruit Unit, Poplarville, MS; *Phomopsis viticola* Sacc. and *P. obscurans* (Ellis & Everh.) Sutton were obtained from M. Ellis, Ohio State University, OH; and *Fusarium oxysporum* Schlechtend.:Fr. and *Botrytis cinerea* Pers.:Fr. were provided by D.E. Wedge, USDA-ARS, Natural Products Utilization Research Unit, University, MS. The strains of *Colletotrichum* spp. and *P. obscurans* were isolated from strawberry (*Fragaria* x *ananassa* Duchesne ex. Rozier). *Phomopsis viticola* and *B. cinerea* were isolated from commercial grape (*Vitis vinifera* L.). *Fusarium oxysporum* was isolated from orchid (*Cycnoches* sp.). Fungi were grown on potato dextrose agar (PDA, Difco, Detroit, MI) in 9-cm petri dishes and incubated in a growth chamber at $24 \pm 2\,°C$ except *B. cinerea* at $18 \pm 2\,°C$, and under cool-white fluorescent lights ($55 \pm 5$ µmols .m$^{-2}$. sec$^{-1}$) with a 12 h photoperiod.

*Conidial Suspension Preparation*

Conidia were harvested from 7 to10 day-old cultures by flooding plates with 10 ml of sterile distilled water and dislodging conidia by softly brushing the colonies with an L-shaped plastic rod. Aqueous conidial suspensions were filtered through sterile

Miracloth (Calbiochem-Novabiochem Corp., La Jolla, CA) to remove hyphae. Conidial

concentrations were determined photometrically from a standard curve based on

absorbency at 625 nm (UV 3101PC Scanning Spectophotometer, Shimadzu Scientific

Instruments, Columbia, MD) (Espinel-Ingroff and Kerkering, 1991; Wedge and Kuhajek,

1998), and suspensions were then adjusted with sterile water to a concentration of $10^6$

conidia/ml.

The effect of washing the conidia on germination time was determined for each

fungal species. The conidial matrix which inhibits germination was removed from the

conidial suspension by washing the spores three times. The procedure involved

centrifuging the conidial suspension at 3,000 rpm (Sorvall® RC 5C Plus Centrifuge,

Asheville, NC) for 10 min at 4°C, discarding the supernatant, and resuspending the

conidial pellet in sterile distilled water by vortexing for 10 sec (VWR Voxtexer 2,

Scientific Industries, Inc., Bohemia, NY). Conidial suspensions were refrigerated

overnight at 4°C (Kenmore, Hoffman Estatets, IL). Conidial germination times were

evaluated in trials with washed and unwashed conidia noting the time at which ~90%

germination was achieved. Each species was replicated three times except *F. oxysporum*

that was replicated twice.

Conidial germination times were evaluated by analysis of variance (ANOVA)

using the general linear model procedure (GLM) of Statistical Analysis Systems (SAS)

software, Ver. 9.1 (SAS Institute, Inc., SAS Campus Drive, Cary, NC 27513-2414).

Means were compared by the least significant difference (LSD) test. A significance level

of $P = 0.05$ was used for LSD and pairwise comparisons.

*Microbioassay*

A 24-well cell culture cluster (Corning Inc., Corning, NY) was used to visualize growth of *B. cinerea*, *C. acutatum*, *C. fragariae*, *C. gloeosporioides*, *F. oxysporum*, *P. viticola*, and *P. obscurans* by direct observation. Technical grade natural product-based fungicides, sampangine, seven sampangine analogs (4-bromosampangine, 4-methoxy-sampangine, benzo[4,5]sampangine, liriodenine Mel AMC-XIII-103, onychine, cryptolepine, and liriodenine CDH-II-37; National Center for Natural Products Research, University, Mississippi) and seven commercial fungicides (benomyl, captan, cyprodinil, fenbuconazole, fenhexamid, iprodione, and kresoxim-methyl; Chem Service, West Chester, PA) were dissolved in 95% ethanol at 12 mM, sonicated for 10 min, and then diluted 1:4 with Roswell Park Memorial Institute mycological liquid medium 1640 (RPMI, 16.2 g/l, Life Technologies™, Grand Island, NY) buffered with MOPS (3-[N-morpholino] propanesulfonic acid] (34.5 g/L; Sigma, St. Louis, MO) to yield 1.2, 0.12, and 0.012 µM concentrations. Eighty microliters of the fungicide solution, 320 µl of RPMI buffer, and 400 µl of conidial suspension (prepared in RPMI) to yield fungicide concentrations of 30, 3.0, and 0.3 µM respectively were added to each well. A positive control containing the conidial suspension and RPMI buffer (but not the fungicide solution) was also run to check for contamination. A sterilized 13 mm diameter cover glass (Fisherbrand®, Pittsburgh, PA) was placed at the bottom of each well. Each 24-well cell culture cluster was incubated in a growth chamber under the same conditions as described in "Fungal Isolates." Conidial germination was assessed by estimating the time at which 90% germination was achieved in a given well (based on the baseline germination previously established during the conidial washing trials) by direct observation of the wells through an IX 70 inverted system microscope (Olympus

America, Inc., Melville, NY). Starting experiments close to 90% germination times was impossible due to the large number of replications handled simultaneously. Permanent slides were made by removing the cover glass from each well, inverting it, placing it in a drop of 1% lactophenol cotton blue (Stevens, 1981), and then sealing it to the microscope slide (Fisherbrand®, Pittsburgh, PA) with transparent fingernail polish. Photographs of the fungi to document changes in morphology were taken with a PM–35 DX Olympus camera (Olympus America, Inc., Melville, NY). Each treatment was run in duplicate and it was repeated independently three times for each fungal species.

*Microtiter Assay*

Treatments were arranged as a split-plot design where whole plots were fungal isolates and sub-plots were fungicides and concentrations. Twenty microliters of the fungicide solution, 80 μl of RPMI buffer, and 100 μl of the conidial suspension were placed in each well of the 96-well cell culture clusters (Nunc MicroWell, untreated; Roskilde, Denmark) at concentrations of 0.3, 3.0, and 30 μM. Each fungicide was evaluated in duplicate against a negative control (reagent blank) containing the fungicide solution and RPMI buffer (but not the conidial suspension) at each concentration. A positive control containing the conidial suspension and RPMI buffer (but not the fungicide solution; unamended media) was also run to check for contamination. Each 96-well cell culture cluster was incubated in a growth chamber (as described in "Fungal Isolates") for 72 h to allow time for mycelial development past the initial germination times. Each treatment was run in duplicate and it was repeated independently three times for each fungal species. Fungal growth was then evaluated by measuring absorbency of each well at 620 nm using a microplate photometer (Packard Spectra Count, Packard

Instrument Co., Downers Grove, IL). Mean absorbency values and standard errors were

used to evaluate fungal growth of *B. cinerea*, *Colletotrichum* spp., and *F. oxysporum* at

48 and 72 h at each fungicide concentration. Mean absorbency values were expressed as

percent inhibition/stimulation using the formula:

$$\% \text{ inhibition/stimulation} = \frac{[(\text{mean sample absorbency}) - (\text{mean unamended absorbency})] \times 100}{(\text{mean unamended absorbency})}$$

Positive numbers indicate stimulation of growth and negative numbers indicate inhibition

of growth compared to the growth of the fungus on unamended media. Differences in

fungal growth *in vitro* demonstrated the sensitivity of fungal plant pathogens to natural

product-based and commercial fungicides with known fungicidal modes of action

(Wedge et al., 2001).

Each concentration and response time was analyzed separately by ANOVA using

Statistical Analysis Systems (SAS) software (version 9.1). Mean separations were

determined based on least significant difference (LSD) test ($P=0.05$). Statistical

comparisons of fungal growth were made both at 48 and 72 h within each concentration

of fungicide and within each concentration of fungicide across fungal growth.

*In Planta* Testing

*Source of Strawberry Plants*

Strawberry plants (cv. Chandler) were purchased from a commercial nursery.

They were planted in 10 x 10 cm plastic pots containing a 1:1 (v/v) mixture of JiffyMix

(JPA, West Chicago, IL) and pasteurized sand. Strawberry plants were grown for a

minimum of 6 weeks in a greenhouse maintained at 30°C day/18°C night ± 6°C with a 16 h photoperiod.

*Leaf Clearing Technique*

Strips of inoculated middle leaflets from strawberry plants (cv. Chandler) were excised on the center of either side of the midrib with a razor blade. Depending on the size of the leaflet, two or three consecutive subsamples from each, the distal, median, and proximal region (from the petiole), were cut with a razorblade. Each subsample measured 3 x 6 mm. Subsamples were placed in a 1:2 absolute ethanol:glacial acetic acid solution for 24 h to clear the leaf (Dhingra and Sinclair, 1995). In order to make conidial counts, samples were stained with 0.05% acid fuchsin for 2 min, rinsed in distilled water, and mounted in a drop of water on glass slides to be observed in a compound microscope (Diéguez-Uribeondo et al., 2003). Photographs of selected sections of the leaflets were taken with a DXC–151A color video camera (Hitachi Instruments, Inc., Houston, TX) attached to a BH–2 Olympus light microscope (Olympus Corporation, Marietta, GA) and captured with Bioquant 98® image analysis software package (R&M Biometrics, Inc., Nashville, TN).

*Detached Leaf Assays for Fungicide Evaluation*

Strawberry plants (cv. Chandler) maintained under standard greenhouse conditions previously mentioned in "Source of Strawberry Plants" were used for this assay. Whole leaves (with petioles) were removed from the plants no more than 4 h before fungicide treatment and/or inoculation. Only the second or third youngest leaves on a plant without visible signs of injury or symptoms of disease were collected. As leaves were collected they were placed in a sealed container at 100% RH and 12°C and

then transferred into sterile dH$_2$O in 10 x 150 mm tissue culture tubes (Pyrex®, Sigma, St. Louis, MO). All three leaflets on a leaf were inoculated by misting the adaxial surface to the point of runoff with a hand pump with a conidial suspension of *C. fragariae* isolate CF-75 prepared as described in "Conidial Suspension Preparation" and adjusted to a final concentration of 7.5x10$^5$ and 1.5x10$^6$ conidia/ml with a hematocytometer (Baxter Scientific Products, McGraw Park, IL) and three spore counts were made to obtain the actual spore concentration. The *C. fragariae* isolate CF-75 was initiated from silica gel cultures maintained by B.J. Smith at the USDA-ARS, Small Fruit Unit, Poplarville, MS. Cultures were grown on potato dextrose agar in 9-cm petri dishes and maintained at room temperature (~25°C) under fluorescent light at 14 h photoperiod. The natural product-based fungicides tested included sampangine and CAY-1 and were prepared at concentrations of 0, 625, 1250, and 2500 ppm and were applied with a vacuum/pressure pump (Welch Pump, Model # 25228-01, Skokie, IL) at 5 psi and a 10 ml TLC sprayer head (Kontes Glass Company, Vineland, NJ). In addition, CAY-1 was tested at 312 ppm. These natural product-based fungicides were evaluated against a commercial fungicide (azoxystrobin) and against two non-inoculated (non-treated) controls containing the natural product-based and commercial fungicides at each concentration. The experiment was repeated twice.

I conducted a pre-inoculation treatment and a post-inoculation treatment. In the pre-inoculation treatment, leaflets were inoculated with the conidial suspension and placed in racks in a dew chamber (Percival Scientific, Model I-60DL, Boone, IA) in complete darkness for 24 h at 30°C. Then leaflets were allowed to air dry, sprayed with the natural product-based and commercial fungicides, and returned to the dew chamber

for an additional 24 h. In the post-treatment, leaflets were sprayed with the natural product-based and commercial fungicides and placed in racks in a dew chamber in complete darkness for 24 h at 30 °C. Then leaflets were allowed to air dry, inoculated with the conidial suspension, and placed in the dew chamber for an additional 48 h. Leaflets in both set of experiments were incubated in the dew chamber for a total of 48 h after inoculation, then they were transferred to 18 gallon sealed plastic containers (65 x 36.1 x 53.1 cm, Rubbermaid®, Wooster, OH) at 100% RH, 28 °C and continuous fluorescent light (Sylvania Super Saver Lite White, 34 W, 16.7 $\mu$mols.m$^{-2}$. sec$^{-1}$; OSRAM Sylvania, Danvers, MA) for an additional three days before assessing disease symptoms. Photographs of each leaflet for posterior macroscopic disease assessment (quantitative) as well as photographs of selected sections of the leaflets for posterior microscopic evaluation of fungal morphology (qualitative) were taken as described in "Leaf Clearing Technique."

Disease severity on the leaflets was assessed as percentage area infected by the fungi expressed in an arbitrary scale of 1 to 3. A score of 0 indicated no symptoms of anthracnose and a score of 3 indicated the most severe anthracnose lesions (where the plant was usually dead). Phytotoxicity was assessed as percentage of necrotic area expressed in an arbitrary scale of 1 to 5. A score of 0 indicated no phytotoxicity and a score of 5 indicated the most severe phytotoxicity (where the plant was usually dead). Disease severity and phytotoxicity were evaluated by analysis of variance (ANOVA) using the general linear model procedure (GLM) of Statistical Analysis Systems (SAS) software (Version 9.1). Means were compared by least significant difference (LSD) test ($P<0.05$).

*In Planta* Screening Techniques

*Inoculation of Plants*

   *Colletotrichum acutatum* isolate Goff maintained on silica gel was provided by

B.J. Smith, USDA-ARS, Small Fruit Unit, Poplarville, MS. Isolates were transferred and

grown on PDA for 14 to 30 days at 25°C under fluorescent light at 14/10 h of light/dark

intervals. The cultures were located approximately 18 cm beneath four fluorescent tubes

(Sylvania Super Saver Lite White, 34 W, 16.7 μmols .m$^{-2}$. sec$^{-1}$; OSRAM Sylvania,

Danvers, MA). Conidial suspensions were prepared as described in "Conidial

Suspension Preparation." Conidial concentrations were estimated from three counts

made with a hematocytometer (Baxter Scientific Products, McGraw Park, IL). Final

concentrations were adjusted to $7.5 \times 10^5$ and $1.5 \times 10^6$ conidia/ml.

   Detached middle leaflets of strawberry cv. Chandler were inoculated by misting to

the point of runoff with 2.5 ml of conidial suspension on the adaxial surfaces. Leaflets

were incubated at 28°C in a sealed 10-gallon tank (ca. 100% relative humidity) for 24 h

and then at reduced relative humidity by removing half of the plastic tank cover for an

additional 12 h (Smith and Black, 1987, 1990). Following incubation, leaflets were

cleared for direct observation as described in "Leaf Clearing Technique." Counts of

ungerminated and germinated conidia of selected areas were registered.

*Accounting for Conidia*

   The following procedure was used to account for all the conidia inoculated to

leaflets of strawberry cv. Chandler. The runoff excess was collected in an empty petri

dish (previously weighted) and the volume was determined by weight. The excess

conidial solution left in the sprayer was transferred to a falcon tube (Falcon®, Becton

Dickinson, NJ) and the volume was determined with a graduated pipet (Kimax®, Fischer

Scientific, Pittsburg, PA). The total volume sprayed on the leaflet surface was calculated

by subtracting the volume of the runoff and the volume left in the sprayer from the initial

volume of 2.5 ml. The volume sprayed on the leaf surface was then used to calculate the

expected number of conidia per unit area.

*Calculation of Conidia per Unit Area*

The total number of ungerminated and germinated conidia and appressoria was

counted in each of the 72 microscope fields (20X) observed on each subsample. These

fields were selected by tracing adjacent fields on each leaflet subsample. The area of the

entire leaflet was calculated with Bioquant 98® image analysis software package (R&M

Biometrics, Inc., Nashville, TN). The number of fields per leaflet was calculated by

dividing the area of the leaflet by the area of a 20X microscope field (738983 $\mu m^2$). The

actual number of conidia per leaflet was obtained by multiplying an average of the

conidia per field (from the right and left side of the leaflet) times the number of fields per

leaflet. The expected number of conidia per leaflet was calculated by multiplying the

initial conidial concentration times the total volume sprayed on the leaflet surface.

