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The use of dimethomorph in
the control of potato late blight
(Phytophthora infestans): Sensitivity survey,
insensitivity generation, and field optimization.

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Jeffrey Michael Stein

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THE USE OF DIMETHOMORPH IN THE CONTROL OF POTATO LATE BLIGHT (*PHYTOPHTHORA INFESTANS*): SENSITIVITY SURVEY, INSENSITIVITY GENERATION, AND FIELD OPTIMIZATION.

By

Jeffrey Michael Stein

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ABSTRACT

THE USE OF DIMETHOMORPH IN THE CONTROL OF POTATO LATE BLIGHT (*PHYTOPHTHORA INFESTANS*): SENSITIVITY SURVEY, INSENSITIVITY GENERATION, AND FIELD OPTIMIZATION.

By

Jeffrey Michael Stein

The sensitivities of 11 isolates of *Phytophthora infestans* to dimethomorph at all stages of the asexual life cycle and when inoculated onto potato leaf disks were examined. Once the sensitivities were determined, the generation of dimethomorph insensitive strains of *P. infestans* was attempted using ethidium bromide and UV light mutagenesis and repeated culturing on amended media. The results from these, and previously reported trials, were used to develop and examine several aspects concerning the field use of dimethomorph for the control of foliar and tuber potato late blight.

Zoospore encystment and cystospore germination were highly inhibited at <0.20 µg dimethomorph ml^{-1}, while direct sporangia germination, and *in vitro* hyphal growth and sporulation were less sensitive. Zoospore release was not sensitive at the highest concentration examined, 10.0 µg dimethomorph ml^{-1}. Dimethomorph applied to leaf disks was most effective at inhibiting symptom development when applied before inoculation. Dimethomorph inhibited

sporulation from leaf disks at concentrations >10.0 μg ml^{-1}, regardless of application timing.

The generation of moderately dimethomorph insensitive strains of *P. infestans* using both ethidium bromide and UV mutagenesis and repeated culturing on sub-lethal amended media was successful. Ethidium bromide and UV mutagenesis created strains with significantly higher EC_{50} values of *in vitro* growth on dimethomorph amended media than sub-lethal culturing, but with lower fitness on un-amended media. Strains created by sub-lethal culturing occasionally had reduced virulence on both leaf disks and in whole tubers.

Rate reduction of the pre-mixed commercial mancozeb and dimethomorph fungicide to 1.34 kg ha^{-1} $week^{-1}$ was as effective at controlling foliar late blight as the full rate program, 1.74 kg ha^{-1} $week^{-1}$. When examined as components of a season-long chlorothalonil program, none of the alternative dimethomorph mixture partner fungicides was significantly more effective at controlling foliar or tuber late blight than the others. Reduced rate dimethomorph and pyraclostrobin, and pyraclostrobin alone, programs offered effective foliar and tuber blight control.

A process was developed that produced late blight usage recommendations for dimethomorph using isolate sensitivity, resistance development, and field evaluations.

Dedicated to:

My parents, who have always supported, encouraged, and respected my choices and to my wife Kimberly, from whom I have learned so much.

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I would like to express my extreme gratitude to the members of my committee: Drs. Willie Kirk, Ray Hammerschmidt, Andy Jarosz, and Joe Vargas, my fellow lab mates, especially Robert Schafer, Brandon Dunlap, and Sarah Shaw for countless hours of assistance, and BASF (was American Cyanamid), who fully funded my research. Other people that deserve appreciation are the staff at the MSU Muck Farm, especially Ron Gnagy, the folks involved in the Michigan Potato Industry, who have been extremely supportive of our program, and my fellow graduate students in the department, with whom I was able discuss my research and de-stress.

To my family and friends, while they may not have understood what I kept doing in school, were always willing to learn about potatoes and talk about what was next.

Finally, I would like to express my appreciation to Michigan State University and the Department of Botany and Plant Pathology for allowing me to have such a wonderful academic experience, especially all of the Professors and Teaching Assistants who taught me so much and guided my education.

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KEY TO SYMBOLS AND ABBREVIATIONS

a.i. = Active Ingredient(s)

ANOVA = Analysis Of Variance

CT = Control

DAI = Days After Inoculation

DAP = Days After Planting

diH_2O = De-ionized H_2O

DNA = Deoxyribonucleic Acid

EBDC = EthyleneBis(DithioCarbamates)

EC_{50} = Effective Concentration for a 50% reduction

FCD = Final Colony Diameter

FFLB = Final Foliar Late Blight

HAI = Hours After Inoculation

HBI = Hours Before Inoculation

LSD = Least Significant Difference

PASS = Pivot-Attached Spray System

RAUDPC = Relative Area Under the Disease Progress Curve

RF = Resistance Factor

RNA = Ribonucleic Acid

SL = Sub-Lethal

USD = U.S. Dollars

UV = Ultraviolet light

WT = Wild-type

DMF

FR

HF

RD

RI

RP

UC

DMR

C

D

F

L

U

DMP

CHL

CHLD

CHLD

CTL

MAD1

MAD2

MED1

MED2

DMR = Dimethomorph / Mancozeb Rate trial

FR = Full Rate, season-long

HF = Half of Full rate, season-long

RD = Rate Decreasing through season

RI = Rate Increasing through season

RP = Rate Peaking at mid-season

UC = Untreated Control

DMRI = Dimethomorph / Mancozeb Rate and Interval trial

C = untreated Control

D = rate Decreasing through season

F = Full Rate season-long

L = Lowest Rate season-long

U = rate increasing (Up) through season

DMP = Dimethomorph Mixture Partner trial

CHL = Chlorothalonil, season-long

CHLD1 = Chlorothalonil / Dimethomorph partial rate

CHLD2 = Chlorothalonil / Dimethomorph partial rate

CTL = untreated Control

MAD1 = Mancozeb / Dimethomorph partial rate

MAD2 = Mancozeb / Dimethomorph full rate

MED1 = Metiram / Dimethomorph partial rate

MED2 = Metiram / Dimethomorph full rate

DSR = Dimethomorph / Pyraclostrobin Rate trial

P25 = Pyraclostrobin 25% of full rate

P50 = Pyraclostrobin 50% of full rate

P75 = Pyraclostrobin 75% of full rate

P100 = Pyraclostrobin at full rate

PD25 = Pyraclostrobin / Dimethomorph 25% of full rate

PD50 = Pyraclostrobin / Dimethomorph 50% of full rate

PD75 = Pyraclostrobin / Dimethomorph 75% of full rate

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CHAPTER ONE

LITERATURE REVIEW

Introduction

The Host

The potato (*Solanum tuberosum* L.) is the most important dicotyledonous and fourth most important food crop in the world (127). Potatoes are consumed by humans as food, fermented for alcohol, processed for starch, used as animal fodder, have a relatively high nutritional value (32,127), and grown in both developed and developing countries. In 2001, world production of potatoes was over 300 million metric tons (48). In 2000, Michigan growers cultivated approximately 20,000 hectares of which about 70% was used for chipping, representing a $100 (USD) million annual market (88). Potato cultivation, both commercial and for home gardens, occurs in a greater variety of environments and locations than any other food crop except maize (74).

The cultivated potato is part of a complex consisting of diploid, triploid and tetraploid forms. The tetraploid form is separated into two subspecies (69): *S. tuberosum* ssp. *andigena* for those cultivated in the Andes and *S. tuberosum* ssp. *tuberosum* for those cultivated elsewhere in

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the world (108). Potatoes are believed to have been introduced into Europe from the region now known as Chile and spread to the rest of the world from Europe (44). Potatoes have been adapted to a variety of climates and selected or bred to flower and produce tubers regardless of day length (day neutral) or according to photoperiod and during long day conditions.

The cultivation of potatoes in regions with weather extremes necessitates the ability to store fresh market and processing potatoes for up to one year at 7-10°C and 90% relative humidity (106). Crops are regenerated from "seed" stored between seasons near 3°C, which inhibits sprout development. Seed pieces are small intact tubers or larger tubers that are sectioned mechanically to multiply the amount of planting material with eyes (sprouts). Following cutting, seed pieces are generally stored at 7-10°C prior to planting to allow for suberization of the cut surface and may be treated with powdered tree bark and/or fungicides to aid in healing and reduce the risk of pathogen infection. The conditions and practices employed to store seed tubers enhance survival of tuber-borne pathogens between growing seasons and promote spread to previously uninfected seed pieces during cutting and after planting (83,92,93,113).

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The Pathogen

The causal agent of potato late blight, *Phytophthora infestans* (Mont.) de Bary, infects both foliar and tuber tissues of the potato. *P. infestans* will also infect a few related species within the family Solanaceae, such as tomato (*Lycopersion esculentum* Mill.) and pear melon (*Solanum muricatum* Ait). The parasitic nature of *P. infestans* was not easily defined because the pathogenic characteristics closely matched those of an obligate biotroph (46) including: sporulation from asymptomatic green tissue as well as lesions, a narrow host range, and intercellular hyphae and haustoria-like structures. In contrast, saprophytic growth occurs on relatively simple defined media, a characteristic of facultative biotrophic pathogens. Compared to other *Phytophthora* species, the saprophytic ability of *P. infestans* was minimal (46). The type of parasitism of *P. infestans* was considered to be of the hemibiotroph type in which the initial interaction between the pathogen and the host results in a delay in cell death until a large degree of infection has occurred (25).