Between 26 and 40 subsamples were counted at each sampling time and the experiment

was repeated three times for each concentration ($7.5 \times 10^5$ and $1.5 \times 10^6$ conidia/ml).

*In Vitro* Screening Techniques

*Simulated Spot Inoculation*

I tested an artificial system to account for the missing conidia in the *in planta*

system I just described. Simulating spot inoculation, I placed 5 μl drops of approximately

$7.5 \times 10^5$ conidia/ml of *C. acutatum* isolate Goff on preweighed, photo-etched coverslips

(23 x 23 mm; Electron Microscopy Sciences, Washington, PA) and made exhaustive counts of conidia. Conidial suspension was prepared as described in "Conidial Suspension Preparation." I determined the weight of the drop by subtracting the weight of the empty coverslip from the weight of the coverslip plus the drop. The expected number of conidia was calculated by multiplying the concentration of conidia in the starting inoculum times the weight of the drop. The experiment was repeated 20 times.

*Pipeting Experiments*

The consistent number of conidia unaccounted for led me to consider the degree to which conidia would stick to the inside of the pipet I used to draw the conidial suspension for the spot inoculation experiments. I designed a set of experiments where I withdrew 50 aliquots from a stock solution (on sets of 5 aliquots) of *C. acutatum* isolate Goff with the same glass pipet. I determined conidial concentration after each set of 5 aliquots by making three counts on the hemocytometer. The stock solution was vortexed between sets of aliquots to homogenize the conidial suspension. Photographs of the pipet tip containing conidia were taken as described in "Detached Leaf Assay for Fungicide Evaluation." The experiment was repeated six times. Data were evaluated by analysis of variance (ANOVA) using the general linear model procedure (GLM) of Statistical Analysis Systems (SAS) software (Version 9.1) and means were compared by the least significance (LSD) test ($P<0.05$).

CHAPTER IV

RESULTS

*In Vitro* Testing

*Microbioassay*

Conidial washings to remove innate germination inhibitors considerably reduced germination times for all seven species (Table 2). Germination times were assessed against an unwashed control at the time when approximately 90% of the conidia in the control had germinated. The reduction of conidial germination time was greatest for the two *Phomopsis* spp. in which the washed conidia germinated more than 20 h earlier than the unwashed conidia. Reducing germination times enhanced germination synchrony of each fungal species, thus improving the precision of the microtiter assays.

The stages of fungal development I recognized included incipient germ tube, germination, hyphal development, and mycelial development. For the purpose of this study, an incipient germ tube is defined as a germ tube being largely surrounded by parental conidial wall (Fig. 1), germination is defined as a germ tube being largely composed of new wall material (Figs. 2–4), and hyphal development indicates a single, possibly branched hypha, that grades into mycelial development with several hyphal branches. Qualitative assessment of fungal development is presented in Table 3. Degrees of development expressed in Table 3 cannot be compared across species, but were assessed at the times of germination for each species with three washes (Table 2).

The most sensitive species were *B. cinerea* and *P. obscurans* with conidial germination inhibited by all commercial fungicides except captan and fenheximide at $\geq 0.3$ µM respectively (Table 3). The least sensitive species was *P. viticola* in which

conidial germination was only inhibited by sampagine at all rates and kresoxim-methyl at

$\geq 30$ µM. Almost as insensitive was *F. oxysporum* in which conidial germination was

inhibited by kresoxim-methyl and sampangine ($\geq 3.0$ µM) and by benomyl and captan

($\geq 30$ µM).

When comparing fungicide efficacy at the $\geq 3.0$ µM concentration, the most

effective commercial fungicide was kresoxim-methyl which prevented germination of all

fungal species except *P. viticola*. Benomyl and fenbuconazole were almost as effective

as kresoxim-methyl. Captan and fenhexamide prevented conidial germination of *B.*

*cinerea*, *C. fragariae*, *C. gloeosporioides*, and *P. obscurans* (Table 3). Iprodione was the

least effective commercial fungicides for inhibiting germination.

The natural product sampangine at $\geq 3.0$ µM inhibited conidial germination in all

fungal species except *C. acutatum* (Table 3), which was inhibited from further growth

when assessed in the microtiter assay at 48 hours. The analog, 4-bromosampangine, at

$\geq 3.0$ µM only inhibited germination in *P. obscurans*, but it was the only fungicide

observed to affect subsequent germ tube development causing two morphological

anomalies in *B. cinerea* and *C. fragariae* during the time frame of this study. Germ tubes

in *B. cinerea* splayed and branched in the presence of this analog at approximately 18 h

(< 10% of the conidia, qualitative observation; Fig. 4B). In addition, *Colletotrichum* spp.

occasionally produce two germ tubes, but in the presence of this sampangine analog, two

or more germ tubes were observed more commonly than the usual for *C. fragariae* at

approximately 10 h (< 10% of the conidia, qualitative observation; Fig. 3B ).

Morphological anomalies were not observed in *C. acutatum*, *C. gloeosporioides*, *F.*

*oxysporum*, *P. obscurans*, or *P. viticola*.

The analogs of sampangine were generally less effective than sampangine itself or the commercial fungicides (Table 3). *Colletotrichum acutatum* was the most resistant of the seven species tested since only 4-methoxysampangine at $\geq 30$ μM and liriodenine CDH-II-37 at $\geq 3.0$ μM inhibited conidial germination. *Phomopsis viticola* was the most resistant of the species challenged with natural product-based fungicides since only kresoxim-methyl at its highest concentration inhibited conidial germination. In contrast, germination of *P. obscurans* was inhibited 100% by most sampangine analogs except 4-methoxysampangine at $\geq 0.3$ μM and onychine at $\geq 0.3$ μM, suggesting that this species was the most sensitive of those tested. Cryptolepine and liriodenine CDH-II-37 inhibited germination of *B. cinerea* conidia at $\geq 3.0$ μM. The most effective sampangine analog was liriodenine CDH-II-37 which inhibited germination of *B. cinerea*, *C. acutatum*, *C. fragariae*, *C. gloeosporioides*, and *P. viticola* at $\geq 3.0$ μM and completely inhibited conidial germination of *P. obscurans*. Onychine was the least effective sampangine analog since it only prevented conidial germination of *B. cinerea* at 30 μM.

*Microtiter Assay*

Data for fungicide efficacy of *Phomopsis* spp. against natural product-based and commercial fungicides are not reported because the data exhibited unacceptably high standard errors. Neither species achieved an acceptable level of synchronous growth or achieved 100% germination during the time frame of this study. Among isolates of the other species, there was a significant interaction between fungal isolate and fungicide concentrations.

The sensitivity of the remaining five species to sampangine, 4-bromosampangine, and seven commercial fungicides was assessed by measuring fungal growth *in vitro*

relative to their growth in unamended controls. After 72 h growth of the most sensitive

fungus, *B. cinerea*, was inhibited by benomyl, cyprodinil, fenbuconazole, fenheximid,

and kresoxim-methyl (Table 4). All three species of *Colletotrichum* were inhibited by

captan and sampangine. In addition, *C. fragariae* was sensitive to benomyl and

cyprodinil. *Colletotrichum gloeosporioides* was sensitive to 4-bromosampangine,

sampangine, and fenbuconazole. *Fusarium oxysporum* was sensitive to kresoxim-methyl.

Comparative efficacy of commercial and natural product-based fungicides on the

growth response of the plant pathogenic fungi were similar between 48 (data not shown)

and 72 h (Table 4), except for kresoxim-methyl which lost efficacy against the three

species of *Colletotrichum* and against *F. oxysporum*, benomyl which lost efficacy against

*F. oxysporum*, and iprodione which actually stimulated all *Colletotrichum* spp. and *F.*

*oxysporum*. Benomyl inhibited growth of *B. cinerea*, *C. fragariae*, and *F. oxysporum*. It

was less effective against *C. gloeosporioides* and actually stimulated *C. acutatum*.

Captan was not highly efficacious against growth of *B. cinerea*, but it inhibited growth of

*F. oxysporum* and the three species of *Colletotrichum*. Cyprodinil inhibited *B. cinerea*

and all three species of *Colletotrichum*. Fenbuconazole inhibited *B. cinerea*, *C.*

*fragariae*, and *C. gloeosporioides*. Fenhexamide, iprodione, and kresoxim-methyl

inhibited growth of *B. cinerea*. The sampangine analog 4-bromosampangine inhibited

growth of *C. gloeosporioides*. Sampangine was effective against all fungi.

Sampangine analogs either inhibited or slightly stimulated fungal growth of each

species. Analysis of these analogs was, therefore, run separately to assess there efficacy

independently of the other fungicides (Table 5). Growth of *C. gloeosporioides* was

inhibited at $\geq 3.0$ μM concentration by benzo[4,5]sampangine, 4-methoxysampangine,

and liriodenine CDH-II-37. Growth inhibition of the other four species by all the analogs was significantly less than that experienced by *C. gloeosporioides*. All concentrations of onychine caused stimulation in all *Colletotrichum* spp. and minimal growth inhibition in *F. oxysporum*. Growth inhibition of *B. cinerea* was less than 50% for all analogs.

<div align="center">*In Planta* Testing</div>

*Leaf Clearing Technique*

This technique also allowed me to explore the details of reproducibility that accompany the commonly used techniques of spraying plants to the point of runoff and spot inoculating specific parts of the plant (e.g., leaves, petioles, stems, etc). I was able to determine the distribution pattern of the inoculum on selected areas of the leaf surface using these techniques. For example, I found evidence that even three conidia in close proximity are less likely to germinate than a solitary conidium on the leaf surface (Fig. 5). I also found that conidia did not adhere to the abaxial surface of the leaves. In addition, the development of the leaf clearing technique had a direct application at the microscopic level as a tool for corroborating the efficacy as disease control agents of several natural product-based fungicides among various research facilities or for greenhouse testing for pathogen resistant strawberry germplasm. Another benefit provided by this technique is that it allowed me to identify the presence of fungal conidia different from the ones that were inoculated on the leaves (Fig. 6).

*Detached Leaf Assay for Fungicide Evaluation*

Sampangine, CAY-1, and azoxystrobin had negligible or no effect on the incidence of anthracnose disease when applied 24 h after the fungal inoculum (pre-inoculation treatment) (Table 6; Fig. 7). In contrast, the natural product-based fungicides

sampangine and CAY-1, and the commercial fungicide azoxystrobin provided protection against anthracnose when applied 24 h before the fungal inoculum (post-inoculation treatment; Table 6; Fig. 8). Direct observation of germination and appressorial formation confirmed that the post-inoculation treatment provided protection (Table 7). Appressorial formation was visible with sampangine (Fig. 9), CAY-1 (Fig. 11), and azoxystrobin (Fig. 13) at their lowest concentration, 625 ppm (Table 7 post-inoculation). Sampangine prevented fungal germination even at its lowest concentration, 625 ppm (Fig. 10; Table 7 post-inoculation). CAY-1 prevented fungal germination at its medium and highest concentration, 1,250 and 2,500 ppm (Fig. 13; Table 7 post-inoculation). Azoxystrobin prevented fungal germination particularly at its medium concentration, 1,250 ppm (Fig. 14; Table 7 post-inoculation). Sporadic phytotoxicity was observed with sampangine, CAY-1, and azoxystrobin on both the pre-inoculation and post-inoculation treatments at all concentrations (Fig. 15).

<div align="center">

*In Planta* Screening Techniques

</div>

*Calculation of Conidia per Unit Area*

Conidia were selectively stained on the cleared leaf surface, which allowed me to calculate conidia per unit area and provide a quantitative assessment of the conidia that attached to the leaflet surface after spraying until run-off (Table 8). The number of conidia unaccounted for range between 217,376 and 901,526 conidia/ml (Table 8).

<div align="center">

*In Vitro* Screening Techniques

</div>

*Simulated Spot Inoculation*

Exhaustive conidial counts on the photo-etched coverslips showed a range of unaccounted conidia (Table 9) between 73 and 1,955 conidia/ml. The potential for losing

conidia in runoff and for leaving conidia in the sprayer was eliminated in this experiment.

The "test for paired differences" test comparing the number of spores on the etched

coverslip indicated that the expected spore count (average 2,512) was significantly greater

(Table 9) than the actual spore count (average 1,647).


*Pipeting Experiments*

The inner wall of the pipet was saturated with conidia by 30 aliquot transfers as

determined by the relative stabilization of the number of conidia delivered in the

simulation of spot inoculation of leaflets (Table 10). Examination of the inner surface of

the pipet at the microscopic level confirmed that the tip was loaded with conidia after 25

aliquot transfers (Fig. 16). The density of conidia and the optics of viewing the inner

curved surface prohibited quantifying this experiment.

Table 1. Initial infection strategies exhibited by some *Colletotrichum* species*.

| Infection strategy | *Colletotrichum* species | Plant host | Author |
|---|---|---|---|
| Intracellular hemibiotrophic | *C. acutatum* | *Fragaria* x *ananassa* | Curry et al. (2002) |
| | *C. destructivum* | *Vigna unguiculata* | Latunde-Dada et al. (1996) |
| | *C. fragariae* | *Fragaria* x *ananassa* | Curry et al. (2002) |
| | *C. gloeosporioides* | *Citrus* spp. | Brown (1977) |
| | | *Medicago sativa* | Churchill et al. (1988) Porto et al. (1988) |
| | | *Populus tremuloides* | Marks et al. (1965) |
| | | *Stylosanthes guianensis* | Ogle et al. (1990) |
| | | *Stylosanthes scabra* | Trevorrow et al. (1988) |
| | *C. graminicola* | *Zea mays* | Politis and Wheeler (1973) |
| | *C. lindemuthianum* 157 | *Vigna unguiculata* | Bailey et al. (1990) |
| | *C. lindemuthianum* | *Phaseolus vulgaris* | Skipp and Deverall (1972) Mercer et al. (1975) Elliston et al. (1976) Landes and Hoffman (1979b) O'Connell et al. (1985) O'Connell and Bailey (1986) |
| | *C. orbiculare* *C. lagenarium* | *Cucumis sativus* | Anderson and Walker (1962) Dargent and Touzé (1974) Stumm and Gessler (1984) Xuei et al. (1988) |
| | *C. sublineolum* | *Sorghum bicolor* | Wharton and Julian (1996) |
| | *C. trifolii* | *Medicago sativa* | Mould et al. (1991) |
| | *C. truncatum* | *Pisum sativum* | Uronu (1989) |
| Subcuticular intramural | *C. capsici* | *Gossypium hirsutum* | Roberts and Snow (1984) |
| | | *Vigna unguiculata* | Bailey et al. unpublished |
| | *C. circinans* | *Allium cepa* | Walker (1921) |
| | *C. gloeosporioides* | *Carica papaya* | Chau and Alvarez (1983) |
| | | *Muscadinia rotundifolia* | Daykin and Milholland (1984) |
| | | *Persea americana* | Prusky et al. (1991) |
| | *C. musae* | *Musa* spp. | Swinburne and Brown (1983) |
| | *C. phomoides* | *Lycopersicon esculentum* | Fulton (1948) |
| Both infection strategies | *C. gloeosporioides* | *Citrus* spp. | Brown (1977) |
| | | *Stylosanthes* spp. | Irwin et al. (1984) Trevorrow et al. (1988) Vinijsanum et al. (1987) |

* Compiled from data presented by Bailey et al., 1992; Perfect et al., 1999; and Curry et al., 2002.

Table 2. Mean conidial germination time $(h)^x$ of seven plant pathogenic fungal species on 24-well cell culture clusters following zero or three washes.

| Fungus | 0 washes | 3 washes | F test[z] |
|---|---|---|---|
| *Botrytis cinerea* | 22.3 a[y] | 18.8 b | *** |
| *Colletotrichum acutatum* | 14.8 a | 8.8 b | *** |
| *C. fragariae* | 14.5 a | 10.8 b | *** |
| *C. gloeosporioides* | 15.3 a | 9.2 b | *** |
| *Fusarium oxysporium* | 17.0 a | 13.0 b | *** |
| *Phomopsis obscurans* | 93.8 a | 68.7 b | *** |
| *P. viticola* | 93.7 a | 72.5 b | *** |

[x]Values are means of three studies with two replications each. Since there was not a significant difference between study and number of washings for any species, data from the three studies were combined for each species. (*Fusarium oxysporium* was run twice with two replications each).