The asexual lifecycle begins with production of multinucleate sporangia borne upon branched sporangiophores that emerge from stomata, lenticels, or cracks in the

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epidermis or periderm (46). Sporangia are disseminated in air currents or water and can germinate and infect directly at 15-25°C (58). However, the optimal temperature range for infection is from 12-18°C because in this range and under conditions of free water, sporangia differentiate into 3-8 biflagellate motile zoospores (9,107,136). Zoospores can remain motile for many hours, demonstrate negative geotaxis and positive chemotaxis towards roots exudates and biological compounds such as amino acids and ethanol (82). Zoospores encyst on contact with a hard surface or agitated. Cysts germinate and penetrate the host directly or through stomata (25). Following penetration, *P. infestans* grows intercellularly and produces haustoria-like structures between the cell walls and plasma membranes within the cells it contacts (25). Optimal temperatures for growth of mycelium *in vitro* is 20°C (46) and 15°C for sexual and asexual sporulation (101). When both mating types are present (A1 with A2) and isolates are compatible, oogonia and amphigynous antheridia are produced and mating occurs, resulting in oospores. Oospore production in the field has only been recently noted in temperate regions (5,61). Excluding oospores, *P. infestans* lacks over-wintering structures. The minimal temperature for growth of *P. infestans* is approximately 3°C (46) and temperatures

below 0°C are tolerated only for short durations of time by non-sexual tissues (118). Because of the lack of over-wintering structures and poor saprophytic ability, *P. infestans* requires a plant host, such as potato tubers, to over-winter in areas with extended periods of freezing.

The genus *Phytophthora* and its relatives are occasionally termed "water molds" or "pseudofungi" because they superficially resemble true fungi in certain aspects of gross morphology. Pseudofungi are in the kingdom Chromista and are unrelated to true fungi in the kingdom Mycota (20,46). Oomycetes have β1,3-glucan and cellulose based cell walls instead of chitin, primitive ribosomes and mitochondria compared to other eukaryotic organisms, substantially different nutritional requirements (71) including a necessity for exogenous sterols and thiamine, and numerous other basic biological activities.

The differences between the two groups complicates chemical control as many of the basic biological activities and structures differ significantly or are lacking completely in *Phytophthora* (143). Fungicides that target processes specific to true fungi, such as demethylation inhibitors of sterol biosynthesis, typically have little effect on pseudofungi and are not used in control programs (115). Multi-site fungicides that affect multiple and non-

specific sites within the pathogen generally have an effect on both true fungi and pseudofungi.

The Disease

Historical Importance

Potatoes were cultivated throughout Europe for over 200 years before the introduction and spread of *P. infestans* during the 1840's (108). The agrarian population of Ireland had become heavily dependent on potatoes as a primary food source because of the high productivity of the crop and nutritional value of the tuber (32). Changes in the structure of Irish society included a population explosion and increased cost of grain and milk resulting in an increased dependence on potatoes (39). Other European countries were less dependent on the potato and thus less affected by late blight epidemics than Ireland (121). The first noted loss to the potato crop from *P. infestans* in Ireland was in the latter portion of the 1845 growing season when an estimated 25% reduction in total yield occurred due to tuber blight and the subsequent rotting (44). In 1846 the epidemic occurred early in the growing season and severely limited tuber production, causing an almost complete loss of the crop. Because of the dependence on potatoes as a base of the diet and a lack of a

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sufficient social support structure, the majority of the population was without adequate nutrition and starvation occurred resulting in a famine that lasted until 1849 (39). During the period of the famine, malnutrition was common and resulted in epidemics of human diseases and death. The combination of a massive number of deaths due to starvation and an emigration of 1.5 million people caused the population of Ireland to be reduced almost in half in the span of five years (44).

Integrated Potato Crop Management

Following the Irish Potato Famine, breeding for resistance to *P. infestans* was initiated in Europe and resulted in the development of resistant varieties. Breeding for high levels of stable resistance has not been fully successful (42) because *P. infestans* has readily overcome vertical resistance (10) and horizontal resistance is incomplete. Current strategies for the control of potato late blight include the use of certified seed potatoes, less susceptible varieties that pass a late blight field tolerance test, removal of cull piles and volunteer potatoes, crop scouting, weather-based disease modeling, and chemical control programs (51,116). Cultural management of the potato crop includes irrigation

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management and hilling of rows to reduce tuber contact with free water, planting and harvesting timing to reduce tuber damage, optimal nutrition management, and desiccation of foliage prior to harvest to eliminate foliar-borne inoculum.

Epidemiology

P. infestans is the most economically important pathogen of potatoes (75). In temperate regions the disease cycle begins each spring with an infected seed tuber, volunteer plant, cull pile, or non-potato host *Solanum* sp. that harbored a *P. infestans* infection between growing seasons. These sources serve as the primary inoculum and the sporangia produced from lesions can then infect neighboring plants and initiate an epidemic. *Phytophthora infestans* grows, reproduces asexually and infects best under cool and humid conditions (9). Under optimal conditions the asexual portion of the life cycle results in a polycyclic epidemic (144) that can completely defoliate the canopy of a susceptible variety through lesion expansion and re-infection. Yield loss occurs indirectly through photosynthetic capacity reduction due to the loss of foliage and directly through tuber infection and the subsequent invasion of the tuber by secondary

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pathogens (108,125). Losses from an epidemic can include the entire crop and the severity can be independent of the foliar epidemic duration as late season infection can result in complete crop loss (76).

Fungicidal Control of *Phytophthora infestans*

Definitions and Fungicide Groups

Fungicidal vs. Fungistatic

The common nomenclature used for the compounds employed to control fungal and pseudofungal plant pathogens is confused in that all are typically called 'fungicides'. Fungicides are compounds that kill fungal organisms. Fungistatic compounds reversibly inhibit growth and/or development. Many of the compounds used in the control of potato late blight can be fungistatic or fungicidal depending on concentration (14,15,26,90,114). For simplicity, the term 'fungicide' will be used in its broadest sense and will cover both true fungicides and fungistats that affect both true fungi and pseudofungal organisms.

Protective vs. Curative

Fungicides can be used to both protect uninfected plant tissue from infection with a fungal plant pathogen or

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to cure or inhibit an already established infection. Biologically, the prevention of a polycyclic epidemic, such as that of late blight of potatoes, is more feasible than attempting to control or halt a previously initiated one. Eliminating or limiting the number of initial infections will reduce the total spore production, and therefore reduce the re-infection capacity and fungicide dose required to limit the rate of epidemic development (142). The majority of fungicides used to control potato late blight are used as protectants. Curative usage of fungicides to control potato late blight is generally ineffective using the currently available fungicides as none of the chemistries can completely halt epidemic development (124).

Biological vs. Physical Mode of Action

The asexual life cycle of *P. infestans* on potatoes can be broken down into two general stages: A) the infection process consisting of zoosporogenesis, release and motility, encystment, germination and penetration, and B) growth inside and sporulation from infected tissue. The effects of fungicides on specific points of the disease cycle are often referred to as the "physical mode(s) of action" (130). Various classes of fungicides can have

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effects on one or both stages, and may only affect a single point within the cycle, e.g. zoospore release. The "biological mode of action" is the specific metabolic process or processes being disrupted by the fungicide, e.g. the disruption of cell wall formation.

Non-systemic Fungicides

The two basic groups of fungicides are defined by the degree of plant uptake that occurs: non-systemic and systemic. Non-systemic, including both residual and contact compounds are not taken up by the plant, are at a higher risk for washing off, and must be present on the surface for activity. Once *P. infestans* penetrates the host, mycelium grows within the leaf or tuber tissue and is not in contact with these compounds and therefore unaffected. Non-systemic fungicide usage can be divided into contact and residual (73). Contact fungicides are used to kill fungal colonies and/or propagules that are present on the plant as well as in the local environment, such as the soil and/or plant debris. Residual fungicides are applied to the plant surface and affect the pathogen on arrival of inoculum in a protectant fashion. In current potato agricultural systems, most non-systemic usage is residual to reduce the cost and environmental impact by

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applying the fungicide to the plant only. Residual fungicides typically interrupt a large number of or a few extremely important metabolic processes and have a wide spectrum of biological activity, including toxicity to plants if infiltrated into tissues. This general disruption of metabolic activity typically causes the inhibition of both direct and indirect sporangia germination, zoospore molitity, and/or cystospore germination and growth (115) resulting in the inhibition of infection. Residual fungicides are generally applied with a protectant strategy, are subject to environmental degradation and therefore have a limited duration of contact with the fungus resulting in a low level of resistance selective pressure (135). To become resistant to a multi-site fungicide, either multiple drastic changes in metabolism or the development of a detoxification or efflux mechanism would be required (134). The combination of a comparatively low resistance selective pressure and the complexity of development of fungal resistance to the fungicide correspond to a small resistance development risk for multi-site fungicides.

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Systemic Fungicides

Systemic fungicides are highly resistant to washing off after absorption by the plant tissue, and remain biologically active until diluted within developing and expanding plant tissue, actively degraded or metabolized. Certain systemic fungicides can be used in both protective and curative schemes as some compounds will affect previously established infections in addition to inhibiting initial infection. Fungicides that limit the growth and sporulation of successful infections can reduce the amount of photosynthetic area lost by limiting lesion expansion, and may reduce resporulation and therefore the re-infection potential, thus altering the rate of epidemic development. In studies of the potato late blight pathosystem, none of the commercially available fungicides completely prevented infection under conducive conditions (51,124).

Systemic fungicides generally have a specific biological mode of action, interrupting one or a few required metabolic processes or specific enzymes. Such targeted biological activity means that often only small groups of related organisms are affected. Activity of systemic fungicides on *P. infestans* may include the disruption of *in planta* growth and resporulation as well as the inhibition of sporangia or cystospore germination

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typical of non-systemic fungicides. Systemic fungicides that affect *in planta* growth or sporulation of the fungal pathogen can be used in a curative fashion and can limit, but typically do not eliminate, previously established infections. The resistance risk for systemic fungicides is higher than that of non-systemic fungicides because their specific biological activity may be overcome by relatively simple changes in target enzyme gene sequence, resulting in the development of resistance. In the true fungus *Mycosphaerella fijiensis*, resistance to the cytochrome bc_1 complex inhibitors such as the strobilurins (Qo inhibitors), results from a change in the DNA sequence of the cytochrome b gene that translates into an alanine at the 143 position in the peptide, instead of glycine (119). The specific activity of many systemic compounds, occasional curative usage by the grower, and prolonged duration of contact between the fungus and fungicide inside the plant results in an increased level of selective pressure applied and a higher resistance development risk in comparison to a multi-site fungicide (134).

Systemic fungicides range in systemicity depending on the type of movement (102). Compounds that move within the plant cells, or symplast, are generally termed "fully systemic" because application of the compound to any

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portion of the plant generally provides protection for the entire plant, including leaves, stems, roots, and tubers. Fully systemic compounds have the potential to move both towards the shoot (acropetal movement) and towards the roots (basipetal movement) (26,115). Fungicides that move only within the intercellular spaces of the plant, or apoplast, can move locally or acropetally. Minimal apoplastic systemicity is termed translaminar, in which application of the compound to one surface of the foliage results in the infiltration and movement throughout the entire leaf and protects both leaf surfaces from infection. The maximum degree of apoplastic systemicity is complete acropetal movement, in which application of the compound to the root system confers protection to all aerial portions of the plant.

Fungicide Development for Commercial Release

Fungicide development in the current agrochemical industry is a multi-step process typically involving initial discovery, early assessment of activity, initial field trials, public release, additional examination of activity and resistance risk assessment, refinement of field usage, and registration for commercial use. Fungicide discovery typically involves the mass screening

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of compounds with unknown properties on representative species from various groups of plant pathogens, e.g. Basidiomycetes, Ascomycotes, Oomycetes, etc. Following discovery, compounds are assessed both *in vitro* and under controlled environment conditions for activity. This assessment often examines both the biological and physical mode(s) of action of the fungicide, but typically uses a limited number of isolates and species. Initial field trials are always conducted at industry research facilities, and may be continued and expanded at external institutions, such as universities. Public release generally occurs at an international plant pathology or crop protection meeting, such as with dimethomorph at the Brighton Crop Protection Conference in 1988 (1). Following public release, additional research on the fungicide is conducted under *in vitro, in vivo,* and field conditions and the specifics of activity and resistance risk assessments on the target organisms are determined, e.g. dimethomorph efficacy on downy mildew of grapes (137) and dimethomorph resistance in *P. parasitica* Dast. (21). The refinement of field activity occurs in various regions and climates, often on multiple crops and is followed by the registration process. Following registration, the fungicide is available for commercial use and additional research often

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continues on various topics, e.g. dimethomorph activity on other *Phytophthora* species (97).

Contemporary Fungicide Usage for Potato Late Blight Control

Fungicide Programs

Following the migration of phenylamide fungicide resistant populations of *P. infestans* from Mexico to Europe, the rest of the Americas, and other parts of the world (61), control recommendations for potato late blight changed. This migration necessitated crop protection schemes that relied primarily on non-systemic residual foliar fungicide applications and a transition in systemic fungicides away from phenylamide usage (115,116). In potato growing regions, three basic types of foliar fungicide programs exist: full-protectant, weather-based protectant and curative (124). Full-protectant foliar fungicide programs begin before the onset of potato late blight, consist primarily of residual fungicide applications that continue throughout the growing season at regular intervals, and may be initiated by weather-based potato late blight prediction models (77,95). Protectant fungicide programs that are fully weather-based are initiated like full-protectant programs, but the application interval and/or product rate(s) vary depending

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on meteorological events (87,126). Weather modeled protectant programs can potentially reduce fungicide usage without increased foliar disease (95). Failure occurs because many of the currently available potato late blight models in the United States were based on the displaced clonal US1/A1 lineage (101), are generally region specific (63) and may not account for local climatic variations (77). Curative programs are initiated following the discovery or predicted onset of potato late blight (51). These can have the lowest level of fungicide input, but have the highest potential for failure. Disease detection aptitude varies and current fungicide products are less effective with curative usage (115,116,124). Attempts at controlling potato late blight curatively following establishment and spread may result in extensive fungicide application of more expensive products (78), increased fungicide resistance selection pressures for systemic products (13,134), and potential yield loss.

Application Methodology

Depending on the growing region, four types of foliar fungicide application methods are used: chemigation, pivot-attached sprayer systems (PASS), aerial and ground application. Chemigation through the existing irrigation

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system requires minor modification of the equipment, and can deliver a more uniform fungicide distribution because of the larger water volume used in delivery (67). Chemigation with certain systemic products can result in acropetal redistribution of the fungicide via root absorption and protection of the entire plant tissue. However, the enormous volume of water used in this form of application may dilute and/or waste fungicide product and result in sub-active concentrations being distributed upon the leaf surface (56). PASS application requires additional modification of the irrigation equipment but does not dilute the fungicide to the same extent as chemigation and offers adequate distribution (56,129). Aerial application systems use substantially less water and rely on rain or irrigation mediated redistribution of fungicides. Further advantages of aerial application include the minimization of physical crop disruption and soil compaction. In comparison to other forms of application, fungicide distribution is poor with aerial application; without water mediated redistribution and frequent applications the majority of the foliar surface remains inadequately protected (67). Ground application delivers appropriate coverage to the crop and is the most uniform in distribution (67). However, because of the

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frequency of application required and the small area that can be treated with each sprayer pass, soil compaction and plant disruption is greatest with this method. Additionally, pathogens can be transferred between fields on equipment. Michigan growers use primarily ground and aerial applications, depending on region and farm size (88). Fungicides may also be applied as seed treatments prior to planting or in furrow at planting.

Application Rates

The rate of active ingredient (a.i.) applied depends on a variety of factors including: the bioactivity of the fungicide, the control program being followed, application methodology, maximum product usage limit, meteorological conditions, the proximity of the nearest inoculum source, and the point within the growing season and/or crop maturity (11,18,23,51,52,70,78,105,133). As product labels dictate in the United States, a standard protectant late blight fungicide program using a residual non-systemic fungicide will typically consist of applications of 0.5 kg ha^{-1} to 1.8 kg ha^{-1} active ingredient with an application interval varying between five to 14 or more days between applications, depending on disease conditions and crop maturity. Incorporation of systemic fungicides into a late

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blight control program permits a decrease in non-systemic fungicide rates (47,124), a possible lengthening of application interval without a loss of biological activity (84), and provides complimentary biological modes of action that may be synergistic (47,57). Rates for systemic fungicides vary between 0.1 kg ha^{-1} to 1.0 kg ha^{-1} and require similar application intervals as non-systemic fungicides, depending on fungicide.

Regional Usage

Fungicide programs employed by growers for the control of potato late blight in Michigan have not changed substantially since grower adaptation to the immigration of the phenylamide insensitive strains and the subsequent failure of phenylamide-based fungicide programs during the middle of the 1990's. The current standard late blight control program consists of regular application of organic non-systemic protectant fungicides commencing before canopy closure and generally continuing until desiccation. In the absence of late blight, minimal systemic fungicide usage occurs and growers typically target such usage to correspond to row closure, tuber initiation, immediately prior to desiccation, and/or during conducive weather periods. In the 2000 growing season, both ground and

aerial application methods were employed and the majority of growers applied nine to 15 applications (88). The average ground application carrier (water) volume ranged from 150 - 280 l ha^{-1} while aerial applications typically used 10 - 47 l ha^{-1}. The average yearly costs for fungicide application during a late blight conducive season, such as 2000, were $100 - 250 (USD) ha^{-1}, not including application costs (88). In locations with active late blight in the crop, a substantial number of systemic fungicide applications occurred as growers used combination applications of a protectant non-systemic and a systemic product in order to apply additional pressure to the late blight epidemic. Such programs are typically more expensive and are likely to only retard the epidemic rate (51,124).

Non-systemic Fungicides Currently In Use

Inorganic Protectants - Copper

Chemical control of potato late blight began with the use of copper compounds, such as the Bordeaux mixture (100), towards the end of the 19th Century. The Bordeaux mixture is considered the initiation of chemical control for crop pests (115) and was extremely successful because of its fungicidal efficacy, tenacity and distribution on

the leaf surface, and relative ease of application. Additionally, the Bordeaux mixture was produced from copper sulphate and lime, two relatively simple, inexpensive, and readily available compounds that could be applied to the crop as a dust or in water. The Bordeaux mixture can be phytotoxic and the development of other copper based fungicides, such as the copper oxides, resulted in the reduction of phytotoxicity (72). All copper fungicides share the copper ion (Cu^{+} or Cu^{+2}) as the biologically active component of the compound and have a certain level of phytoxicity that can result in yield reduction when used in the absence of late blight (72). Fungitoxicity occurs because the copper ions interact with sulphhydryl, carboxylic acid and hydroxyl groups of proteins causing the denaturing of multiple enzymes, thus interfering with fungal metabolism and disrupting both direct and indirect sporangia and cystospore germination (73). Copper-based fungicides gained widespread and extensive usage in many crop systems and were the dominant chemistry in potato late blight control until the development of the organic protectants (115).

Organic Protectants

Dithiocarbamates

With the discovery and development of the dithiocarbamate compounds (40) copper was eventually displaced as the dominant fungicide for the control of potato late blight in the United States (73) and the organic fungicide era began. The dithiocarbamates were less phytotoxic than copper compounds and easier to transport and apply. For the control of potato late blight, the ethylenebis(dithiocarbamates), or EBDCs, were found to be the most fungicidally active members of the group and are still used as a base for control programs (115,116). As with coppers, EBDCs are non-systemic, residual fungicides that function by disrupting spore germination and preventing penetration of the host by the pathogen (15). The active component is the dithiocarbamate anion which was thought to disrupt metal-containing enzymes or interact with thiol groups of enzymes (132). The EBDCs have a moderate level of non-target toxicity, are considered possible human carcinogens by the Environmental Protection Agency (EPA) and their use may eventually be restricted (62).