[y]Values in the same row that do not share a common letter were significantly different in a mean separation test using Fisher's protected LSD ($P < 0.05$). [z]F test: *** significant at $P < 0.0001$.

Table 3. Effects of sampangine, seven of its analogs, and seven commercial fungicides on growth and development of selected plant pathogenic fungi in an *in vitro* microbioassay.

| | *Botrytis cinerea* | | | *Colletotrichum acutatum* | | | *Colletotrichum fragariae* | | | *Colletotrichum gloeosporioides* | | | *Fusarium oxysporum* | | | *Phomopsis obscurans* | | | *Phomopsis viticola* | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Concentration (µM)[y] | 0.3 | 3.0 | 30 | 0.3 | 3.0 | 30 | 0.3 | 3.0 | 30 | 0.3 | 3.0 | 30 | 0.3 | 3.0 | 30 | 0.3 | 3.0 | 30 | 0.3 | 3.0 | 30 |
| Control[x] | | +[z] | | | + | | | + | | | ++ | | | + | | | +++ | | | ++ | |
| Sampangine | - | - | - | + | + | + | + | - | - | + | - | - | + | - | - | - | - | - | - | - | - |
| 4-bromosampangine | + | + | - | ++ | ++ | ++ | + | + | + | + | - | - | + | + | + | - | - | - | + | + | + |
| 4-methoxysampangine | + | + | - | + | + | - | + | + | + | ++ | - | - | + | + | - | +++ | - | - | ++ | + | + |
| Benzo[4,5]sampangine | + | + | + | ++ | ++ | ++ | + | + | + | + | - | - | + | + | + | - | - | - | ++ | + | + |
| Cryptolepine | ++ | - | - | ++ | + | + | + | + | ~ | ++ | + | - | + | + | - | - | - | - | + | + | + |
| Liriodenine, AMC-XIII-103 | + | + | - | + | + | + | + | - | - | + | - | - | + | - | - | - | - | - | ++ | ++ | + |
| Liriodenine, CDH-II-37 | + | - | - | + | - | - | + | - | - | ++ | - | - | + | + | - | - | - | - | + | - | - |
| Onychine | ++ | + | - | ++ | + | + | ++ | ++ | + | ++ | ++ | ++ | + | + | + | +++ | +++ | ~ | +++ | ++ | + |
| Benomyl | - | - | - | - | - | - | ~ | - | - | ~ | - | - | + | + | - | - | - | - | + | + | + |
| Captan | + | - | - | + | - | - | + | - | - | ++ | ~ | - | ++ | + | + | - | - | - | ++ | ++ | ++ |
| Cyprodinil | - | - | - | - | - | - | ~ | ~ | ~ | ~ | ~ | - | ++ | + | + | - | - | - | +++ | + | + |
| Fenbuconazole | - | - | - | - | + | - | ~ | ~ | - | - | - | - | + | + | + | - | - | - | + | + | + |
| Fenhexamid | - | - | - | + | + | - | + | - | ~ | + | - | - | ++ | ++ | + | + | - | - | +++ | ++ | + |
| Iprodione | - | - | - | + | + | - | + | + | - | + | + | - | ++ | + | + | - | - | - | +++ | ++ | + |
| Kresoxim-methyl | - | - | - | - | - | - | - | - | - | - | - | - | + | - | - | - | - | - | ++ | + | - |

[x]Growth medium without any fungicide.
[y]Concentration of fungicides incorporated into growth medium.
[z]Growth is indicated by the following symbols: − = no germination, ~ = germination < 90%, + = germination approximately 90%, ++ = germination > 90% but less than 100%, +++ = virtually all condia germinated. Note that qualitative levels of germination cannot be compared across species due to inherent species differences in growth rates and delayed times for beginning observations (due to the large number of simultaneous observations required).

Table 4. Sensitivity of fungi to fungicides. Comparative growth response[w] of five plant pathogens in growth solution amended with three concentration levels[x] of conventional and natural product-based fungicides compared to growth in unamended solution after 72 h.

| Concentration Fungicide | Botrytis cinerea | Colletotrichum acutatum | Colletotrichum fragariae | Colletotrichum gloeosporioides | Fusarium oxysporum | LSD$_{0.05}$ |
|---|---|---|---|---|---|---|
| 0.3 µM | | | | | | |
| 4-Bromosampangine | -48c[y], B[z] | -7cd, A | -15b, A | -99d, C | -3ab, A | 24 |
| Sampangine | -18b, AB | -6cd, A | -33bc, B | -33b, B | -5ab, A | 22 |
| Benomyl | -97d, D | 8bc, A | -37c, C | -16b, B | 2a, AB | 18 |
| Captan | 25a, A | 10abc, B | 15a, AB | 15a, AB | 5a, B | 14 |
| Cyprodinil | -90d, C | -85e, C | -83d, C | -56c, B | 5a, A | 9 |
| Fenbuconazole | -83d, D | 16ab, A | 19a, A | -59c, C | -10b, B | 19 |
| Fenhexamid | -98d, C | 20ab, A | 13a, AB | 11a, AB | 0ab, B | 17 |
| Iprodione | 24a, A | 27a, A | 17a, A | 18a, A | 2a, A | 35 |
| Kresoxim-methyl | -97d, C | -18d, A | -36c, A | -59c, B | -30c, A | 18 |
| LSD$_{0.05}$ | 19 | 18 | 18 | 20 | 2 | |

Table 4 (cont'd).

| Concentration Fungicide | Botrytis cinerea | Colletotrichum acutatum | Colletotrichum fragariae | Colletotrichum gloeosporioides | Fusarium oxysporum | LSD$_{0.05}$ |
|---|---|---|---|---|---|---|
| 3.0 µM | | | | | | |
| 4-Bromosampangine | -59c, C | -25b, AB | -46c, BC | -99d, D | -14bc, A | 23 |
| Sampangine | -79d, B | -90d, BC | -97f, C | -99d, C | -43e, A | 11 |
| Benomyl | -94e, C | 15a, A | -93ef, C | -60b, B | -1a, A | 19 |
| Captan | -2a, A | -99d, C | -98f, C | -98d, C | -24cd, B | 20 |
| Cyprodinil | -97e, C | -95d, C | -84e, B | -84cd, B | -6ab, A | 7 |
| Fenbuconazole | -93e, C | 6a, A | 5b, A | -98d, C | -29d, B | 20 |
| Fenhexamid | -97e, C | 11a, A | 10b, AB | 16a, A | -4ab, B | 14 |
| Iprodione | -40b, C | 22a, A | 24a, A | 31a, A | 4a, B | 17 |
| Kresoxim-methyl | -97e, D | -48c, A | -72d, B | -83c, C | -66f, B | 9 |
| LSD$_{0.05}$ | 12 | 20 | 12 | 15 | 12 | |

Table 4 (cont'd)

| Concentration<br>Fungicide | Botrytis<br>cinerea | Colletotrichum<br>acutatum | Colletotrichum<br>fragariae | Colletotrichum<br>gloeosporioides | Fusarium<br>oxysporum | LSD$_{0.05}$ |
|---|---|---|---|---|---|---|
| 30 μM | | | | | | |
| 4-Bromosampangine | -32a, A | -11cd, A | -27b, A | -100d, B | -9b, A | 25 |
| Sampangine | -98b, A | -100f, B | -100c, B | -100d, B | -100f, B | 1 |
| Benomyl | -96b, C | 15b, A | -94c, C | -45c, B | -77e, C | 20 |
| Captan | -28a, A | -100f, B | -100c, B | -100d, B | -100f, B | 6 |
| Cyprodinil | -98b, B | -98f, B | -89c, B | -95d, B | -31c, A | 10 |
| Fenbuconazole | -98b, C | -22d, A | -98c, C | -100d, C | -45d, B | 13 |
| Fenhexamid | -100b, C | -2c, A | -20b, AB | -7b, AB | -21bc, B | 18 |
| Iprodione | -98b, C | 65a, A | 58a, A | 17a, B | 11a, B | 34 |
| Kresoxim-methyl | -99b, B | -70e, A | -80c, B | -90d, C | -70e, A | 7 |
| LSD$_{0.05}$ | 4 | 16 | 21 | 18 | 12 | |

[w]Growth response calculated as ((Mean Sample Absorbency) - (Mean Unamended Absorbency) - (Mean Unamended Absorbency) x 100)/(Mean Unamended Absorbency) = % Inhibition/Stimulation. Positive numbers indicate a stimulation of growth and negative numbers indicate an inhibition of growth compared to the growth of the fungus on unamended medium.

[x]Concentration of fungicides incorporated into growth medium.

[y]Mean values followed by different lower case letters in the same column within a concentration within a fungus are significantly (*P* = 0.05) different as determined by least significant difference (LSD).

[z]Mean values followed by different upper case letters in the same row are significantly (*P* = 0.05) different as determined by LSD.

74

Table 5. Sensitivity of fungi to natural product-based analog fungicides. Comparative growth response[w] of five plant pathogens in growth solution amended with three concentration levels[x] of natural product-based analog fungicides compared to growth in unamended solution after 72 h.

| Concentration Fungicide | Botrytis cinerea | Colletotrichum acutatum | Colletotrichum fragariae | Colletotrichum gloeosporioides | Fusarium oxysporum | LSD$_{0.05}$ |
|---|---|---|---|---|---|---|
| 0.3 µM | | | | | | |
| 4-Methoxysampangine | -5a[y], A[z] | 0c, A | -3b, A | -16b, A | 3a, A | 26 |
| Benzo[4,5]Sampangine | -55b, B | -26d, A | -20c, A | -100c, C | -17b, A | 26 |
| Cryptolepine | 1a, A | 18a, A | 11a, A | 22a, A | 0a, A | 23 |
| Liriodenine, CDH-II-37 | -12a, B | 20a, A | 5ab, AB | -6b, AB | 1a, AB | 27 |
| Liriodenine, Mel AMC-XII-103 | -15a, B | 11b, A | 4ab, AB | -4b, AB | 0a, AB | 24 |
| Onychine | -8a, A | 20a, A | 5ab, AB | -6b, AB | 1a, AB | 27 |
| LSD$_{0.05}$ | 28 | 7 | 10 | 14 | 6 | |

Table 5 (cont'd)

| Concentration Fungicide | Botrytis cinerea | Colletotrichum acutatum | Colletotrichum fragariae | Colletotrichum gloeosporioides | Fusarium oxysporum | LSD$_{0.05}$ |
|---|---|---|---|---|---|---|
| 3.0 μM | | | | | | |
| 4-Methoxysampangine | -18a, A | -44c, B | -44cd, B | -91c, C | -34b, AB | 24 |
| Benzo[4,5]Sampangine | -47a, A | -46c, A | -55d, A | -99c, B | -42b, A | 41 |
| Cryptolepine | -44a, B | 13a, A | -18ab, AB | 9a, A | -6a, A | 33 |
| Liriodenine, CDH-II-37 | -59a, B | -12b, A | -30bcd, AB | -98c, C | -38b, AB | 29 |
| Liriodenine, Mel AMC-XII-103 | -27a, AB | -6ab, A | -27bc, AB | -54b, C | -33b, BC | 22 |
| Onychine | -21a, B | 13a, A | 5a, AB | 8a, AB | -3a, AB | 33 |
| LSD$_{0.05}$ | 67 | 22 | 27 | 16 | 14 | |

Table 5 (cont'd)

| Concentration Fungicide | Botrytis cinerea | Colletotrichum acutatum | Colletotrichum fragariae | Colletotrichum gloeosporioides | Fusarium oxysporum | LSD$_{0.05}$ |
|---|---|---|---|---|---|---|
| 30 μM | | | | | | |
| 4-Methoxysampangine | 19a, A | -25b, B | -40b, B | -79b, C | -24b, B | 23 |
| Benzo[4,5]Sampangine | -23ab, A | -39c, A | -29ab, A | -100c, B | -27b, A | 30 |
| Cryptolepine | -57bcd, A | -58d, A | -97c, B | -99c, B | -62c, A | 31 |
| Liriodenine, CDH-II-37 | -92d, A | -99e, B | -98c, B | -99c, B | -99d, B | 3 |
| Liriodenine, Mel AMC-XII-103 | -87cd, A | -98e, B | -93c, AB | -96c, AB | -99d, B | 10 |
| Onychine | -45bc, B | 0a, A | -11a, A | 3a, A | -11a, A | 21 |
| LSD$_{0.05}$ | 45 | 11 | 21 | 14 | 7 | |

[w]Growth response calculated as ((Mean Sample Absorbency) - (Mean Unamended Absorbency) x 100)/(Mean Unamended Absorbency) = % Inhibition/Stimulation. Positive numbers indicate a stimulation of growth and negative numbers indicate an inhibition of growth compared to the growth of the fungus on unamended medium.

[x]Concentration of fungicides incorporated into growth medium.

[y]Mean values followed by different lower case letters in the same column within a concentration within a fungus are significantly ($P = 0.05$) different as determined by least significant difference (LSD).

[z]Mean values followed by different upper case letters in the same row are significantly ($P = 0.05$) different as determined by LSD.

Table 6. Effects of natural product-based and commercial fungicides during pre- and post-inoculation greenhouse treatments.

| | Azoxystrobin | | | | Sampangine | | | |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| | Pre-inoculation | | Post-inoculation | | Pre-inoculation | | Post-inoculation | |
| | Anthracnose | Phytotoxicity | Anthracnose | Phytotoxicity | Anthracnose | Phytotoxicity | Anthracnose | Phytotoxicity |
| None | 0.58a | 0.08b | 0.75a | 0.33a | 0.58a | 0.08b | 0.75a | 0.33a |
| 50% Etoh | 0.25a | 0.33a | 0.83a | 0.00b | 0.25a | 0.33ab | 0.83a | 0.00b |
| 625 ppm | 0.33a | 0.08b | 0.42ab | 0.08b | 0.75a | 0.08b | 0.25b | 0.00b |
| 1,250 ppm | 0.83a | 0.08b | 0.08b | 0.17ab | 0.75a | 0.08b | 0.17b | 0.00b |
| 2,500 ppm | 0.67a | 0.08b | 0.42ab | 0.00b | 0.58a | 0.50a | 0.29b | 0.00b |
| LSD | 0.60 | 0.17 | 0.54 | 0.23 | 0.58 | 0.36 | 0.41 | 0.17 |
| $Pr > F$ | 0.27 | 0.14 | 0.06 | 0.04 | 0.36 | 0.08 | 0.01 | 0.00 |

Table 6 (cont'd).

| | CAY-1 | | | |
| --- | --- | --- | --- | --- |
| | Pre-inoculation | | Post-inoculation | |
| | Anthracnose | Phytotoxicity | Anthracnose | Phytotoxicity |
| None | 0.58abc[x] | 0.08b | 0.75ab | 0.33a |
| 50% EtOH | 0.25c | 0.33ab | 0.83 a | 0.00b |
| 312 ppm | 0.42bc | 0.25ab | 0.42abc | 0.00b |
| 625 ppm | 0.92ab | 0.58a | 0.33bc | 0.00b |
| 1,250 ppm | 1.00a | 0.25ab | 0.21 c | 0.00b |
| 2,500 ppm | 0.83ab | 0.08b | 0.21 c | 0.08b |
| LSD | 0.23 | 0.47 | 0.42 | 0.17 |
| $Pr > F$ | 0.04 | 0.28 | 0.02 | 0.00 |

[x]Mean values followed by different letters in the same column are significantly ($P = 0.05$) different as determined by least significant difference (LSD).