Phthalonitriles

Chlorothalonil is the only commercially available product for late blight control from this group. Its biological activity is thought to be similar to that of the EBDCs because reaction with thiol groups resulted in the disruption of metabolism (31,54), thus preventing spore germination and penetration (128). Chlorothalonil is a non-systemic fungicide that must be present on the leaf surface for activity. In the 2000 growing season, Michigan growers used chlorothalonil as the primary late blight fungicide (88). The application, distribution, and redistribution patterns of chlorothalonil for various application methodologies have been studied thoroughly (16,67). As with the EBDCs, chlorothalonil has a moderate non-target impact and is considered a possible human carcinogen by the EPA, thus usage may eventually be restricted (62).

Organic Tins

Two triphenyltin compounds represent the organic tin fungicides: triphenyltin hydroxide and triphenyltin acetate. Both compounds are primarily used in a protectant manner and share the triphenyltin group as the active component (31). Unlike the other classes of protectant

fungicides, triphenyltin compounds have slight translaminar systemicity and an antisporulation activity (115). As with the copper fungicides, a low level of phytotoxicity occurs when applied to developing foliage. Triphenyltins are typically mixed with EBDC fungicides and applied in the latter portion of the season or when an active late blight epidemic is occurring. The biological mode of action is not completely understood but is thought to be the disruption of oxidative phosphorylation (31). The physical mode of action results in the inhibition of spore germination, zoospore motility, and vegetative growth (31). Non-host, and specifically mammalian (4,22) toxicity of triphenyltin fungicides is high and this group is at a high risk of usage restriction.

Pyridinamines

Fluazinam is the only commercially available product from this class for the control of potato late blight. The biological mode of action is not fully understood but an uncoupling of oxidative-phosphorylation has been reported (12,65) on both true fungi and pseudofungi (86). Fluazinam is biologically active in relatively small concentrations, inhibits spore germination and infection (3), and reduces the duration of zoospore motility (97). Fluazinam is non-

systemic and must be used on a regular schedule for disease control. Non-host toxicity is low compared to the other contact fungicides. Fluazinam can be successfully incorporated into a control program using partial host resistance and varying fungicide rates and application intervals in order to reduce the amount of season-long fungicide application (84).

Systemic Fungicides Currently in Use

First Generation Systemics

The initial development of systemic fungicides for the control of true fungi occurred during the 1960s with the introduction of the benzimidazoles (133) and was followed later by additional groups. Systemic fungicides for the control of pseudofungi were not available until the almost simultaneous introduction of the cyanoacetamide-oximes, phosphonate, carbamates, and phenylamides in the late 1970s (115). Only two fungicides: cymoxanil (a cyanoacetamide-oxime) and metalaxyl (a phenylamide) had initial widespread and intensive usage for the control of *P. infestans*.

Cyanoacetamide-oximes

As the single commercially available member of the cyanoacetamide-oxime fungicide group, cymoxanil is active

on certain members of the Peronosporales (27,115) and has both translaminar systemicity and a high level of acropetal transport via root uptake (28). Breakdown *in planta* into glycine can occur within days (28) and is temperature dependent (98). When used for the control of potato late blight, cymoxanil is typically mixed with a non-systemic contact fungicide, providing a synergism of activities (47,57). The biological mode of action has not yet been elucidated but disruption of RNA synthesis was considered a possible target (38). Insensitivity within the related Oomycete *Plasmopara viticola* (Berk. & Curt.) Berl. & de Toni has developed *in situ* within certain populations (78) but no change in sensitivity has been detected to cymoxanil within *P. infestans* populations studied (66). Its primary physical mode of action is similar to the non-systemic contact fungicides in that zoospore release, germination and appressorium formation were inhibited (115). Cymoxanil also provided moderate post infection curative activity due to the partial inhibition of hyphal growth *in planta* (78).

Phosphonate Compounds

All of the compounds within this group breakdown *in planta* which releases the phosphonate anion, the active component of the fungicide (64). The alkyl phosphonates

may have additional fungicidal activity from their metal cation. Fungicidal activity on foliar infections of *P. infestans* is sporadic and often very limited, resulting in little commercial use worldwide (64). The application of phosphonate fungicides to infected crops resulted in a reduction of tuber infection and demonstrated the full systemic movement of the compound *in planta* (30). Phosphonates are more important for the control of *Phytophthora* diseases affecting tree roots than for *P. infestans* in potatoes (46).

Carbamates

Propamocarb and prothiocarb are both carbamate fungicides and highly specific to members of the Peronosporales. Propamocarb has received the most research and field use for the control of *P. infestans*. The biological mode of action has not been elucidated but a disruption of the cellular membrane and/or function has been observed (114). The biological activity of propamocarb is relatively low compared to other semi-systemic late blight fungicides in rate comparisons and large amounts must be applied for comparable activity (115). Propamocarb has local systemicity movement only (110). The physical mode of action is proposed to be a

combination between a moderate effect on inhibition of sporangial germination and a greater effect on hyphal growth disruption (110). Post-infection activity is limited, therefore propamocarb is most useful when applied in a protectant fashion (78,110).

Phenylamides

The phenylamide fungicide group is highly specific for members of the Peronosporales and is further divided into two groups: the acylalanines and the butyrolactones. Metalaxyl, a member of the former group, has been the most important systemic fungicide for the control of *P. infestans* on potatoes in the United States. Discovery of the phenylamides occurred during the investigation of the structurally related chloroacetanilide herbicides (36). Metalaxyl has acropetal systemicity and application of the fungicide to roots or lower foliar portions of the plant often confers protection to the rest of the foliage, including new growth (26). Protection of tubers from foliar applications has also been reported (104,117). The biological mode of action is not completely understood but the inhibition of ribosomal RNA biosynthesis occurred via the putative disruption of the RNA polymerase I complex (36). Metalaxyl inhibited both fungal growth and

sporulation (33,34,35). The development of resistance to metalaxyl in *P. infestans* can be induced with ultraviolet light irradiation (17), selected for *in vitro* through repeated culturing (139) and is thought to occur from a DNA sequence mutation at the gene for the target site of the fungicide (36). Cross-resistance to other phenylamide fungicides occurred (21). Metalaxyl has high biological activity and unlike the non-systemic fungicides, does not affect zoosporogenesis or germination but instead inhibits fungal growth (14). The high level of systemicity and inhibition of hyphal growth allowed for effective post-infection usage of metalaxyl to control previously infected potato plants. Unfortunately, frequent usage, especially in a curative fashion results in high selective pressure placed on the pathogen, resulting in the development and spread of resistance.

Second Generation Systemics

Many potato-growing regions around the world became heavily dependent on phenylamide fungicides for control of *P. infestans* on potatoes and growers adopted fungicide application patterns that deviated from strict protectant programs, such as post-infection application programs in order to eradicate existing infections. The phenylamide

insensitive strains of *P. infestans* that migrated from Mexico to other potato growing regions in the early 1990's disrupted crop protection programs and resulted in severe crop losses (53). Additionally, these strains were often comparatively more aggressive than displaced strains (37,75), had different physiologies resulting in enhanced fitness (8), and often were of the complimentary mating type allowing for possible sexual recombination and the putative generation of new genotypes (55). Following the migration of these phenylamide insensitive genotypes and subsequent crop losses, the development of existing and novel fungicides for the control of *P. infestans* was accelerated.

Cytochrome bc_1 Complex Inhibitors

Two fungicide groups, the strobilurins and oxazolidinediones share a common biological site of activity and are effective against both true fungi and pseudofungi. The only two currently commercially available strobilurin fungicides for the control of *P. infestans* on potatoes within the United States are azoxystrobin and pyraclostrobin (F500), with trifloxystrobin and others under development for possible future release. The strobilurin fungicides were derived from strobilurin A,

which is produced by the Basidiomycete *Strobilurus tenacellus* (54).

Famoxadone is an oxazolidinedione and is the only member of this group that is close to registration for use on potatoes in the United States. For both groups the biological mode of action is the inhibition of respiration through the binding of the cytochrome bc_1 complex (80,131). Because of the single site of activity, the development of resistance within the target organism is probable, and at least two mechanisms for insensitivity to these fungicides have been found (119). The first mechanism is the induction of the alternative oxidase pathway. With this type of insensitivity, the fungus is still able to respire, but at a reduced rate resulting in lowered fitness. The second type of insensitivity results from a change in the nucleic acid sequence of the target peptide gene and yields total resistance to the fungicide. The most common mutation resulting in the resistance of true fungi to fungicides of this group results in the replacement of glycine with alanine at the 143 amino acid position (119). The physical mode of action of both groups is identical in the disruption of zoosporogenesis, zoospore motility, and hyphal growth. Compounds within these groups are locally systemic and are applied in a protectant fashion with

limited applications per season to reduce the resistance development risk.

Benzamides

The only commercially available member of this group for the control of *P. infestans* on potatoes is zoxamide. Zoxamide binds β-tubulin and disrupts microtubule assembly, similar to the benzimidazole fungicides, resulting in the inhibition of mitosis (140). Attempts to generate *P. infestans* mutants resistant to zoxamide were unsuccessful and the resistance development risk was considered lower than that of the phenylamides (139,141). The physical mode of action for zoxamide is the disruption of mitosis and most stages of the life cycle were affected (140). The zoospore stages, including zoosporogenesis, encystment and germination were unaffected, as mitosis is not known to occur during these portions of the life cycle (25).