Table 7. Semi-quantitative analysis at the microscopic level of the effects of natural product-based and commercial fungicides on fungal germination *in planta*. Strawberry leaves cv. Chandler inoculated with *C. fragariae* isolate CF-75.

| Treatment | Fungicide | Concentration (ppm) | Observations | | |
|---|---|---|---|---|---|
| | | | Ungerminated conidia | Germinated conidia | Appressoria |
| | None | 0 | 4 | 2 | 5 |
| | 50% EtOH | 0 | 4 | 4 | 3 |
| | Sampangine | 625 | 3 | 2 | 4 |
| | | 1,250 | 3 | 3 | 5 |
| | | 2,500 | 3 | 3 | 5 |
| Pre-inoculation | CAY-1 | 312 | 1 | 1 | 2 |
| | | 625 | 3 | 2 | 4 |
| | | 1,250 | 2 | 1 | 4 |
| | | 2,500 | 2 | 2 | 3 |
| | Azoxystrobin | 625 | 3 | 3 | 3 |
| | | 1,250 | 2 | 2 | 4 |
| | | 2,500 | 2 | 2 | 3 |

81

Table 7. (cont'd)

| Treatment | Fungicide | Concentration (ppm) | Observations | | |
|---|---|---|---|---|---|
| | | | Ungerminated conidia | Germinated conidia | Appressoria |
| | None | 0 | 3 | 3 | 5 |
| | 50% EtOH | 0 | 3 | 2 | 5 |
| | Sampangine | 625 | 4 | 2 | 1 |
| | | 1,250 | 2 | 2 | 1 |
| | | 2,500 | 2 | 2 | 1 |
| Post-inoculation | CAY-1 | 312 | 1 | 1 | 1 |
| | | 625 | 1 | 1 | 1 |
| | | 1,250 | 1 | 1 | 1 |
| | | 2,500 | 1 | 1 | 1 |
| | Azoxystrobin | 625 | 1 | 1 | 1 |
| | | 1,250 | 1 | 1 | 1 |
| | | 2,500 | 1 | 1 | 1 |

Scale: 1 = very few; 2 = few; 3 = some; 4 = many; 5 = lots

Table 8. Estimates of conidial inoculation of strawberry leaflets (cv. Chandler) with *Colletotrichum acutatum*.

| Conidial conc. (conidia/ml) | Inoculum delivered (ml) | Conidia counted | Fields counted | Conidia/field | Total leaflet area (m²)[a] | Total fields/ leaflet[b] | Actual conidia/ leaflet[c] | Expected conidia/ leaflet[d] | Unac- counted conidia[e] |
|---|---|---|---|---|---|---|---|---|---|
| 600,000 | 0.68 | 2,226 | 162 | 13.72 | 1,828,451,345 | 2,474 | 34,018 | 408,000 | 373,982 |
| 780,000 | 0.36 | 1,014 | 144 | 7.05 | 1,636,601,483 | 2,215 | 15,616 | 280,800 | 265,184 |
| 650,000 | 0.36 | 1,155 | 126 | 9.2 | 1,335,178,009 | 1,807 | 16,624 | 234,000 | 217,376 |
| 1,450,000 | 0.65 | 2,967 | 162 | 18.3 | 1,654,558,253 | 2,239 | 40,974 | 942,500 | 901,526 |
| 1,520,000 | 0.38 | 2,544 | 144 | 17.7 | 1,445,388,231 | 1,956 | 34,621 | 577,600 | 542,979 |
| 1,420,000 | 0.39 | 1,522 | 135 | 11.3 | 1,663,734,206 | 2,251 | 25,436 | 553,800 | 528,364 |

[a]Calculated using Bioquant 98®
[b]Total fields/leaflet = Total leaflet area/area of one microscope field (738,982 μm²).
[c]Actual conidia/leaflet = conidia/field x total fields/leaflet
[d]Conc. x inoculum delivered
[e]Expected conidia/leaf - actual conidia/leaflet

Table 9. Counts of conidia on photo-etched coverslips simulating spot inoculation of a strawberry leaflet.

| Actual conidial count[a] | Inoculum (750,000 conidia/ml)[b] | Conidial concentration (conidia/ml) | Expected conidial count[a,c] | Unaccounted conidia[d] |
|---|---|---|---|---|
| 2,528 | 34,000 | 640,000 | 2,176 | 358 |
| 2,332 | 35,000 | 640,000 | 2,240 | 92 |
| 1,400 | 40,000 | 682,500 | 2,730 | 1,330 |
| 1,441 | 40,000 | 682,500 | 2,730 | 1,289 |
| 2,760 | 43,000 | 625,000 | 2,687 | 73 |
| 3,451 | 49,000 | 625,000 | 3,066 | 388 |
| 1,475 | 30,000 | 715,000 | 2,145 | 670 |
| 1,318 | 30,000 | 715,000 | 2,145 | 827 |
| 1,343 | 34,000 | 707,500 | 2,405 | 1,062 |
| 1,441 | 35,000 | 707,500 | 2,476 | 1,035 |
| 1,333 | 30,000 | 675,000 | 2,025 | 692 |
| 1,461 | 40,000 | 675,000 | 2,700 | 1,239 |
| 1,282 | 37,000 | 700,000 | 2,590 | 1,308 |
| 1,125 | 44,000 | 700,000 | 3,080 | 1,955 |
| 1,593 | 40,000 | 675,000 | 2,700 | 1,107 |
| 1,659 | 40,000 | 675,000 | 2,700 | 1,041 |
| 1,346 | 32,000 | 725,000 | 2,320 | 974 |
| 1,239 | 35,000 | 725,000 | 2,537 | 1,298 |
| 1,107 | 40,000 | 600,000 | 2,400 | 1,293 |
| 1,305 | 40,000 | 600,000 | 2,400 | 1,095 |

[a]Mean of the difference (actual - expected) = 865.45; Std Dev 625.41; Std Error 139.85; t value 6.19; Pr < 0.0001
[b]Originally measured by weight.
[c]Inoculum x conidial concentration
[d]Actual conidia/leaf - expected conidia/leaflet

Table 10. The effect of repeated use of a single pipet to transfer conidia of *Colletotrichum acutatum* isolate Goff to a hemocytometer. Counts were made after each set of five transfers.

| Number of transfers | Number of observations | Average concentration in hemocytometer (conidia/ml)[a, b] | Concentration in pipet (conidia/ml) |
|---|---|---|---|
| 05 | 6 | 487,500 a | 262,500 |
| 10 | 6 | 417,500 a | 332,500 |
| 15 | 6 | 287,500 b | 462,500 |
| 20 | 6 | 245,000 b | 505,000 |
| 25 | 6 | 217,500 b | 532,500 |
| 30 | 6 | 107,500 c | 642,500 |
| 35 | 6 | 112,500 c | 637,500 |
| 40 | 6 | 100,000 c | 650,000 |
| 45 | 6 | 62,500 c | 687,500 |
| 50 | 6 | 87,500 c | 662,500 |

[a]Starting concentration is 750,000 conidia/ml.
[b]LSD = 77,500
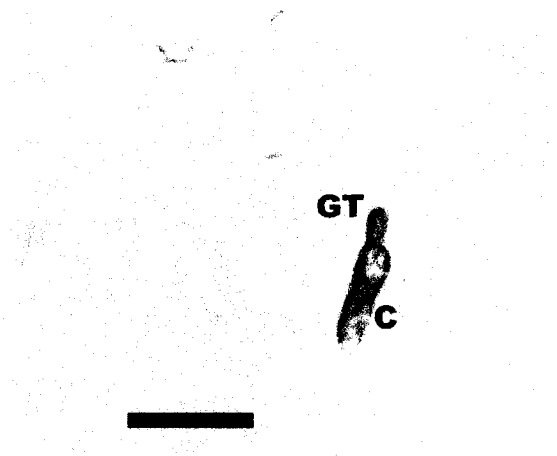
Figure 1. Incipient conidial germination of *Colletotrichum acutatum* challenged with iprodione at a concentration of 3.0 µM. C = conidium, GT = germ tube. Bar = 10 µm



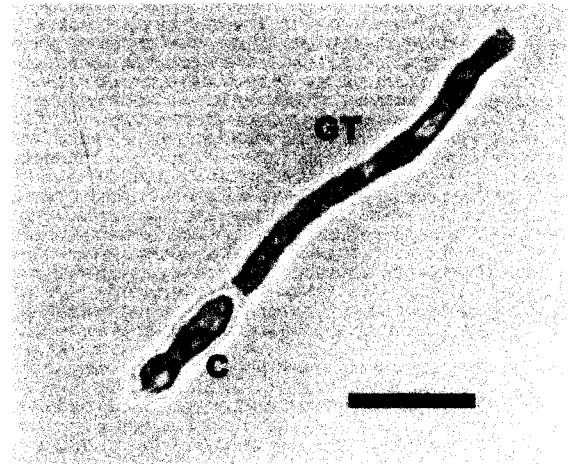Figure 2. Normal conidial germination of *Colletotrichum gloeosporioides* challenged with captan at a concentration of 0.3 µM. C = conidium, GT = germ tube. Bar = 10 µm
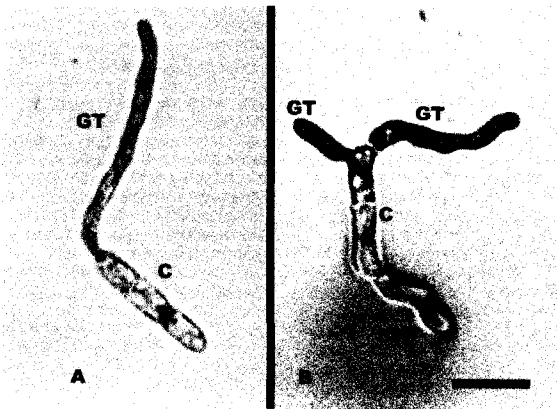


Figure 3. Effect of 4-bromosampangine on germination of *Colletotrichum fragariae* at a concentration of 0.3 µM. A. Normal germ tube development. B. Anomalous germ tube branching. C = conidium, GT = germ tube. Bar = 10 µm
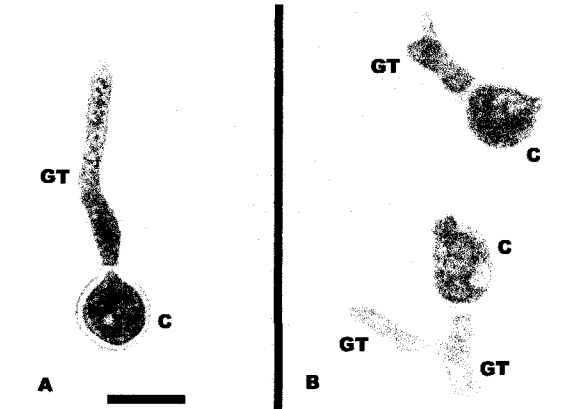


Figure 4. Effect of 4-bromosampangine on the germination of *Botrytis cinerea* at a concentration of 0.3 µM. A. Normal germ tube. B. Splayed (upper) and branched (lower) germ tubes. C = conidium, GT = germ tube. Bar = 10 µm

Figure 5. Cleared strawberry leaflet from cv. Chandler inoculated with 1.5x10$^6$ conidia/ml of *Colletotrichum fragariae* isolate CF-75 showing ungerminated and germinated conidia.



Figure 6. Cleared strawberry leaflet from cv. Chandler inoculated with 1.5x10$^6$ conidial/ml of *Colletotrichum fragariae* isolate CF-75 showing an unidentified germinated fungal conidium.



Figure 7. Pre-inoculation treatment. Strawberry leaflet from cv. Chandler challenged with CAY-1 at 1,250 ppm.



Figure 8. Post-inoculation treatment. Strawberry leaflet from cv. Chandler challenged with sampangine at 1,250 ppm.

Figure 9. Pre-inoculation treatment. Cleared strawberry leaflet from cv. Chandler inoculated with $1.5 \times 10^6$ conidia/ml of *Colletotrichum fragariae* isolate CF-75 and challenged with sampangine at 625 ppm. Arrows point to representative appressoria.



Figure 10. Post-inoculation treatment. Cleared strawberry leaflet from cv. Chandler challenged with sampangine at 625 ppm and inoculated with $1.5 \times 10^6$ conidia/ml of *Colletotrichum fragariae* isolate CF-75.
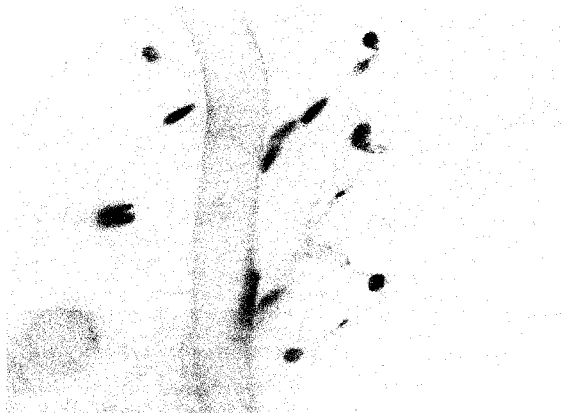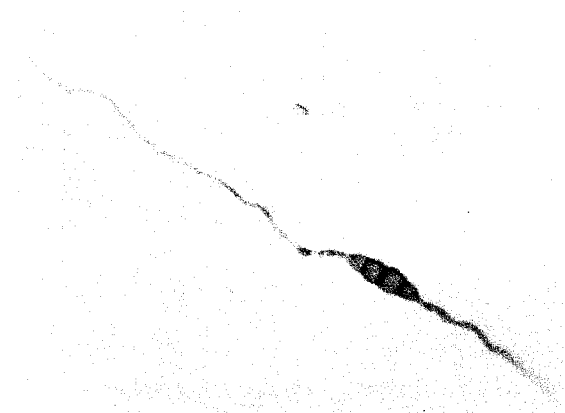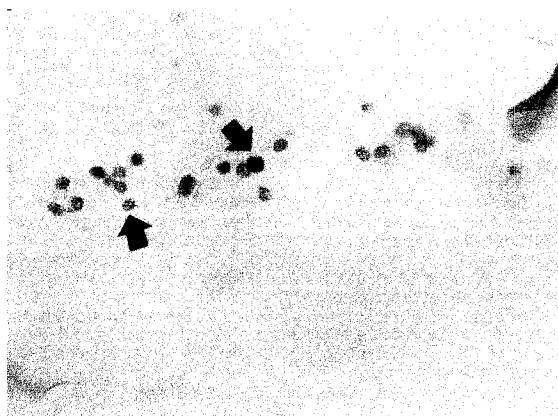


Figure 11. Pre-inoculation treatment. Cleared strawberry leaflet from cv. Chandler inoculated with $1.5 \times 10^6$ conidia/ml of *Colletotrichum fragariae* isolate CF-75 and challenged with CAY-1 at 625 ppm. Arrows point to representative appressoria.



Figure 12. Post-inoculation treatment. Cleared strawberry leaflet from cv. Chandler challenged with CAY-1 at 2,500 ppm and inoculated with $1.5 \times 10^6$ conidia/ml of *Colletotrichum fragariae* isolate CF-75.
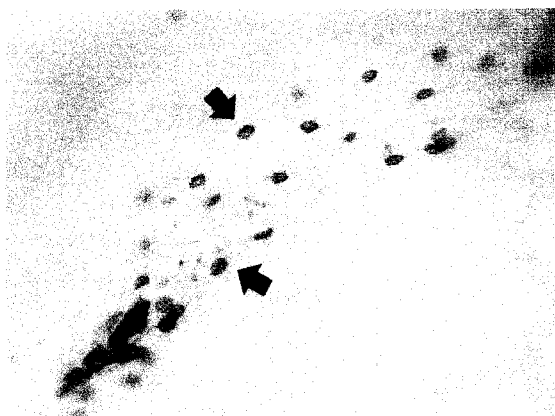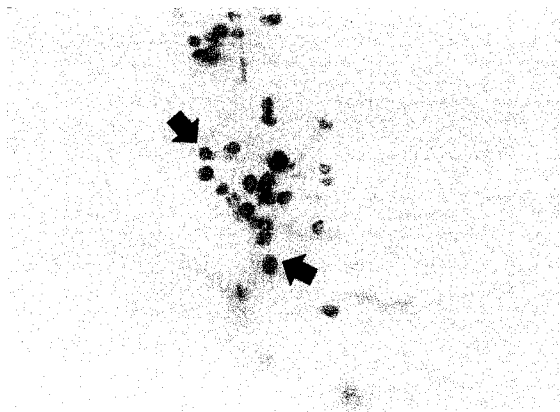
Figure 13. Pre-inoculation treatment. Cleared strawberry leaflet from cv. Chandler inoculated with 1.5x10$^6$ conidia/ml of *Colletotrichum fragariae* isolate CF-75 and challenged with azoxystrobin at 625 ppm. Arrows point to representative appressoria.
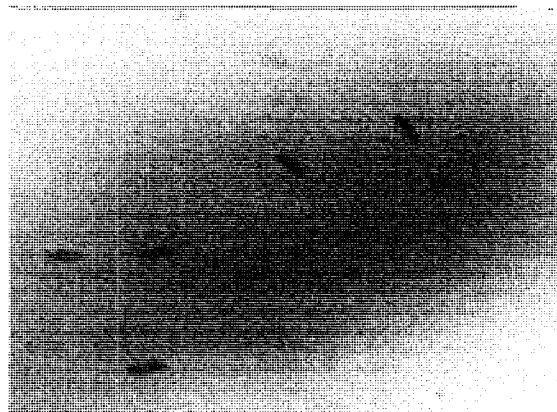


Figure 14. Post-inoculation treatment. Cleared strawberry leaflet from cv. Chandler challenged with azoxystrobin at 1,250 ppm and inoculated with 1.5x10$^6$ conidia/ml of *Colletotrichum fragariae* isolate CF-75.