Morpholines

Dimethomorph is the only commercially available member of the morpholine fungicide group that is used to control late blight. It was derived from cinnamic acid, showed translaminar and acropetal systemicity, and was specific against the genus *Phytophthora* and certain members of the

Peronosporaceae (1). Dimethomorph was most effective when used as a protectant fungicide (29), however post-infection activity also occurred (2). Dimethomorph caused disruption of cell wall formation (1,90) and inhibition of sporangia formation in *P. infestans* when applied to normally developing lesions (2,29). The biological mode of action has not been elucidated, no activity on protoplasts was found, and dimethomorph was considered to have a single site of activity (2). Dimethomorph exhibited a lower resistance development risk than metalaxyl in *P. parasitica* (139) and in *P. capsici* and *P. infestans* (141) using single isolates. Dimethomorph negatively affected all stages of the *P. infestans* life cycle except zoosporogenesis and zoospore release as these stages do not involve cell wall biosynthesis (2,29,90).

Dimethomorph was originally released for emergency use on potatoes in the United States as a pre-mixed wettable powder with mancozeb as its mixture partner (9% dimethomorph / 60% mancozeb by weight) to reduce the resistance development risk. The product label was later modified and dimethomorph was re-released for full non-emergency usage as a 50% dimethomorph single active ingredient product. Tank mixing dimethomorph with a protectant fungicide is mandatory according to the label

and allows growers more flexibility in mixture partner product choice. Rate and interval optimization and mixture partner assessment for dimethomorph within a Michigan potato system have not been performed.

Future Fungicides

Following the development and spread of phenylamide resistance in many of the Oomycete plant pathogens, the market for Oomycete fungicides has undergone a resurgence. Agrochemical companies are in the process of both active discovery of new compounds and further development and registration of existing chemistries. The cost of late blight control is currently one of the limiting factors in potato production (79,88) and none of the currently available late blight fungicides were as efficacious and had the same level of post-infection activity as metalaxyl did on sensitive strains (97). A search for suitable replacement products is a high priority in many industry research and development programs. The recent release of the strobilurins, fluazinam, and zoxamide for late blight management in the United States demonstrates the grower demand for suitable products with disease control comparable to currently used fungicides, but with reduced environmental impact.

Research Objectives

The objectives of this study were to 1) the examine the sensitivity to dimethomorph of isolates of *P. infestans* from various genetic backgrounds at multiple stages within the asexual lifecycle and *in vivo*, 2) attempt to generate insensitivity to dimethomorph within *P. infestans* and characterize the insensitivity if found, 3) optimize the field efficacy of dimethomorph within a potato late blight program by examining mixture partners, rate, and application intervals.

CHAPTER TWO

DIMETHOMORPH SENSITIVITY IN *PHYTOPHTHORA INFESTANS*

Abstract

The sensitivities of 11 isolates of *Phytophthora infestans* to dimethomorph at all stages of the asexual life cycle and when inoculated onto potato leaf disks were examined. Zoospore encystment and cystospore germination were both highly sensitive to dimethomorph with EC_{50} values for most isolates <0.20 μg ml^{-1}, while direct sporangia germination and *in vitro* hyphal growth and sporulation were less so. Zoosporogenesis was not significantly inhibited at the maximum dimethomorph concentration examined, 10.0 μg ml^{-1}. EC_{50} values between isolates were significantly different (Fischer's LSD, $\alpha = 0.05$) for all stages of the asexual life cycle, except direct sporangia germination and zoosporogenesis. Application of 1000.0 μg ml^{-1} dimethomorph to potato leaf disks at 24 or 48 hours before inoculation completely inhibited symptom development for most isolates, while application after inoculation generally was not significantly different from the untreated control, regardless of concentration. Sporulation from leaf disks

treated with dimethomorph at 24 or 48 hours after inoculation was completely inhibited at 1000.0 μg ml^{-1}.

Introduction

Dimethomorph, a cinnamic acid derivative, was one of the fungicides released in response to the migration (60) or spontaneous development (141) of phenylamide resistant strains of *Phytophthora infestans*. Initial studies with dimethomorph demonstrated a specificity of activity towards the genus *Phytophthora* and certain members of the Peronosporaceae (1). Dimethomorph was most effective when used as a protectant fungicide (29), however a degree of curative activity also occurred (1,29). A moderate amount of translaminar and acropetal systemicity was also noted (1,29). One of the most interesting aspects of dimethomorph was the inhibition of *P. infestans* sporangia formation when applied to normally developing lesions under controlled conditions (2,29), also known as "antisporulation" activity.

The specific biological mode of action of dimethomorph has not yet been elucidated but a disruption of cell wall formation, specifically the organization and not the synthesis of wall components, was noted (1,90). Dimethomorph disrupted all stages of the asexual life cycle

of *P. infestans* except zoosporogenesis, zoospore release, and motility as these stages do not involve cell wall biosynthesis (2,29,90). The activity of dimethomorph on other *Phytophthora* species was examined (96) and found to be similar to that in *P. infestans*. However, differences in the effective concentration for a 50% reduction relative to the untreated control (EC_{50}) for mycelial growth and cystospore germination were apparent between species.

The majority of studies examining dimethomorph activity against *Phytophthora* species typically consisted of only one isolate per species. The single study that compared the sensitivity of several isolates of *P. infestans* to dimethomorph examined only the *in vivo* activity and not the effects on the different stages of the asexual life cycle, nor antisporulation activity (29). Varying sensitivity to fungicides is present in other Oomycete plant pathogens (138) and it is possible that such variation is present in *P. infestans* to dimethomorph.

The objective of this study was to examine the sensitivity of isolates of *P. infestans* from various genetic backgrounds to dimethomorph 1) at multiple stages in the potato late blight disease cycle (asexual life cycle of *P. infestans*) and 2) protectant, curative, and

antisporulation activity of dimethomorph when inoculated onto potato leaves.

MATERIALS AND METHODS

Preparation of Fungicide Stock Solutions and Amended Media

Assessment of the inhibition of *in vitro* hyphal growth rates was performed on modified rye B agar (2,19) consisting of the filtrate of pre-rinsed rye (*Secale cereale* L.) seeds (100.0 g L^{-1}) boiled for 1 hour, de-ionized (di) H_2O added to a final volume of 1.0 L, glucose (8.0 g L^{-1}), β-sitosterol (0.05 g L^{-1}) and agar (15.0 g L^{-1}). All plates for each replication of the experiment were prepared from the same batch of media in order to reduce variability.

A dimethomorph stock solution was prepared by dissolving technical grade (95% pure) dimethomorph into 95% ethanol and performing serial dilutions as required. For solid media, the fungicide stock solutions were added to molten media at 10.0 ml L^{-1} when the temperature was 55°C. Sterility was obtained by filter sterilizing the fungicide solution through a 0.22 µm syringe driven filter (Millipore Corp., Bedford, MA, U.S.A.).

Production of Viable Sporangia

To produce viable sporangia of similar age, potato tubers (cv. Russet Burbank) were surface sterilized with 0.50% sodium hypochlorite in diH_2O (10% commercial bleach solution) for 30 minutes, rinsed three times in sterile diH_2O, and allowed to dry. Tubers were sliced into 7.0 mm sections and placed into sterile 150 mm plastic petri dishes on top of 1.0 cm^2 agar sections previously colonized by *P. infestans*. Plates were sealed with Parafilm and incubated at 18°C / 15°C (12 hours light / 12 hours dark) until at least 50% of the tuber surface was covered by mycelia (typically five days). Sporangia were harvested by gently removing the mycelium with a plastic culture spreader, transferred into sterile micro-centrifuge tubes, and 1.0 ml sterile diH_2O was added. Sporangia were counted with a hemacytometer and the concentration was adjusted to 1.0×10^4 sporangia ml^{-1}.

Inhibition of Hyphal Growth and Sporulation *in vitro*

Previously characterized (Table 1) isolates of *P. infestans* that had been sub-cultured once after re-isolation from infected potato leaves were cultured on rye B agar for 21 days. Colonized plugs, 4.0 mm diameter, were transferred from the margin of the colony onto fungicide

Table 1. *P. infestans* Isolate ID, mating type, genotype, and State in which isolated (U.S.A.).

Isolate ID	**Mating Type**	**Genotype**[1]	**Origin**
Pi88	A1	US1	ND
Pi95-5	A1	US1	MI
Pi671	A1	US14	WA
Pi458	A2	US17	ID
Pi670	A2	US7	OR
Pi213	A2	US8	CO
Pi94-4	A2	US8	MI
Pi95-7	A2	US8	MI
Pi97-2	A2	US8	MI
Pi98-1	A2	US8	MI
Pi98-2	A2	US8	MI

1. Allozyme-based genotype[59].

amended rye B media in 60 mm diameter plastic petri dishes and incubated at 21°C. The fungicide concentrations used were 0.0, 0.01, 0.1, 1.0, 10.0 μg ml^{-1}, with three replicate plates per concentration. Colony diameter was measured 11 days after inoculation (DAI). Percent inhibition of radial growth was calculated with respect to the mean colony diameter of the non-amended plates within each isolate. Percent inhibition values were then transformed using probits, i.e. the inverse of the standard normal distribution (89), and expressed (49) as a function of the $\log_{10}$ of concentration. Curve equation parameters were then determined using linear regression (SigmaPlot, SPSS Inc., Chicago, IL, U.S.A.) and the EC_{50} for hyphal growth (diameter) was calculated and reverse transformed for each isolate. The experiment was repeated three times and the EC_{50} values for each isolate were used as replicates for an analysis of variance (Proc GLM - SAS/Stat, SAS Institute, Cary, NC, U.S.A.) at $\alpha = 0.05$ by pair-wise comparisons using Fisher's LSD.

The effects of dimethomorph on sporangia production *in vitro* were examined by using a modification of a previously described method of sporangia quantification (19). Ten colonized agar plugs, 0.1 mm diameter, were randomly excised from each replicate plate of the *in vitro*

sensitivity assay, five plugs 2.0 mm from the colony margin, and five 2.0 mm from the initially transferred inoculum. The plugs were then placed into a 1.5 ml micro-centrifuge tube with 1.0 ml of sterile de-ionized H_2O (diH_2O) and agitated to dislodge the sporangia. Sporangia were counted with a hemacytometer. Percent inhibition and EC_{50} values were calculated and analyzed as described.

Inhibition of Direct and Indirect Germination

To assess the effects of dimethomorph on direct germination, aliquots of the previously prepared sporangial suspensions from inoculated tuber slices were transferred to 96 well polystyrene culture plates (Costar, Corning Incorporated) and dimethomorph stock solutions were added for a final concentration of 0.0, 0.01, 0.1, 1.0, and 10.0 $\mu g\ ml^{-1}$ dimethomorph, in each of three replicate wells per isolate. Sporangia/fungicide solutions were incubated for 72 hours at 21°C and the numbers of total and germinated sporangia were counted. To examine indirect germination and zoospore encystment, sporangia/fungicide solutions were incubated at 8°C for five hours to induce zoosporogenesis and the number of motile zoospores was counted. The solutions were then incubated at 21°C and the number of total cystospores was counted after 24 hours. To examine

cystospore germination, zoospore suspensions were prepared as described and incubated at 21°C for 24 hours to allow for zoospore encystment. Fungicide solutions were added, the solutions were incubated at 21°C for 48 hours, and the number of total and germinated cystospores was counted. Percent inhibition and EC_{50} values were calculated and analyzed as described.

Isolate Sensitivity Ranking and Correlations of the Asexual Life Cycle Stages

To compare the overall sensitivity of each isolate to dimethomorph, the isolates were ranked within each stage of the asexual life cycle using the least significant mean EC_{50} values generated from each analysis of variance. An analysis of variance on ranks (89) was performed from these values and a mean rank of sensitivity was generated for each isolate and compared. Zoosporogenesis was not included in this analysis because of the lack of sensitivity of this process to dimethomorph. Pearson's Product Moment Correlation (89) was used to examine the correlation of the asexual life cycle stages of the *P. infestans* isolates used to detail any trends between stages.

Protectant, Curative, and Antisporulation Activity of Dimethomorph *in vivo*

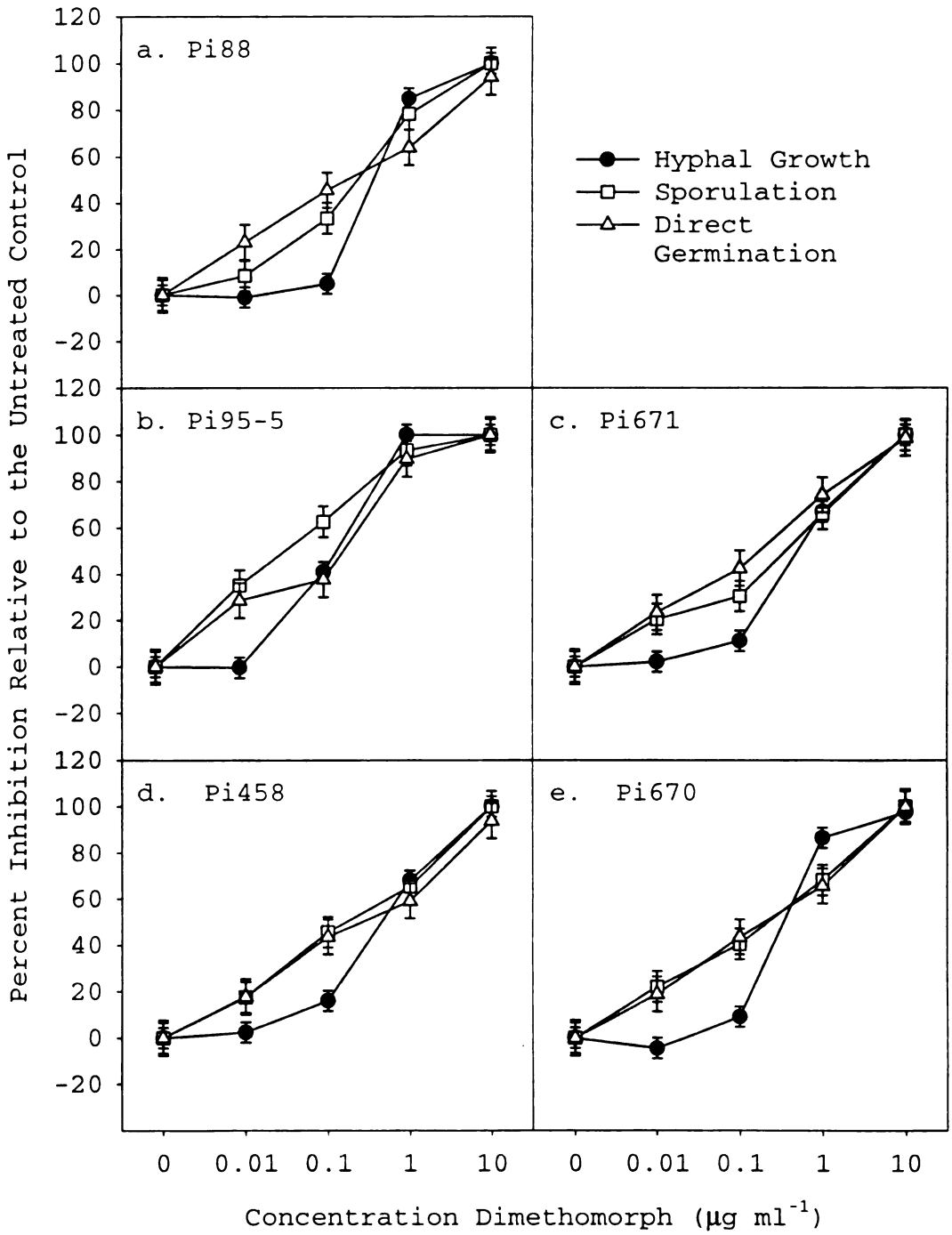
The *in vivo* activity of dimethomorph was assessed relative to the inoculation event by removing fully expanded leaflets of similar age from greenhouse grown potato plants (cv. Snowden) and surface sterilizing them with 0.50% sodium hypochlorite in diH_2O (10% commercial bleach solution) for one minute. Leaflets were then rinsed three times in sterile diH_2O, allowed to dry, and cut into 20 mm diameter leaf disks with a sterilized core borer. Leaf disks were placed onto water agar (15.0 g L^{-1}) amended with rifamycin (37.5 mg l^{-1}), ampicillin (10 mg l^{-1}), and nystatin (37.5 mg l^{-1}) which was previously dissolved in 1.0 ml dimethylsulfoxide, stored frozen in the dark, and added to the molten media following sterilization. Leaf disks were temporarily removed from the agar for treatment and dimethomorph was applied until run-off using the formulated commercial product at 0.0, 1.0, 10.0, 100.0, and 1000.0 µg ml^{-1} at 24 or 48 hours before (HBI) or after (HAI) inoculation. Twelve leaf disks were inoculated per experiment, and the experiment was repeated three times. Following inoculation, leaf disks were incubated at 21°C light / 18°C dark (12 hour cycles) and assessed for infection by *P. infestans* at 96 hours after inoculation for

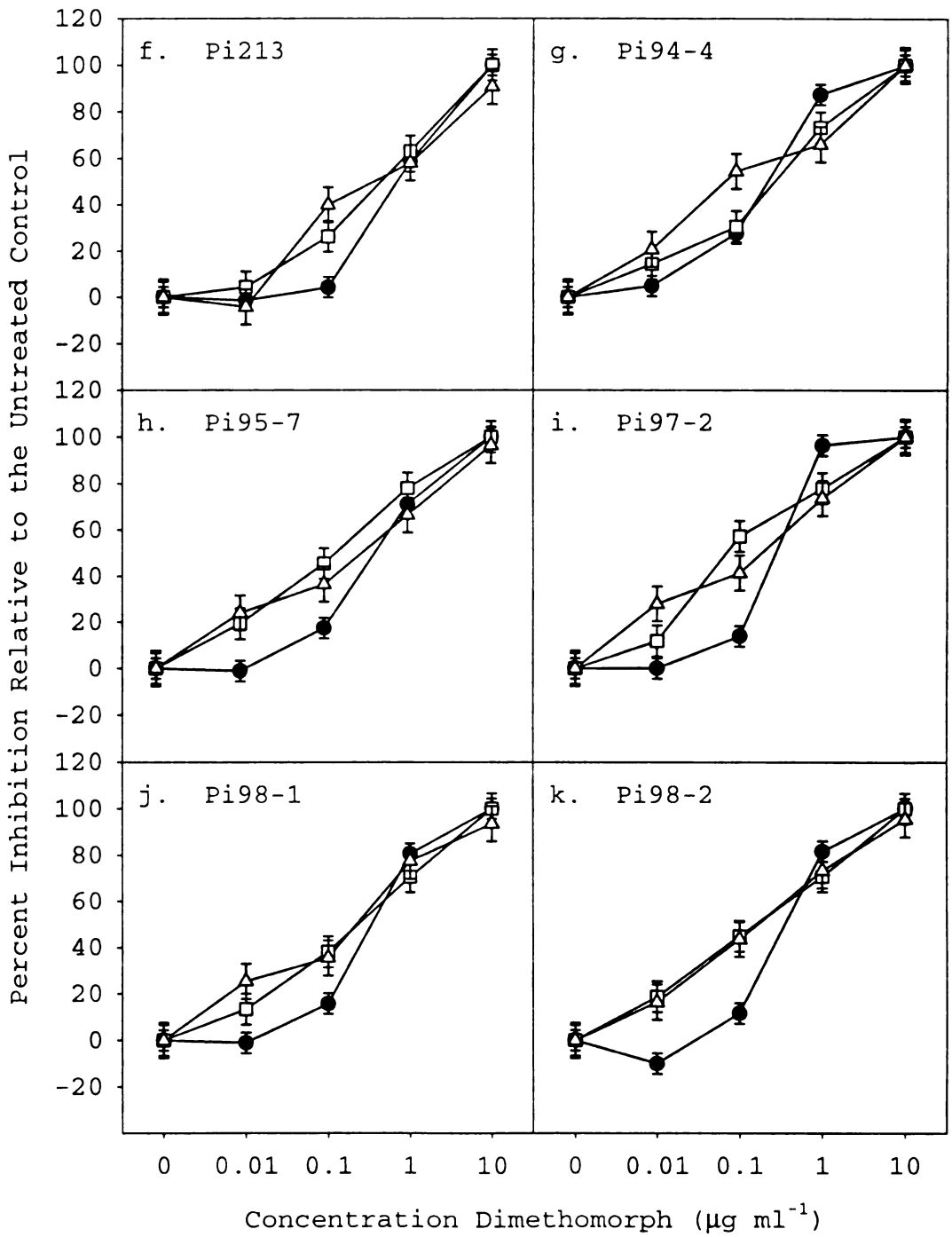
symptoms and signs of infection by *P. infestans*, such as necrosis and sporulation. To assay for the inhibition of sporulation, the four leaf disks from each replicate were placed into a 15.0 ml centrifuge tube containing 4.0 ml of diH_2O, agitated, and sporangia were counted with a hemacytometer. Percent inhibition and EC_{50} values were calculated and analyzed as described for both incidence of symptom development and the number of sporangia produced per leaf disk area (cm^2).