Figure 15. Pre-inoculation treatment. Strawberry leaflet from cv. Chandler challenged with sampangine at 2,500 ppm.
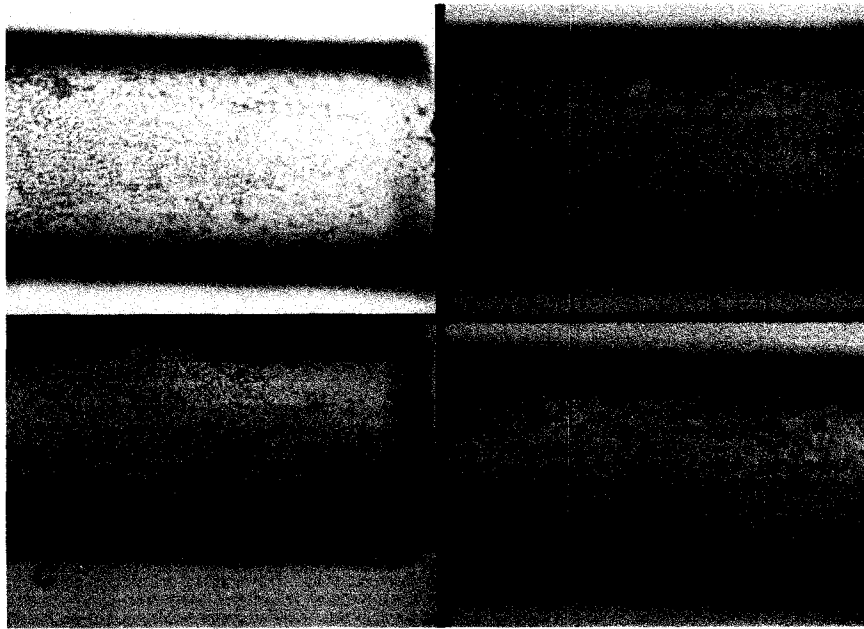
Figure 16. Representative glass pipet tips showing the accumulation of conidia of *Colletotrichum acutatum* isolate Goff on the inner wall through repeated aliquot transfers. (A) 20 transfers, (B) 30 transfers, (C) 40 transfers, and (D) 50 transfers.

CHAPTER V

DISCUSSION

*In Vitro* Testing

*Microbioassay*

Several modifications to assay methods reduced variability in percent germination

and the time to maximum germination which proved beneficial for screening fungicides

*in vitro*. Conidial concentrations between $10^5$ and $10^6$ conidia/ml ensured the appropriate

amount of fungal inoculum to assess germination levels of selected plant pathogenic

fungi precisely and accurately. The incorporation of Roswell Park Memorial Institute

mycological liquid medium 1640 (RPMI) as the carbon source provided adequate

germination levels in a reasonable time plus it provided the advantage of knowing the

exact composition of the medium. My preliminary studies (data not presented) revealed

the importance of introducing the washing of conidia towards obtaining reproducible

germination levels of fungal conidia in screening experiments. Seemingly minor points

such as not washing the conidial inoculum can lead to misinterpreted results. The level of

conidial germination when spores are not washed is dramatically lower than after

washing. But without knowing the level of germination in a screening test, the efficacy

of a developmental fungicide might be mistakenly rated high, when in fact, the fungus

just failed to germinate at infectious levels for other reasons. Washing conidia and over

night refrigeration prior to using a conidial suspension as inoculum removed natural

germination inhibitors contained in the conidial matrix (Arpin and Bouillant, 1981; Leite

and Nicholson, 1992; Louis and Cooke, 1985a; Seebach et al., 1989), reduced

germination times, and increased cultural synchrony of all fungal species. This improved

the reliability and reproducibility of the microtiter assays and reduced experimental error associated with growth measurement or error bias. Furthermore, fungal species were selected in early testing of fungicides in microtiter assays based on observed response patterns. Finally, the novel use of glass cover slips in each well of the 24-well cell culture clusters provided a convenient method for achieving high quality microscopic observations that allowed me to document the effect of fungicides on fungal developmental morphology. Photometric readings are sensitive to percent germination that resulted from increased biomass, but those readings cannot distinguish developmental stages of fungal growth (i.e., conidial germination, germ tube elongation, appressorial formation, hyphal growth, and mycelial development).

Sampangine is an effective natural product-based fungicide inhibiting germination in all test fungi at 3.0 and 30 µM except *C. acutatum*. It out-performed all of its analogs and was more effective against *F. oxysporum* and *P. viticola* than most of the commercial fungicides tested. These data indicate that sampangine would be a welcome addition to the arsenal against plant pathogenic fungi.

The physical mode of action of sampangine is apparently to inhibit conidial germination in *B. cinerea*, *C. fragariae*, *C. gloeosporioides*, *F. oxysporum*, *P. obscurans*, and *P. viticola*. This apparent physical mode of action is comparable to some of the commercial fungicides tested including captan, iprodione, and kresoxim-methyl that also inhibit conidial germination on several hosts (Edlich and Lyr, 1995; Leroux, 1996; Pommer and Lorenz, 1982; Ypema and Gold, 1999). Köller (1994) describes captan as a multi-site fungal inhibitor, consistent with my observations that captan (and sampangine)

did not inhibit conidial germination in *C. acutatum* at about 9 h, although both fungicides prevented subsequent growth measured at 48 h (microtiter data not shown).

The sampangine analog, 4-bromosampangine, was the most interesting natural product-based fungicide tested. Direct microscopic observations of the effects of this sampangine analog indicated that germ tube development was affected not only by the presence of distorted germ tubes that branched more frequently than those of untreated conidia, but also by the emergence of multiple germ tubes suggesting the possibility that this compound may protect the host after fungi have already established themselves in or on the host. This analog provides a new avenue to further explore in detail its physical mode of action given that it induces different effects in the germ tubes of two different species suggesting a non-specific site to provide control of fungal pathogens.

Anomalies caused by 4-bromosampangine in germ tube development suggest a physical mode of action similar to the action of commercial fungicides benomyl, cyprodinil, and fenhexamid where conidial germination is not inhibited, but subsequent developmental stages such as germ tube elongation and hyphal growth are inhibited (Duben et al., 2002; Hänßler and Pontzen, 1999; Latorre et al., 2002; Leroux et al., 1998; Richmond and Pring 1971; Rosslenbroich and Stuebler, 2000). In addition, observations of germinating conidia of *B. cinerea* (Hänßler and Pontzen, 1999) and *B. fabae* (Richmond and Pring 1971), when exposed to fenhexamid and benomyl respectively, produced swollen, branched germ tubes. My observations of the effects of 4-bromosampangine coincide with the results of those studies, and therefore, a testable hypothesis for the physical mode of action would postulate that germ tube elongation and hyphal growth, but not conidial germination, is inhibited by 4-bromosampangine.

Benomyl binds to β-tubulin disrupting microtubule function (Hippe, 1991). Microtubules constitute the cytoskeleton of the cell and maintain the integrity of the Spitzenkörper which contains the material necessary for cell wall synthesis and indicates the direction of cell expansion (Howard and Aist, 1980; Richmond and Pring, 1971). My observations of germ tube morphological anomalies may have resulted from the disruption of microtubule mediated transport of cell wall material (Howard and Aist, 1980), and therefore, a reasonable testable hypothesis for the biochemical mode of action would postulate that microtubules in the cytoskeleton of growing fungal tips are disrupted by 4-bromosampangine.

The most obvious effects of sampangine and the commercial fungicides tested were to inhibit and/or delay germination which raises an interesting question, not tested in the scope of this research, regarding the duration of inhibition. Richmond and Pring (1971) reported that conidia of *B. fabae* that had been completely inhibited from germinating in the presence of benomyl germinated normally when transferred to a medium free of benomyl. In addition they reported that when exposed again to benomyl, germinating conidia of *B. fabae* produced swollen and distorted germ tubes that branched more frequently than the usual. However, conidia of *B. fabae* resumed normal growth when transferred to a medium free of benomyl. Furthermore, the abnormal germ tubes produced in the presence of benomyl formed a single hypha and grew normally in the absence of the fungicide. The technique I developed allowed me to document not only the presence of distorted germ tubes that branched more frequently than those of normal conidia but also the emergence of multiple germ tubes caused by 4-bromosampangine in *B. cinerea* and *C. fragariae*.

The interaction between fungal pathogens and fungicides described in this study is the first detailed investigation on fungal germination of the two new natural product-based synthesized fungicides sampangine and 4-bomosampangine against the anamorphs of seven economically important Ascomycetes and may serve as a model for other similar interactions. Microbioassays add a new dimension to the accurate, sensitive, and rapid detection system of fungal sensitivities provided by the microtiter assays since it incorporates visualization of fungal anomalies at various developmental stages caused by the fungicides and would allow targeting specific compounds from a pool of tested compounds. Moreover, since the microbioassay is corroborated by data obtained from the microtiter assay, combining both assays provides a reasonable approach to better understand fungicide-pathogen interactions and to investigate the physical and biochemical modes of action of fungicides.

*Microtiter Assay*

*Fusarium oxysporum* was the least sensitive species challenged by the commercial fungicides. In view of the increasing insensitivity exhibited by many fungi, sampangine represents an alternative to commercial fungicides now used to control economically important plant pathogenic fungi. The potential benefits and broad spectrum antifungal activity exhibited by sampangine make it a promising candidate for further greenhouse testing and even field studies. Results from preliminary greenhouse trials demonstrated the protectant activity of sampangine by reducing leaf lesion severity on strawberry when applied to the host prior to inoculation with *C. fragariae* (Wedge, unpublished data).

*Phomopsis* spp. were not included in the microtiter assay due to their asynchronous germination behavior which prevented reliable absorbency readings by the

microplate photometer. Hyphal growth after conidial germination for these species

requires several days, and as a result, the sensitivity and reproducibility of the method

(absorbency) is diminished, since only a proportion of the growing population can be

measured. *Phomopsis* spp. proved not to be suitable organisms for testing the efficacy of

fungicides. On the other hand, *B. cinerea, C. acutatum, C. fragariae, C. gloeosporioides,*

and *F. oxysporum* represent excellent examples of organisms suitable for this type of

assay.

I focused through *in vitro* assays on the efficacy of the natural product-based

fungicide, sampangine, and its analogs, by observing their effects on fungal growth. The

antifungal activity of sampangine is comparable in efficacy to commercial fungicides at

inhibiting fungal growth and is more promising than any of its analogs. The inhibitory

effects of sampangine on fungal growth is relevant when considering the potential

reduction of the initial infection and dispersal of the pathogens (Ypema and Gold, 1999).

*In Planta* Testing

*Leaf Clearing Technique*

The development of this technique has proven valuable in support of the

greenhouse screening experiments I conducted for testing the efficacy of natural product-

based and commercial fungicides *in planta*. Microscopic observations of the effects of

sampangine and CAY-1 indicated that germ tube development was affected during the

post-inoculation treatments which is relevant regarding their potential use as fungicides.

Thus, the development of the leaf clearing technique confirms a proper direction for

exploring the mode of action of natural product-based fungicides suggested by the

detached leaf assays. Sampangine and CAY-1 exhibit a similar mode of action to that

documented for azoxystrobin to control anthracnose on the surface of treated strawberry leaflets by preventing conidial germination, therefore acting as protectant fungicides (Bertelsen et al., 2001; Wong and Wilcox, 2001).

In addition, this technique allowed me to correlate disease symptoms at the macroscopic level with fungal growth at the microscopic level ruling out the possibility of "false–positive" results. "False– positive results" are due to the fact that the fungus might not be on the leaf surface, or might be at such low concentrations that it does not cause disease symptoms, or the fungus may fail to infect. Finally, this technique allowed me to identify fungal spores different from the ones sprayed to the host and establish that the presence of such unexpected spores may or may not contribute to some extent to the severity of the disease. Therefore it let me directly relate anthracnose lesions with the presence of the fungal pathogen *C. fragariae* isolate CF-75 that I had inoculated onto the strawberry leaf.

*Detached Leaf Assay for Fungicide Evaluation*

A persistent problem for *in planta* assays is the uncertainty of whether the resistance of the plant or the efficacy of the fungicide is causally related to "false–positive" results or to the failure of the fungus to perform on cue. Conducting *in planta* tests to confirm fungal sensitivity *in vitro* is essential to reduce the level of uncertainty generated by these variables. Furthermore, the fact that resistance to several fungicide classes has occurred in some *Colletotrichum* especies (Peres et al., 2002, 2004) makes an assessment of the level of the efficacy of new fungicides necessary. To accomplish this, methods that allow the baseline sensitivity for these fungicides are needed. However, current *in vitro* methods used to evaluate the effects of protectant

fungicides (radial growth measurements in the presence of different fungicide concentrations and determination of the minimal inhibitory fungicide concentration, among others) by inhibiting spore germination are inappropriate when the mechanism of the fungicide to be tested is related to infection or growth within the plant (Kunz et al., 1998).

To measure the inhibition of germination the plant host is not required and sensitivity tests can be performed on microscopic slides, agar plates, or cell culture clusters (Peres et al., 2004; Petsikos-Panayotarou et al., 2003; Wedge and Kuhajek, 1998; Wedge et al., 2001). However, a disadvantage of this method is that inhibitory fungicide concentrations *in vitro* are smaller than those used in the field and no conclusions can be drawn regarding fungicide efficacy in the field. On the other hand, even though sensitivity assays on whole plants or detached leaves are labor intensive *in planta* assays are particularly suited to analyze fungal sensitivity against protectant and curative fungicides since fungal virulence and susceptibility of the plant tissue contribute to the results (Kunz et al., 1998).

*In vitro* assays may not reflect field conditions and sensitive isolates could be inaccurately classified as resistant since infection structures are not differentiated *in vitro* (Birchmore et al., 1996). *Colletotrichum* species exhibit a targeted secretion of cutinolytic enzymes only during differentiation of infection structures (Podila et al., 1995). However, some fungi do not differentiate infection structures during growth on agar plates and therefore, secretion of infection related enzymes is not required. As a consequence, *in planta* tests involving the plant host constitute an absolute necessity to corroborate sensitivity of fungal populations to new fungicides *in vitro*.

My results show that the natural product-based fungicides sampangine, CAY-1, and the commercial fungicide azoxystrobin provided protection against anthracnose when applied 24 h before the fungal inoculum, but they had negligible or no effect on the incidence of the disease when applied 24 h after the fungal inoculum. These results support the findings of Wong and Wilcox (2001) who report protectant activity of azoxystrobin when applied before fungal inoculation, but had little effect on the incidence of disease when applied after fungal inoculation. These results obtained during the greenhouse screening trials suggest a protectant physical mode of action for the natural product-based fungicides sampangine and CAY-1 against *C. fragariae* isolate CF–75 similar to that documented for the commercial fungicide azoxystrobin which entails the inhibition of conidial germination (Bertelsen et al., 2001; Wong and Wilcox, 2001). Promising results from the greenhouse studies suggest that field studies of these two natural product-based fungicides is warranted since ultimately screening under field conditions is the final stage in a natural product discovery program before going into commercial production.

*In Planta* Screening Techniques

*Calculation of Conidia per Unit Area*

The level of inoculum delivered to a leaf surface was consistently less than expected. This consistent error can confound interpretation of *in planta* screening studies. Without the microscopic analysis, an investigator would have expected their inoculum to be about 10-fold higher than delivered. The significance of this finding has practical applications. If a fungicide was being tested and the inoculum delivered was insufficient to elicit disease symptoms, it would lead to the assumption that the fungicide

was effective. Likewise, if a susceptible cultivar of strawberry was being tested and the inoculum was insufficient to elicit disease symptoms, it would lead to the assumption that the cultivar was resistant. An estimate of the amount of inoculum that actually lands on the host would be important for a correct interpretation of the results obtained during screening trials.

<p align="center">*In Vitro* Screening Techniques</p>

*Simulated Spot Inoculation*

The photo-etched coverslip experiments eliminated the potential experimental errors from the *in planta* experiments including conidia not attaching to the leaflet surface, and thus in the runoff, and conidia settling on the inner tube of the sprayer. Photo-etched coverslips allowed me to obtain an exhaustive count of the conidia.

*Pipeting Experiments*

Adsorbance to the pipet tips accounted for the missing conidia, suggesting that the results of screening procedures should be interpreted with caution since the number of conidia landing on the plant surface is consistently less than the initial inoculum concentration. *In planta* assays will carry the uncertainty of whether the resistance of the plant or the efficacy of the fungicide is causally related to "positive" results or to an insufficient amount of inoculum delivered. A measure of the inoculum which actually arrives on the plant is rarely assessed or reported which opens the possibility of evaluating a fungicide as efficacious when in fact the amount of inoculum delivered to the plant was insufficient to induce disease symptoms.

REFERENCES

Abdel-Gawad, M.M., S.M. El-Amin, H. Ohigashi, Y. Watanabe, N. Takeda, H. Sugiyama, and M. Kawanaka. 2000. Molluscicidal saponins from *Anagallis arvensis* against schistosome intermediate hosts. Japanese Journal of Infectious Diseases 53:17–19.

Agrios, G.N. 2005. Plant Pathology. 5th ed., Elsevier Academic Press, San Diego, CA. 922 p.

Alexander, H.M. 1992. Evolution of disease resistance in natural plant populations. Pages 326–344 *in* R.S. Fritz and E.L. Simms, eds., Plant Resistance to Herbivores and Pathogens: Ecology, Evolution, and Genetics. The University of Chicago Press, Chicago.

Anesiadis, T., G.S. Karaoglanidis, and K. Tzavella-Klonari. 2003. Protective, curative, and eradicant activity of the strobilurin fungicide azoxystrobin against *Cercospora beticola* and *Erysiphe betae*. Journal of Phytopathology 151:647–651.

Arpin, N., and M.L. Bouillant. 1981. Light and mycosporines. Pages 435–454 *in* G. Turian and H.R. Hohl, eds., The Fungal Spore, Morphogenetic Controls. Academic Press, London.

Atti, S.A-E., H.A Ammar, C.H. Phoebe, P.L. Schiff, and D.J. Slatkin. 1982. Journal of Natural Products 45(4):476–480.

Bailey, J.A., R.J. O'Conell, R.J. Pring, and C. Nash. 1992. Infection strategies of *Colletotrichum* species. Pages 88–120 *in* J.A. Bailey and M.J. Jegerm, eds., *Colletotrichum:* Biology, Pathology, and Control. CAB International Walling-ford, United Kingdom.

Baldwin, B.C., J.M. Clough, C.R.A. Godfrey, J.R Godwin, and T.E. Wiggins. 1996. The discovery and mode of action of ICIA 5504. Pages 69–78 *in* H. Lyr, P.E. Russel, and H.D. Sisler, eds., Modern Fungicides and Antifungal Compounds. Intercept Ltd., Andover, U.K.

Baxter, A.P., G.C.A. Van der Westhuizen, and A. Eric. 1983. Morphology and taxonomy of South African isolates of *Colletotrichum*. South African Journal of Botany 2:259–289.

Bergstrom, G.C., and R.L. Nicholson. 1981. Invertase in the spore matrix of *Colletotrichum graminicola*. Phytopathology 102:139–147.

Bertelsen, J.R., E. de Neergaard, and V. Smedegaard-Petersen. 2001. Fungicidal effects of azoxystrobin and epiconazole on phyllosphere fungi, senescence and yield of winter wheat. Plant Pathology 50:190–205.

Birchmore, R.J., R.J. Williams, R.J. Milling, and P.E. Russell. 1996. Development of methods for monitoring populations of *Botrytis cinerea* for sensitivity to pyrimethanil. Pages 377–385 *in* H. Lyr, P.E. Russel, and H.D. Sisler, eds., Modern Fungicides and Antifungal Compounds. Intercept Ltd., Andover, U.K.

Bonnen, A.M., and R. Hammerschmidt. 1989a. Cutinolytic enzymes from *Colletotrichum lagenarium*. Physiological and Molecular Plant Pathology 35:463–474.

Bonnen, A.M., and R. Hammerschmidt. 1989b. Role of cutinolytic enzymes in infection of cucumber by *Colletotrichum lagenarium*. Physiological and Molecular Plant Pathology 35:475–481.

Bos, L., and J.E. Parlevliet. 1995. Concepts and Terminology on Plant/Pest Relationships: Toward consensus in plant pathology and crop protection. Annual Review of Phytopathology 33:29–102.

Bourett, T.M., and R.J. Howard. 1991. Ultrastructural immunolocalization of actin in a fungus. Protoplasm 163:199–202.

Brent, K.J. 1996. Pathways to success in fungicide research. Pages 3–15 *in* H. Lyr, P.E. Russell, and H.D. Sisler, eds, Modern Fungicides and Antifungal Compounds, Intercept Limited, Andover, Hants, U.K.

Brown, G.E. 1977. Ultrastructure of penetration of ethylene-degreened Robinson tangerines by *Colletotrichum gloeosporioides*. Phytopathology 67:315–320.

Buchenauer, H. 1990. Physiological reactions in the inhibition of plant pathogenic fungi. Pages 217–292 *in* G. Haug and H. Hoffmann, eds., Chemistry of Plant Protection. Springer-Verlag, Berlin, Germany.

Chaky, J., K. Anderson, M. Moss, and L. Vaillancourt. 2001. Surface hydrophobicity and surface rigidity induce spore germination in *Colletotrichum graminicola*. Phytopathology 91:558–564.

Cooke, R. 1977. The Biology of Symbiotic Fungi. John Wiley & Sons. 282 p.

Cox, M.L., and J.A.G. Irwin. 1988. Conidium and appressorium variation in Australian isolates of the *Colletotrichum gloeosporioides* group and closely related species. Australian Systematic Botany 1:139–149.

Curry, K.J., M. Abril, J.B. Avant, and B.J. Smith. 2002. Strawberry anthracnose: Histopathology of *Colletotrichum acutatum* and *Colletotrichum fragariae*. Phytopathology 92:1055–1063.

Dahmen, H., H.C. Hoch, and T. Staub. 1988. Differential effects of sterol inhibitors on growth, cell membrane permeability, and ultrastructure of two target fungi. Phytopathology 78:1033–1042.

Davidse, L.C. 1986. Benzimidazole fungicides: Mechanism of action and biological impact. Annual Review of Phytopathology 24:43–65.

Davidse, L.C. 1987. Biochemical aspects of benzimidazole fungicides - Action and resistance. Pages 245–258 *in* H. Lyr, ed., Modern Selective Fungicides–Properties, Applications, Mechanisms of Action. Gustav Fisher Verlag, New York.

Dekker, J. 1995. Development of resistance to modern fungicides and strategies for its avoidance. Pages 23–38 *in* H. Lyr, ed., Modern Selective Fungicides–Properties, Applications, Mechanisms of Action. Gustav Fisher Verlag, New York.

Delp, C.J. 1987. Benzimidazoles and related fungicides. Pages 233–244 *in* H. Lyr, ed., Modern Selective Fungicides–Properties, Applications, Mechanisms of Action. Gustav Fisher Verlag, New York.

Delp, B.R., and R.D. Milholland. 1980. Evaluating strawberry plants for resistance to *Colletotrichum fragariae*. Plant Disease 64:1071–1073.

De Lapeyre de Bellaire, L., M. Chillet, and X. Mourichon. 2000. Elaboration of an early quantification method of quiescent infections of *Colletotrichum musae* on bananas. Plant Disease 84:128–133.

De Lucca, A.J., J.M. Bland, C.B. Vigo, M. Cushion, C.P. Selitrennikoff, J. Peter, and T.J. Walsh. 2002. CAY-1, a fungicidal saponin from *Capsicum* sp. fruit. Medical Mycology 40:131–137.

Denoyes-Rothan, B., M. Lafargue, G. Guerin, and M. Clerjeau. 1999. Fruit resistance to *Colletotrichum acutatum* in strawberries. Plant Disease 83:549–553.

Dey, P.K. 1933. Studies in the physiology of the appressorium of *Colletotrichum gloeosporioides*. Annals of Botany 47:305–312.

Dhingra, O.D., and Sinclair, J.B. 1995. Basic Plant Pathology Methods. Second edition. Lewis Publishers, FL, USA. 434 p.

Diéguez-Uribeondo, J., H. Förster, and J.E. Adaskaveg. 2003. Digital image analysis of internal light spots of appressoria of *Colletotrichum acutatum*. Phytopathology 93:923–930.

Duben, J., Rosslenbroich, H.J., and Jenner, G. 2002. Teldor® (Fenhexamid) – A new specific fungicide for the control of *Botrytis cinerea* and related pathogens on *Rubus*, *Ribes*, and other crops. Acta Horticulturae 585:325–329.

Edlich, W., and H. Lyr. 1995. Mechanism of action of dicarboximide fungicides. Pages 119–131 *in* H. Lyr, ed., Modern Selective Fungicides–Properties, Applications, Mechanisms of Action. Gustav Fisher Verlag, New York.

Egley, G.H. 1994. Substrate influences upon germination of *Colletotrichum truncatum* conidia. Canadian Journal of Botany 72:1758–1765.

Ehr, R.J., G. Kemmitt, and D. Ouimett. 2005. Periodic Table of Fungicides 2.1. Dow Agrosciences LLC. Indianapolis, IN. L01-818-017 (3/05) BOD; 010-30143.

Emmett, R.W., and D.G. Parbery. 1975. Appressoria. Annual Review of Phytopathology 13:147–167.

Espinel-Ingrof, A., and Kerkering, T.M. 1991. Spectrophotometric method of inoculum preparation for the *in vitro* susceptibility testing of filamentous fungi. Journal of Clinic Microbiology 29:393-394.

Freeman, S., S. Horowitz, and A. Sharon. 2001. Pathogenic and non-pathogenic lifestyles in *Colletotrichum acutatum* from strawberry and other plants. Phytopathology 91:986–992.

Foster, B., and T. Staub. 1996. Basis for use strategies of anilinopyrimidine and phenylpyrrole fungicides against *Botrytis cinerea*. Crop Protection 15(6):529–537.

Goes, A., and H. Kimati. 1994. Efeito de benomyl no crescimento micelial de *Colletrotrichum gloeosporioides*, agente causal da queda prematura de frutos jovens de citros. Summa Phytopathology 24:246–253.

Gold, R.E., E. Ammermann, H. Köhle, G.M.E. Leinhos, G. Lorenz, J.B. Speakman, M. Stark-Urnau, and H. Sauter. 1996. The synthetic strobilurin BAS 490 F: Profile of a modern fungicide. Pages 79–92 *in* H. Lyr, P.E. Russell, and H.D. Sisler, eds, Modern Fungicides and Antifungal Compounds, Intercept Limited, Andover, Hants, U.K.

Green, J.R., N.A. Pain, M.E. Cannell, G.L. Jones, C.L. Leckie, S. McCready, K. Mendgen, A.J. Mitchell, J.A. Callow, and R.J. O'Connell. 1995. Analysis of differentiation and development of the specialized infection structures formed by biotrophic fungal plant pathogens using monoclonal antibodies. Canadian Journal of Botany 73:S408-S417.

Gunnell, P.S., and W.D. Gubler. 1992. Taxonomy and morphology of *Colletotrichum* species pathogenic to strawberry. Mycologia 84(2):157–165.

Hamer, J.E., R.J. Howard, F.G. Chumley, and B. Valent. 1988. A mechanism for surfacce attachment in spores of a plant pathogenic fungus. Science 239:288–290.

Hänßler, G., and R. Pontzen. 1999. Effect of fenhexamid on the development of *Botrytis cinerea*. Pflanzenschutz-Nachrichten Bayer 52(2):158–176.

Hardham, A.R. 1992. Cell biology of pathogenesis. Annual Review of Plant Physiology and Plant Molecular Biology 43:491–526.

Heath, I.B. 1990. The roles of actin in tip growth of fungi. International Review of Cytology 123:95–127.

Hewitt, H.G. 1998. Fungicide resistance. Pages 155–181 *in* H.G. Hewitt, ed., Crop Protection. Centre for Agriculture and Biosciences International, Wallingford, UK.

Heye, U.J., J. Speich, H. Siegle, A. Steinemann, B. Forster, G. Knauf-Beiter, J. Herzog, and A. Hubele. 1994. CGA 219417: a novel broad-spectrum fungicide. Crop Protection 13:541–549.

Hickey, K.D., and K.S. Yoder. 1981. Field performance of sterol-inhibiting fungicides against apple powdery mildew in the mid-Atlantic apple growing region. Plant Disease 65:1002–1006.

Hicks, B.R. (ed.) 2001. DuPont abandoning Benlate. Crop Protection, April 28, 2001, Issue # 137, page 11. Retrieved June 3, 2007, from Crop Protection Web site http://www.crop-protection-monthly.co.uk/Archives/CPMApr2001.doc#_ Toc65669388.

Hippe, S. 1991. Influence of fungicides on fungal fine structure. Pages 265–331 *in* K. Mendgen and D.E. Lesemann, eds., Electron Microscopy of Plant Pathogens. Springer-Verlag, Berlin, Germany.

Hisada, Y., T. Kato, and Y. Kawase. 1977. Systematic movement in cucumber plants and control of cucumber grey mould by a new fungicide, S. 7131. Netherland Journal of Plant Pathology 83(S1):71–78.

Hoch, H.C., and R.C. Staples. 1991. Signaling for infection structure fromation in fungi. Pages 25-46 *in* G.T. Cole and H.C. Hoch, eds., The Fungal Spore and Disease Initiation in Plants and Animals. Plenum Press, New York.

Horowitz, S., S. Freeman, and A. Sharon. 2002. Use of green fluorescent protein-transgenic strains to study pathogenic and nonpathogenic lifestyles in *Colletotrichum acutatum*. Phytopathology 92:743–749.

Howard, R.J., and J.R. Aist. 1980. Cytoplasmic microtubules and fungal morphogenesis: Ultrastructural effects of methyl benzimidazol-2-yl carbamate determined by freeze-substitution of hyphal tip cells. Journal of Cellular Biology 87:55–64.

Hufford, C.D., S. Liu, and A.M. Clark. 1987. Anticandidal activity of eupolauridine and onychine, alkaloids from *Cleistopholis patens*. Journal of Natural Products 50(5):961–964.

Irwin, J.A.G., D.F. Cameron, and J.M. Lenné. 1984. Responses of *Stylosanthes* to anthracnose. Pages 295–310 *in* H. Stace and L.A. Edye, eds., The Biology and Agronomy of *Stylosanthes*. Academic Press, Australia.

Ishida, N., and S. Akai. 1969. Relation of temperature to germination of conidia and appressorium formation in *Colletotrichum lagenarium*. Mycologia 61:382–386.

Ishii, H., B.A. Fraaije, T. Sugiyama, K. Noguchi, K. Nishimura, T. Takeda, T. Amano, and D.W. Hollomon. 2001. Occurrence and molecular characterization of strobilurin resistance in cucumber powdery mildew and downey mildew. Phytopathology 91:1166–1171.