Results

For all isolates, no significant difference (Fischer's LSD, α = 0.05) was observed in the percent inhibition of hyphal growth on media amended with 0.0 and 0.01 $\mu g\ ml^{-1}$ dimethomorph (Figure 1). At 0.1 $\mu g\ ml^{-1}$ dimethomorph, all isolates exhibited less than 20% inhibition of hyphal growth except Pi94-4 and Pi95-5, which were 28% and 41% respectively. At 1.0 $\mu g\ ml^{-1}$ dimethomorph, more than 50% inhibition of hyphal growth occurred in all isolates, with Pi95-5 being completely inhibited, while 10.0 $\mu g\ ml^{-1}$ dimethomorph completely inhibited hyphal growth of all isolates. Most isolates had a sigmoidal shaped sensitivity curve when percent inhibition was plotted against the $\log_{10}$

Figure 1 (a-k). The percent inhibition, relative to the untreated control, of *in vitro* hyphal growth, sporulation, and direct sporangia germination, for *P. infestans* vs. dimethomorph concentration (0.0, 0.01, 0.1, 1.0, and 10.0 $\mu g\ ml^{-1}$). Error bars represent Fischer's LSD ($\alpha = 0.05$).


a. Pi88
b. Pi95-5
c. Pi671
d. Pi458
e. Pi670
Hyphal Growth
Sporulation
Direct Germination
Percent Inhibition Relative to the Untreated Control
120
100
80
60
40
20
0
-20
0
0.01
0.1
1
10
Concentration Dimethomorph (µg ml-1)


f. Pi213
g. Pi94-4
h. Pi95-7
i. Pi97-2
j. Pi98-1
k. Pi98-2
Percent Inhibition Relative to the Untreated Control
120
100
80
60
40
20
0
-20
0
0.01
0.1
1
10
Concentration Dimethomorph (µg ml-1)

of concentration. The calculated EC_{50} values for inhibition of hyphal growth ranged from 0.131 to 0.800 μg ml^{-1} dimethomorph (Table 2). Five non-overlapping significance categories were present with the isolate Pi213 having a significantly higher EC_{50} value than all others.

Regardless of the concentration of dimethomorph in amended media, almost all isolates produced significantly less sporangia per colony area (cm^2) than the untreated control (Figure 1) and Pi95-5 exhibited a 35% inhibition at a concentration of only 0.01 μg ml^{-1} dimethomorph. Sensitivity of *in vitro* sporulation was more variable at 0.01 and 0.1 μg ml^{-1} dimethomorph than was hyphal. Sensitivity curves tended to be more linear than sigmoidal, unlike those of hyphal growth. The calculated EC_{50} values ranged from 0.036 to 0.437 μg ml^{-1} dimethomorph (Table 2). The isolate Pi213 had a significantly higher EC_{50} than Pi95-5, Pi97-2, and Pi98-2. However, distinct trends in sensitivity and genetic background were not apparent.

For all isolates except Pi213, 0.01 μg ml^{-1} dimethomorph significantly inhibited direct sporangia germination in comparison to the untreated control (Figure 1). For most isolates, inhibition trends were similar to those of *in vitro* sporulation and sensitivity curves were

Table 2. The effective concentration of dimethomorph (μg ml^{-1}) required for a 50% reduction of *in vitro* hyphal growth (colony diameter) and sporulation for *P. infestans* isolates.

Isolate ID	*in vitro* EC_{50} (μg ml^{-1})[1]			
	Hyphal Growth[2]		Sporangia Production[3]	
Pi88	0.425	c[4]	0.199	abc
Pi95-5	0.131	e	0.036	c
Pi671	0.625	b	0.320	ab
Pi458	0.585	b	0.203	abc
Pi670	0.392	c	0.236	abc
Pi213	0.800	a	0.437	a
Pi94-4	0.270	d	0.247	abc
Pi95-7	0.533	b	0.171	abc
Pi97-2	0.291	d	0.115	bc
Pi98-1	0.419	c	0.246	abc
Pi98-2	0.429	c	0.163	bc

1. Effective concentration for a 50% reduction, calculated using probit transformation of the percent inhibition relative to the untreated control.
2. Calculated from colony diameter on rye B media amended with 0, 0.01, 0.1, 1.0, or 10 μg ml^{-1} dimethomorph.
3. Production of sporangia per cm^2 colony area on rye B media amended with 0, 0.01, 0.1, 1.0, or 10 μg ml^{-1} dimethomorph.
4. Means followed by the same letter are not significantly different using Fischer's LSD ($\alpha = 0.05$).

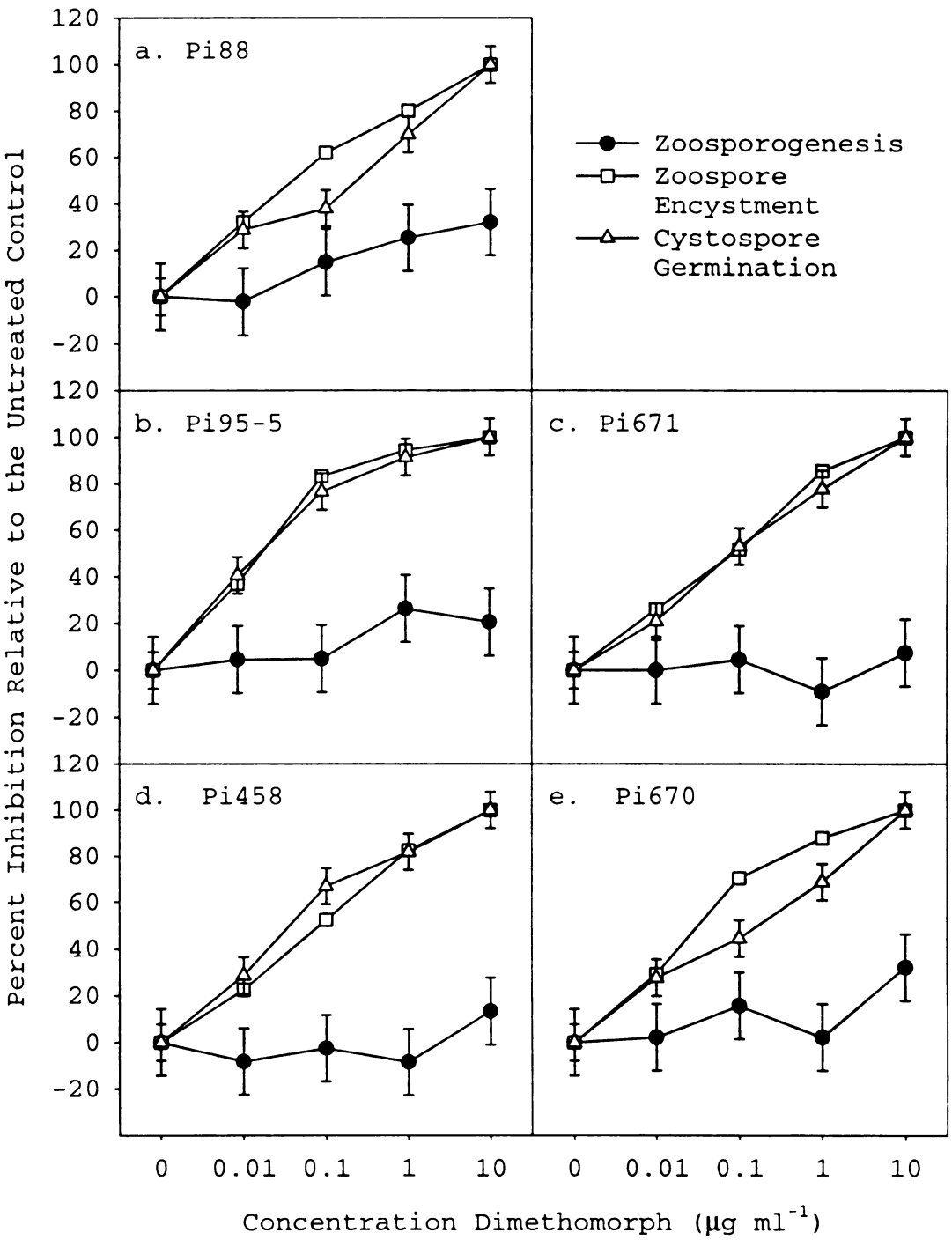
primarily linear. No significant differences were calculated between isolates for the EC_{50} of inhibition of direct sporangia germination (data not shown). EC_{50} values ranged from 0.096 to 0.231, with a mean of 0.163 $\mu g\ ml^{-1}$ dimethomorph.