Jones, A.L. 1981. Fungicide resistance: Past experience with benomyl and dodine and future concerns with sterol inhibitors. Plant Disease 65:990–992.

Kato, T., K. Suzuki, J. Takahashi, and K. Kamoshita. 1984. Negatively correlated cross-resistance between benzimidazole fungicides and methyl N-(3,5-dichlorophenyl) carbamate. Journal of Pesticide Science 9:489–495.

Kerrigan, J., and C.W. Mims. 1993. Ultrastructural and cytochemical investigations of the fungus *Colletotrichum gloeosporioides*, causal organism of pecan anthracnose. The BEAM Newsletter of the Southeastern Electron Microscopy Society 9:21–25.

Khan, A., and T. Hsiang. 2003. The infection process of *Colletotrichum graminicola* and relative aggressiveness on four turfgrass species. Canadian Journal of Microbiology 49:433–442.

Knauf-Beiter, G., H. Dahmen, U. Heye, and T. Staub. 1995. Activity of cyprodinil: Optimal treatment timing and site of action. Plant Disease 79:1098–1103.

Kolattukudy, P.E. 1985. Enzymatic penetration of the plant cuticle by fungal pathogens. Anuual Review of Phytopathology 23:223–250.

Kolattukudy, P.E., L.M. Rogers, D. Li, C-S Hwang, and M. Flaishman. 1995. Surface signalling in pathogenesis. Proceedings of the National Academy of Sciences of the United States 92:4080–4087.

Köller, W. 1991. The plant cuticle - A barrier to be overcome by fungal plant pathogens. Pages 219–246 *in* G.T. Cole and H.C. Hoch, eds., The Fungal Spore and Disease Initiation in Plants and Animals. Plenum Press, New York.

Köller, W. 1994. Chemical control of apple scab - status quo and future. Norwegian Journal of Agricultural Sciences S17:149–170.

Kunz, S., B. Lutz, H. Deising, and K. Mendgen. 1998. Assessment of sensitivities to anilinopyrimidine- and strobilurin-fungicides in populations of the apple scab fungus *Venturia inaequalis*. Journal of Phytopathology 146:231–238.

Kuo, K.C. 1999. Germination and appressorium formation in *Colletotrichum gloeosporioides*. Proceedings of the National Science Council, Republic of China, Part B 23:126–132.

Lalitha, T., and L.V. Venkataraman. 1991. Antifungal activity and mode of action of saponins from *Madhuca butyracea* Mach. Indian Journal of Experimental Biology 29:558–562.

Landes, M., and G.M. Hoffmann. 1979a. Germination and infection behaviour of *Colletotrichum lindemuthianum* on *Phaseolus vulgaris*. Phytopathologische Zeitschrift 95:259–273.

Landes, M., and G.M. Hoffmann. 1979b. Ultrahistological investigations of the interactions in compatible and incompatible systems of *Phaseolus vulgaris* and *Colletotrichum lindemuthianum*. Phytopathologische Zeitschrift 96:330–351.

Latorre, B.A., I. Spadaro, and M.E. Rioja. 2002. Occurrence of resistant strains of *Botrytis cinerea* to anilinopyrimide fungicides in table grapes in Chile. Crop Protection 21:957–961.

Latunde-Dada, A.O., R.J. O'Connell, C. Nash, R.J. Pring, J.A. Lucas, and J.A. Bailey. 1996. Infection process and identity of the hemibiotrophic anthracnose fungus (*Colletotrichum destructivum* O'Gara) from cowpea (*Vigna unguiculata* (L.) Walp.). Mycological Research 100:1133–1141.

Leandro, L.F.S., M.L. Gleason, F.W. Nutter, Jr., S.N. Wegulo, and P.M. Dixon. 2001. Germination and sporulation of *Colletotrichum acutatum* on symptomless strawberry leaves. Phytopathology 91:659–664.

Leandro, L.F.S., M.L. Gleason, F.W. Nutter, Jr., S.N. Wegulo, and P.M. Dixon. 2003. Strawberry plant extracts stimulate secondary conidiation by *Colletotrichum acutatum* on symptomless strawberry leaves. Phytopathology 93:1285–1291.

Legard, D.E., C.L. Xiao, J.C. Mertely, and C.K. Chandler. 2001. Management of Botrytis fruit rot in annual winter strawberry using captan, thiram, and iprodione. Plant Disease 85:31–39.

Leinhos, M.E., R.E. Gold, M. Düggelin, and R. Guggenheim. 1997. Development and morphology of *Uncinula necator* following treatment with the fungicides kresoxim-methyl and penconazole. Mycological Research 101:1033–1046.

Leite, B., and R.L. Nicholson. 1992. Mycosporine-Alanine: A self-inhibitor of germination from the conidial mucilage of *Colletotrichum graminicola*. Experimental Mycology 16:76–86.

Lenné, J.M., R.M. Sonoda, and D.G. Parbery. 1984. Production of conidia by setae of *Colletotrichum* species. Mycologia 76:359–362.

Leroux, P. 1996. Recent developments in the mode of action of fungicides. Pesticide Science 47:191–197.

Leroux, P., and M. Gredt. 1995. Etude *in vitro* de la résistance de *Botrytis cinerea* aux fungicides anilinopyrimidines. Agronomie 15:367–370.

Leroux P., F.Chapeland, A. Arnold, and M. Gredt. 1998. Résistance de *Botrytis cinerea* aux fungicides. Du laboratoire au vignoble et vice versa. Phytoma 504:62–67.

Li, G., C. He, X. Wang, and X. Wang. 1998. Extraction of *Phytolacca acinose* and its mulloscacidal effects. Journal Tongji Medical University 18:69–71.

Lipps, P.E. 1983. Survival of *Colletotrichum graminicola* in infested corn residues in Ohio. Plant Disease 67:102–104.

López-Franco, R., R.J. Howard, and C.E. Bracker. 1995. Satellite Spitzenkörper in growing hyphal tips. Protoplasma 188:85–103.

Louis, I., and R.C. Cooke. 1985a. Conidial matrix and spore germination in some plant pathogens. Transactions of the British Mycological Society 84:661–667.

Louis, I., and R.C. Cooke. 1985b. Enzymes in conidial matrix of *Colletotrichum gloeosporioides* and *Mycosphaerella pinodes*. Transactions of the British Mycological Society 84:742–745.

Louis, I., A. Chew, and G. Lim. 1988. Influence of spore density and extracellular conidial matrix on spore germination in *Colletotrichum capsici*. Transactions of the British Mycological Society 91:694–697.

Lyr, H. 1995. Selectivity in modern fungicides and its basis. Pages 13–22 *in* H. Lyr, ed., Modern Selective Fungicides–Properties, Applications, Mechanisms of Action. Gustav Fisher Verlag, New York.

Mahfouz, M., S. El-Amin, H. Ohigashi, Y. Watanabe, N. Takeda, H. Sugiyama, and M. Kawanaka. 2000. Mulliscicidal saponins from *Anagallis arvensis* against Schistosome intermediate hosts. Japanese Journal of Infectious Diseases 53:17–19.

Manaut, F., and H. Maraite. 1997. Conidium germination and appressorium penetration of *Colletotrichum gloeosporioides* on *Stylosanthes guianensis*. Journal of Phytopathology 146:19–26.

Masner, P., P. Muster, and J. Schmid. 1994. Possible methionine biosynthesis inhibition by pyrimidineamine fungicides. Pesticide Science 42:163–166.

McRae, C.F., and B.A. Auld. 1988. The influence of environmental factors on anthracnose of *Xanthium spinosum*. Phytopathology 78:1182–1186.

McRae, C.F., and G.R. Stevens. 1990. Role of conidial matrix of *Colletotrichum orbiculare* in pathogenesis of *Xanthium spinosum*. Mycological Research 94:890–896.

Mendgen, K., M. Hahn, and H. Deisign. 1996. Morphogenesis and mechanisms of penetration by plant pathogenic fungi. Annual Review of Phytopathology 34:367–386.

Mercer, P.C., R.K.S. Wood, and A.D. Greenwood. 1971. Initial infection of *Phaseolus vulgaris* by *Colletotrichum lindemuthianum*. Pages 381–389 *in* T.F. Preece, and C.H. Dickinson, eds., Ecology of Leaf Surface Micro-organisms. Academic Press, London.

Mercure, E.W., B. Leite, and R.L. Nicholson. 1994a. Adhesion of ungerminated conidia of *Colletotrichum graminicola* to artificial hydrophobic surfaces. Physiological and Molecular Plant Pathology 45:421–440.

Mercure, E.W., H. Kunoh, and R.L. Nicholson. 1994b. Adhesion of *Colletotrichum graminicola* to corn leaves: A requirement for disease development. Physiological and Molecular Plant Pathology 45:407–420.

Miehle, B.R., and F.L. Lukezic. 1972. Studies on conidial germination and appressorium formation by *Colletotrichum trifolii*. Canadian Journal of Microbiology 18:1263–1269.

Milling, R.J., and C.J. Richardson. 1995. Mode of action of the anilino-pyrimidine fungicide pyremethanil. 2. Effects on enzyme secretion of *Botrytis cinerea*. Pesticide Science 45:34–48.

Miura, I., T. Kamakura, S. Maeno, T. Nagata, S. Hayashi, and I. Yamaguchi. 1994. Effect of mepanipyrim on uptake of various substrates and macromolecular biosynthesis in *Botrytis cinerea*. Pesticide Science 19:103–109.

Mims, C.W. 1991. Using electron microscopy to study plant pathogenic fungi. Mycologia 83:1–19.

Mims, C.W., E.A. Richardson, R.P. Clay, and R.L. Nicholson. 1995. Ultrastructure of conidia and the conidium aging process in the plant pathogenic fungus *Colletotrichum graminicola*. International Journal of Plant Sciences 156:9–18.

Mims, C.W., and L.J. Vaillancourt. 2002. Ultrastuctural characterization of infection and colonization of maize leaves by *Colletotrichum graminicola*, and by a *C. graminicola* pathogenicity mutant. Phytopathology 92:803–812.

Mould, M.J.R., G.J. Boland, and J. Robb. 1991a. Ultrastructure of the *Colletotrichum trifolii–Medicago sativa* pathosystem. I. Pre-penetration events. Physiological and Molecular Plant Pathology 38:179–194.

Mould, M.J.R., G.J. Boland, and J. Robb. 1991b. Ultrastructure of the *Colletotrichum trifolii–Medicago sativa* pathosystem. II. Post-penetration events. Physiological and Molecular Plant Pathology 38:195–210.

Nicholson, R.L. 1992. *Colletotrichum graminicola* and the anthracnose disease of corn and sorghum. Pages 186–202 *in* J.A. Bailey and M.J. Jeger, eds., *Colletotrichum*: Biology, Pathology and Control. CAB International, Wallingford, UK.

Nicholson, R.L. 1996. Adhesion of fungal propagules. Pages 117–134 *in* M. Nicolle and V. Gianinazzi-Pearson, eds., Histology, Ultrastructure and Molecular Cytology of Plant–microorganisms Interactions. Kluwer Academic Publishers, Dordrecht/Norwell, MA.

Nicholson, R.L., and L. Epstein. 1991. Adhesion of fungi to the plant surface: prerequisite for pathogenesis. Pages 3–23 *in* G.T. Cole, and H.C. Hoch, eds., The Fungal Spore and Disease Initiation in Plants and Animals. New York, Plenum Press.

Nicholson, R.L., and W.B.C. Moraes. 1980. Survival of *Colletotrichum graminicola*: Importance of the spore matrix. Phytopathology 70:255-261.

Nicholson, R.L., L.G. Butler, and T.N. Asquith. 1986. Glycoproteins from *Colletotrichum graminicola* that bind phenols: Implications for survival and virulence of phytopathogenic fungi. Phytopathology 70:1315–1318.

Nicholson, R.L., J. Hipskind, and R.M. Hanau. 1989. Protection against phenol toxicity by the spore mucilage of *Colletotrichum graminicola*, an aid to secondary spread. Physiological and Molecular Plant Pathology 35:243–252.

Niks, R.E., and D. Rubiales. 2002. Potentially durable resistance mechanisms in plants to specialized fungal pathogens. Euphytica 124:201–216.

Ntahimpera, N., L.L. Wilson, M.A. Ellis, and L.V. Madden. 1999. Comparison of rain effects on splash dispersal of three *Colletotrichum* species infecting strawberry. Phytopathology 89:555–563.

O'Connell, R.J. 1987. Absence of a specialized interface between intracellular hyphae of *Colletotrichum lindemuthianum* and cells of *Phaseolus vulgaris*. The New Phytologist 107:725–734.

O'Connell, R.J., and J.A. Bailey. 1991. Hemibiotrophy in *Colletotrichum lindemuthianum*. Pages 211–222 *in* K. Mendgen, and E.D. Lesseman, eds., Electron Microscopy of Plant Pathogens, Springer-Verlag, Berlin, Heildelberg.

O'Connell, R.J., J.A. Bailey, and D.V. Richmond. 1985. Cytology and physiology of infection of *Phaseolus vulgaris* by *Colletotrichum lindemuthianum*. Physiological Plant Pathology 27:75-98.

O'Connell, R.J., C. Nash, and J.A. Bailey. 1992. Lectin cytochemistry: a new approach to understanding cell differentiation, pathogenesis and taxonomy in *Colletotrichum*. Pages 67–87 *in* J.A. Bailey, and M.J. Jeger, eds., *Colletotrichum: Biology, Pathology, and Control*, CAB International, Wallingford.

O'Connell, R.J., A.B. Uronu, G. Waksman, C. Nash, J.P.P. Keon, and J.A. Bailey. 1993. Hemibiotrophic infection of *Pisum sativum* by *Colletotrichum truncatum*. Plant Pathology 42:774–783.

O'Connell, R.J., N.A. Pain, K.A. Hutchison, G.L. Jones, and J.R. Green. 1996. Ultrastructure and composition of the cell surfaces of infection structures formed by the fungal plant pathogen *Colletotrichum lindemuthianum*. Journal of Microscopy 181:204-212.

O'Connell, R.J., S. Perfect, B. Hughes, R. Carzaniga, J. Bailey, and J. Green. 2000. Dissecting the cell biology of *Colletotrichum* infection processes. Pages 57–77 *in* D. Prusky, S. Freeman, and M. Dickman, eds., *Colletotrichum:* Host Specificity, Pathology, and Host-Pathogen Interaction. APS Press, St. Paul, MN.

Okunji, C.O., M.M. Iwu, J.E. Jackson, and J.D. Tally. 1996. Biological activity of saponins from two *Dracaena* species. Advances in Experimental Medicine and Biology 404:415–428.

Olaya, G. and Köller, W. 1999. Baseline of *Venturia inaequalis* populations to the strobilurin fungicide kresoxim-methyl. Plant Disease 83:274–278.

Pangga, I.B., S. Chakraborty, and D. Yates. 2004. Canopy size and induced resistance in

Stylosanthes scabra determine antrachnose severity at high $CO_2$. Phytopathology

94:221–227.

Pappas, A.C., and D.H. Fisher. 1979. A comparison of the mechanism of action of

vinclozolin, procymidone, iprodioine, and prochloraz against Botrytis cinerea.

Pesticide Science 10:239–246.

Parbery, D.G. 1996. Trophism and the ecology of fungi associated with plants.

Biological Reviews 71:473–527.

Pasche, J. S., C. M. Wharam, and N. C. Gudmestad. 2004. Shift in Sensitivity of

Alternaria solani in Response to Q(o)I Fungicides. Plant Disiase 88:181–187.

Pascholati, S.F., H. Deising, B. Leite, D. Anderson, and R.L. Nicholson. 1993. Cutinase

and non-specific esterease activities in the conidial mucilage of Colletotrichum

graminicola. Physiological and Molecular Plant Pathology 42:37–51.

Pelah, D., Z. Abramovich, A. Markus, and Z. Wiesman. 2002. The use of commercial

saponin from Quillaja saponaria bark as a natural larvicidal agent against Aedes

aegypti and Culex pippiens. Journal of Ethnopharmacology 81:407–409.

Peres, N.A.R., N.L. Souza, S.E. Zitko, and L.W. Timmer. 2002. Activity of benomyl

for control of postbloom fruit drop of citrus caused by Colletotrichum acutatum.

Plant Disease 86:125–130.

Peres, N.A.R., N.L. Souza, T.L. Peever, and L.W. Timmer, 2004. Benomyl sensitivity of

isolates of Colletotrichum acutatum and C. gloeosporioides from citrus. Plant

Disease 88:125–130.

Perfect, S.E., H.B. Hughes, R.J. O'Connell, and J.R. Green. 1999. *Colletotrichum*: A model genus for studies on pathology and fungal-plant interactions. Fungal Genetics and Biology 27:186–198.

Petsikos-Panayotarou, N., E. Markellou, and A.E. Kalamarakis. 2003. *In vitro* and *in vivo* activity of cyprodinil and pyrimethanil on *Botrytis cinerea* isolates resistant to other botrycides and selection for resistance to pirymethanil in a greenhouse population in Greece. European Journal of Plant Pathology 109:173–182.

Podila, G.K., L.M. Rogers, and P.E. Kolattukudy. 1993. Chemical signals from avocado surface wax trigger germination and appressorium formation in *Colletotrichum gloeosporioides*. Plant Physiology 103:267–272.

Podila, G.K., E. Rosen, M.J.D. San Francisco, and P.E. Kolattukudy. 1995. Targeted secretion of cutinase in *Fusarium solani* f. sp. *pisi* and *Colletotrichum gloeosporioides*. Phytopathology 85:238–242.

Polacheck, I., M. Levy, M. Guizie, U. Zehavi, M. Naim, and R. Evron. 1991. Mode of action of the antimycotic agent G2 isolated from alfalfa roots. International Journal of Medical Microbiology 275:504–512.

Politis, D.J. 1976. Ultrastructure of penetration by *Colletotrichum graminicola* of highly resistant oat leaves. Physiological Plant Pathology 8:117–122.

Politis, D.J., and H. Wheeler. 1973. Ultrastructural study of penetration of maize leaves by *Colletotrichum graminicola*. Physiological Plant Pathology 3:465-471.

Pommer. E.H., and G. Lorenz. 1982. Resistance of *Botrytis cinerea* Pers. to dicarboximide fungicides – a literature review. Crop Protection 1:221–230.

Porter, F.M. 1969. Protease, cellulase, and differential localization of endo- and exopolygalacturonase in conidia and conidial matrix of *Colletotrichum orbiculare*. Phytopathology 59:1209–1213.

Rahe, J.E., and J. Kuć. 1970. Metabolic nature of the infection limiting effect of heat on bean anthracnose. Phytopathology 60:1005–1009.

Ramadoss, C.S., J. Uhlig, D.M. Carlson, L.G. Butler, and R.L. Nicholson. 1985. Composition of the mucilaginous spore matrix of *Colletotrichum graminicola*, a pathogen of corn, sorghum, and other grasses. Journal of Agricultural Food Chemistry 33:728–732.

Renault, S., A.J. DeLucca, S. Boue, J.M. Bland, C.B. Vigo, and C.P. Selitrennikoff. 2003. CAY-1, a novel antifungal compound from cayenne pepper. Medical Mycology 41:1–7.

Richmond, D.V., and R.J. Pring. 1971. The effect of benomyl on the fine structure of *Botrytis fabae*. Journal of General Microbiology 66:79–94.

Rimelspach, J.W., and M.J. Boehm. 2005. Terms and Concepts for Fungicides. Retrieved February 7, 2007, from The Ohio State University, Department of Plant Pathology Web site http://www.turf.msu.edu/docs/74th_Conference/Boehm_ Fungicides_Concepts.pdf

Roberson, R.W., and M.M. Vargas. 1994. The tubulin cytoskeleton and its sites of nucleation in hyphal tips of *Allomycetes macrogynus*. Protoplasma 182:19–31.

Rosenberg, D.A., F.W. Meyer, and C.V. Cecilia. 1979. Fungicide strategies for control of benomyl-tolerant *Penicillum expansum* in apple storages. Plant Disease Reporter 63:1033–1037.

Rosslenbroich, H.J., and D. Stuebler. 2000. *Botrytis cinerea* – History of chemical control and novel fungicides for its management. Crop Protection 19:557–561.

Schadeck, R.J.G., B. Leite, and D.F. Buchi. 1998. Lipid mobilization and acid phosphatase activity in lytic compartments during conidium dormancy and appressorium formation of *Colletotrichum graminicola*. Cell Structure and Function 23:333–340.

Schadeck, R.J.G., M.A.F. Randi, D.F. Buchi, and B. Leite. 2003. Vacuolar system of ungerminated *Colletotrichum graminicola* cinidia: convergence of autophagic and endocytic pathways. FEMS Microbiology Letters 218:277–283.

Seebach, D., G. Adam, C. von dem Bussche-Hunnefeld, U. Gisi, and H. Binder. 1989. EPC-Synthesis and fungistatic activity of a gloeosporone analog with an ω-hydroxy-butyl instead of the pentyl side chain on the macrocyclic ring. Liebigs Annalen der Chemie (12):1007–1012.

Segal, R., and E. Schlösser. 1975. Role of glycosidases in the membranolytic, antifungal action of saponins. Arch. Microbiol. 104:147–150.

Seidel, V., F. Bailleul, and P.G. Waterman. 1999. Partially acetylated tri- and tetrarhamnoside dodecanyl ether derivatives from *Cleistopholis patens*. Phytochemistry 52:465–472.

Sela-Buurlage, M.B., L. Epstein, and R.J. Rodriguez. 1991. Adhesion of ungerminated *Colletotrichum musae* conidia. Physiological and Molecular Plant Pathology 39:345–352.

Shabi, E., S. Elisha, and Y. Zelig. 1981. Control of pear and apple disease in Israel with sterol-inhibiting fungicides. Plant Disease 65:992–994.

Siegel, M.R. 1981. Sterol-inhibiting fungicides: Effects on sterol biosynthesis. Plant

Disease 65:986–989.

Simpson, D.W., C.Q. Winterbottom, J.A. Bell, and M.L. Maltoni. 1994. Resistance to a

single UK isolate of *Colletotrichum acutatum* in strawberry germplasm from

Northern Europe. Euphytica 77:161–164.

Sisler, H.D. 1996. Why study modes of action of fungicides? Pages 35–41 *in* H. Lyr,

P.E. Russell, and H.D. Sisler, eds, Modern Fungicides and Antifungal

Compounds, Intercept Limited, Andover, Hants, U.K.

Skoropad, W.P. 1967. Effect of temperature on the ability of *Colletotrichum*

*graminicola* to form appressoria and penetrate barley leaves. Canadian Journal of

Plant Science 47:431–434.

Smith, B.J. 1998. Botrytis blossom blight of southern blueberries: Cultivar susceptibility

and effect of chemical treatments. Plant Disease 82:924–927.

Smith, B.J., and L.L. Black. 1987. Resistance of strawberry plants to *Colletotrichum*

*fragariae* affected by environmental conditions. Plant Disease 71:834–837.

Smith, B.J., and L.L. Black. 1990. Morphological, cultural, and pathogenic variation

among *Colletotrichum* species isolated from strawberry. Plant Disease 74:69–76.

Smith, B.J., and L.L. Black. 1993a. *In vitro* activity of fungicides against *Colletotrichum*

*fragariae*. Acta Horticulturae 348:509–512.

Smith, B.J., and L.L. Black. 1993b. *In vitro* fungicide studies show the occurrence of

benomyl-resistant *Colletotrichum* spp. from strawberry. Advances of Strawberry

Research 12:42–48.

Smith, B.J., L.L. Black, and G.J. Galleta. 1990. Resistance to *Colletotrichum fragariae* in strawberry affected by seedling age and inoculation method. Plant Disease 74:1016–1021.

Smith, B.J., and C.L. Gupton. 1993. Calcium applications before harvest affect the severity of anthracnose fruit rot on greenhouse-grown strawberries. Acta Horticulturae 348:477–482.

Smith, K. (ed.). 1999. Iprodione (Rovral) RED. USDA Pesticide re-registration notification network, Volume 9, Number 1, January 21, 1999. Retrieved June 3, 2007, from Purdue University, The Center for Environmental and Regulatory Information Systems Web site:http://ceris.purdue.edu/info/usda_rnn/rnn5-7.html.

Smith, J.E., L. Korsten, and T.A.S. Aveling. 1999. Infection process of *Colletotrichum dematium* on cowpea stems. Mycological Research 103:230–234.

Soto-Estrada, A., H. Förster, J. Hasey, and J.E. Adaskaveg. 2003. New fungicides and application strategies based on inoculum and precipitation for managing stone fruit rust on peach in California. Plant Disease 87:1094–1101.

Stakman, E.C., and J.G. Harrar. 1957. Principles of Plant Pathology. The Ronald Press, Co., New York. 581 p.

Staples, R.C., L. Laccetti, and Z. Yaniv. 1976. Appressorium formation and nuclear division in *Colletotrichum truncatum*. Archives of Microbiology 109:75–84.

Stevens, R.B., (ed.). 1981. Mycology Guidebook. Washington University Press, Washington. 712 pp.

Sutton, B.C. 1966. The appressoria of *Colletotrichum graminicola* and *Colletotrichum falcatum*. Canadian Journal of Botany 44:887–897.

Swinburne, T.R. 1976. Stimulants of germination and appressoria formation by

Colletotrichum musae (Berk. and Curt.) Arx in banana leachate.

Phytopathologische Zeitschrift 87:74–90.

Szkolnic, M. 1981. Physical modes of action of sterol-inhibiting fungicides against

apple diseases. Plant Disease 65:981–985.

Talbot, N.J., D.J. Ebbole, and J.E. Hamer. 1993. Identification and characterization of

MPG1, a gene involved in pathogenicity from the rice blast fungus Magnaporthe

grisea. Plant Cell 5:1575–1590.

Talbot, N.J., M. Kershaw, G.E. Wakley, O.M.H. de Vries, J.G.H. Wessels, and J.E.

Hamer. 1996. MPG1 encodes a fungal hydrophobin involved in surface

interactions during infection-related development of the rice blast fungus

Magnaporthe grisea. Plant Cell 8:985–999.

Van Dyke, C.G., and C.W. Mims. 1991. Ultrastructure of conidia, conidium

germination, and appressorium development in the plant pathogenic fungus

Colletotrichum truncatum. Canadian Journal of Botany 69:2455–2467.

Vinijsanun, T., J.A.G. Irwin, and D.F. Cameron. 1987. Host range of three strains of

Colletotrichum gloeosporioides from tropical pasture legumes, and comparative

histological studies of interactions between type B disease-producing strains and

Stylosanthes scabra (non host) and S. guianensis (host). Australian Journal of

Botany 35:665–677.

Vizvary, M.A., and H.L. Warren. 1982. Survival of Colletotrichum graminicola in soil.

Phytopathology 72:522–525.

Waterman, P.G., and I. Muhammad. 1985. Sesquiterpenes and alkaloids from *Cleistopholis patens*. Phytochemistry 24:523–527.

Wedge, D.E., and M.A. Kuhajek. 1998. A microbioassay for fungicide discovery. SAAS Bulletin of Biochemistry and Biotechnology 11:1-7.

Wedge, D.E., and D.G. Nagle. 2005. Fungicidal properties of sampangine and its analogs to agriculturally important fungal plant pathogens. United States Patent # 6,844,353B2; January 18, 2005.

Wedge, D.E., K.J. Curry, J. Boudreaux, P. Pace, and B.J. Smith. 2001. A microtiter assay demonstrates sensitivity and resistance profiles for *Botrytis cinerea* isolates from Louisiana strawberry farms. Advances in Strawberry Research 20:27–33.

Wessels, J.G.H. 1990. Role of the cell wall architecture in fungal tip growth generation. Pages 1–29 *in* I.B. Heath, ed., Tip Growth in Plant and Fungal Cells. San Diego, Academic Press, Inc.

Wessels, J.G.H. 1993. Wall growth, protein excretion and morphogenesis in fungi. New Phytopathology 123:397–413.

Wessels, J.G.H. 1994. Developmental regulation of fungal cell wall formation. Annual Review of Phytopathology 32:413–437.

Wessels, J.G.H. 1997. Hydrophobins: proteins that change the nature of the fungal surface. Advances in Microbial Physiology 38:1–45.

Wharton, P.S., and A.M. Julian. 1996. A cytological study of compatible and incompatible interactions between *Sorgum bicolor* and *Colletotrichum sublineolum*. New Phytologist 14:25–34.

Wharton, P.S., A.M. Julian, and R.J. O'Connell. 2001. Ultrastructure of the infection of *Sorghum bicolor* by *Colletotrichum sublineolum*. Phytopathology 91:149–158.

Whiteside, J.O. 1980a. Tolerance of *Mycosphaerella citri* to benomyl in Florida citrus groves. Plant Disease 64:300–302.

Whiteside, J.O. 1980b. Detection of benomyl tolerant strains of *Elsinoe fawcettii* in Florida citrus groves and nurseries. Plant Disease 64:871–872.

Wicks, T. 1977. Tolerance to benzimidazole fungicides in blue mold (*Penicillum expansum*) on pears. Plant Disease Reporter 61:447–449.

Wilson, L.L., L.V. Madden, and M.A. Ellis. 1990. Influence of temperature and wetness duration on infection of immature and mature strawberry fruit by *Colletotrichum acutatum*. Phytopathology 80:111–116.

Wolkow, P.M., H.D. Sisler, and E.L. Vigil. 1983. Effect of inhibitors of melanin biosynthesis on structure and function of appressoria of *Colletotrichum lindemuthianum*. Physiological Plant Pathology 22:55–71.

Wong, F.P., and W.F. Wilcox. 2000. Distribution of baseline sensitivities to azoxystrobin among isolates of *Plasmopara viticola*. Plant Disease 84:275–281.

Wong, F.P., and W.F. Wilcox. 2001. Comparative physical modes of action of azoxystrobin, mancozeb, and metalaxyl against *Plasmopara viticola* (grapevine downy mildew). Pant Disease 85:649–656.

Wong, F.P., and W.F. Wilcox. 2002. Sensitivity to azoxystrobin among isolates of *Uncinula necator*: Baseline distribution and relationship to myclobutanil sensitivity. Plant Disease 86:394–404.

Wösten, H.A.B., S.A. Asgeirsdóttir, J.H. Krook, J.H. Drenth, and J.G.H. Wessels. 1994. The fungal hydrophobin Sc3p self-assembles at the surface of aerial hyphae as a protein membrane constituing the hydrophobic rodlet layer. European Journal of Cell Biology 63:122–129.

Wösten, H.A.B., and J.G.H. Wessels. 1997. Hydrophobins, from molecular structure to multiple functions in fungal development. Mycoscience 38:363–374.

Xuei, X.L., U. Järlfors, and J. Kuć. 1988. Ultrastructural changes associated with induced systemic resistance of cucumber to disease: host response and development of *Colletotrichum lagenarium* in systemically protected leaves. Canadian Journal of Botany 66:1028–1038.

Yang, X., L.L. Wilson, L.V. Madden, and M.A. Ellis. 1990. Rain splash dispersal of *Colletotrichum acutatum* from infected strawberry fruit. Phytopathology 80:590–595.

Young, D.H., and H. Kauss. 1984. Adhesion of *Colletotrichum lindemuthianum* spores to *Phaseolus vulgaris* hypocotyls and to polystyrene. Applied and Environmental Microbiology 47:616–619.

Yourman, L., and S.N. Jeffers. 1999. Resistance to benzimidazoles and dicarboximide fungicides in greenhouse isolates of *Botrytis cinerea*. Plant Disease 83:569–567.

Ypema, H.L., and R.E. Gold. 1999. Kresoxim-methyl: Modification of a neturally occurring compound to produce a new fungicide. Plant Disease 83:4–19.

Zuck, M.G., and W.E. MacHardy. 1981. Recent experience in timing sprays for control of apple sacb: Equipment and test results. Plant Disease 65:995–998.

Zulfiqar, M., R.H. Brlansky, and L.W. Timmer. 1996. Infection of flower and vegetative tissues of citrus by *Colletotrichum acutatum* and *C. gloeosporioides*. Mycologia 88:121–128.