Zoosporogenesis was not inhibited at any of the concentrations examined, up to 10.0 $\mu g\ ml^{-1}$ dimethomorph (Figure 2). No significant differences existed in EC_{50} values between isolates, and all exceeded 10.0 $\mu g\ ml^{-1}$ dimethomorph (data not shown).

Zoospore encystment and cystospore germination inhibition trends were similar and most isolates exhibited significant inhibition of both factors at 0.01 $\mu g\ ml^{-1}$ dimethomorph (Figure 2). For both factors, sensitivity trends were generally linear. The isolate Pi213 had a significantly higher EC_{50} value for zoospore encystment, than Pi88, Pi95-5, Pi94-4, and Pi97-2, while other distinct trends were not apparent (Table 3). For cystospore germination, the EC_{50} values for Pi670 and Pi88 were significantly higher than five of the 11 isolates, but distinct trends were not apparent.

No correlations were noted between EC_{50} value and isolate mating type, genotype, or location of isolation.

Figure 2 (a-k). The percent inhibition, relative to the untreated control, of *in vitro* zoosporogenesis, zoospore encystment, and cystospore germination for *P. infestans* vs. dimethomorph concentration (0.0, 0.01, 0.1, 1.0, and 10.0 $\mu g\ ml^{-1}$). Error bars represent Fischer's LSD (α = 0.05).


a. Pi88
b. Pi95-5
c. Pi671
d. Pi458
e. Pi670
Zoosporogenesis
Zoospore Encystment
Cystospore Germination
Percent Inhibition Relative to the Untreated Control
120
100
80
60
40
20
0
-20
0
0.01
0.1
1
10
Concentration Dimethomorph (µg ml-1)

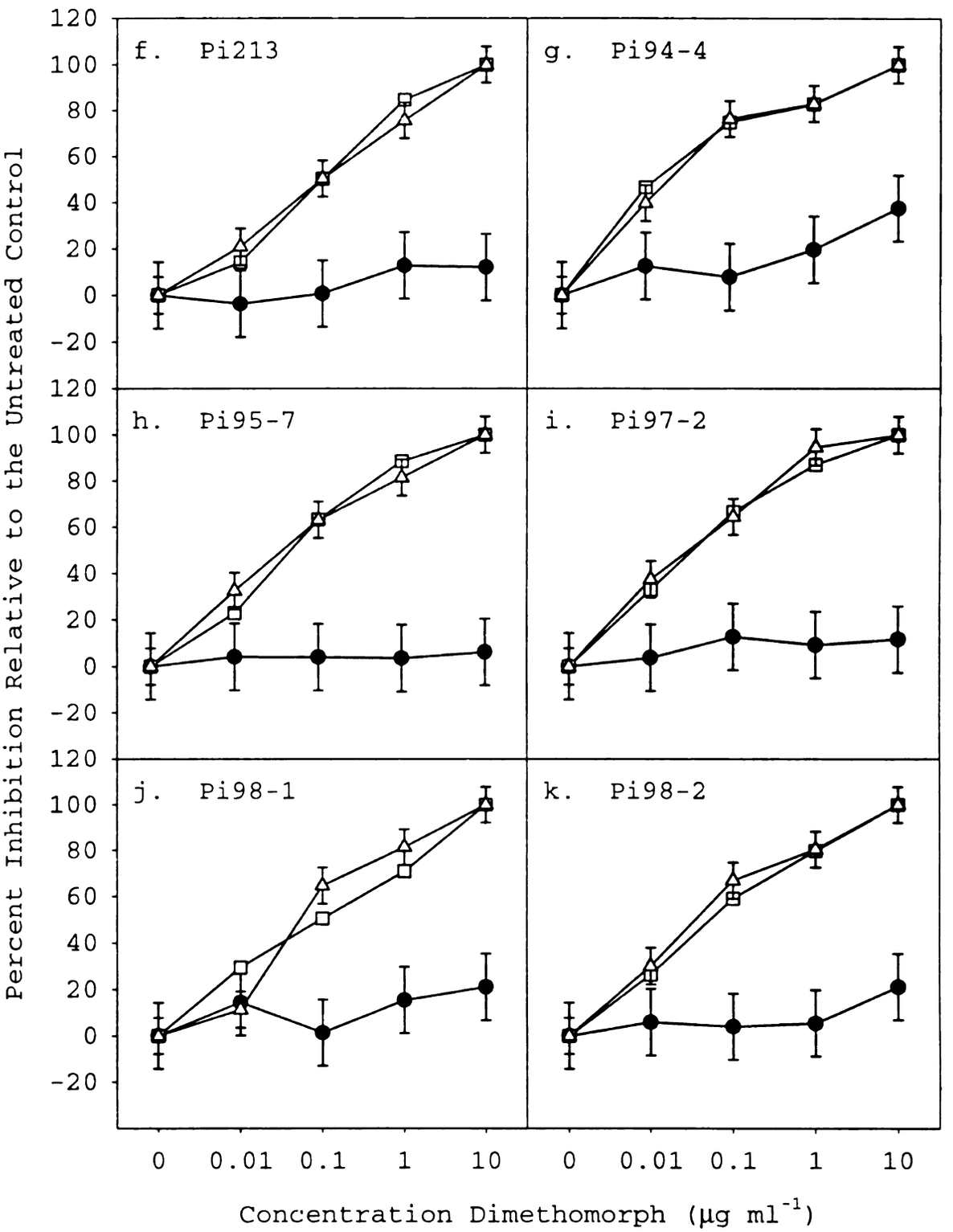

f. Pi213
g. Pi94-4
h. Pi95-7
i. Pi97-2
j. Pi98-1
k. Pi98-2
Percent Inhibition Relative to the Untreated Control
120
100
80
60
40
20
0
-20
0
0.01
0.1
1
10
Concentration Dimethomorph (µg ml-1)

Table 3. The effective concentration of dimethomorph (μg ml^{-1}) required for a 50% reduction of zoospore encystment, cystospore germination, and direct sporangia germination for *P. infestans* isolates.

Isolate ID	*in vitro* EC_{50} (μg ml^{-1})[1]	
	Zoospore[2] Encystment	Germination Cystospore[3]
Pi88	0.047 bc[4]	0.169 a
Pi95-5	0.016 bc	0.016 d
Pi671	0.067 abc	0.117 ab
Pi458	0.084 abc	0.047 bcd
Pi670	0.036 bc	0.145 a
Pi213	0.136 a	0.114 abc
Pi94-4	0.011 c	0.018 d
Pi95-7	0.053 abc	0.042 bcd
Pi97-2	0.033 bc	0.027 cd
Pi98-1	0.098 ab	0.071 abcd
Pi98-2	0.063 abc	0.046 bcd

1. Effective concentration for a 50% reduction, calculated using probit transformation of the percent inhibition relative to the untreated control.
2. Encystment of zoospores in sterile diH_2O amended with 0, 0.01, 0.1, 1.0, or 10 μg ml^{-1} dimethomorph.
3. Germination of encysted zoospores in sterile diH_2O amended with 0, 0.01, 0.1, 1.0, or 10 μg ml^{-1} dimethomorph.
4. Means followed by the same letter are not significantly different using Fischer's LSD (α = 0.05).

The most sensitive isolate overall was Pi95-5, an A1/US1 strain (Table 4). However, the sensitivity of the other A1/US1 strain, Pi88, was near the average of the isolates examined. The isolate with the lowest overall sensitivity was Pi213. The isolates, Pi458, and Pi671 had significantly lower mean sensitivity than all other isolates, except Pi98-1. All stages of the asexual life cycle were positively and significantly correlated with each other except cystospore germination, which was not significantly correlated with inhibition of any other stage (Table 5).

No significant difference between isolates was observed for the percent inhibition of the incidence of leaf disk symptom development at 0.0, 1.0, and 10.0 $\mu g\ ml^{-1}$ dimethomorph, regardless of application timing (Figure 3). When dimethomorph was applied prior to inoculation, there were no significant differences in the inhibition of symptom incidence between application timings, at any concentration. Sensitivity curves for dimethomorph application prior to inoculation were sigmoidal and complete inhibition of symptom development occurred at 1000.0 $\mu g\ ml^{-1}$ dimethomorph. No significant inhibition of symptom development occurred when dimethomorph was applied after inoculation, regardless of timing, although the

Table 4. Median rank of isolate sensitivity when EC_{50} values were ranked within hyphal growth, sporulation (*in vitro*), direct sporangia germination, zoospore encystment, and cystospore germination and compared with an analysis of variance on ranks.

Isolate ID	Mating Type / Genotype[1]	Median Rank of Isolate Sensitivity	
Pi88	A1/US1	6[2]	b[3]
Pi95-5	A1/US1	1	d
Pi671	A1/US14	9	a
Pi458	A2/US17	9	a
Pi670	A2/US7	7	b
Pi213	A2/US8	11	a
Pi94-4	A2/US8	2	cd
Pi95-7	A2/US8	6	bc
Pi97-2	A2/US8	3	d
Pi98-1	A2/US8	8	ab
Pi98-2	A2/US8	5	bc

1. Allozyme-based genotype[59]
2. Sensitivity ranking is from most sensitivity (lowest) to least sensitivity (highest).
3. Means followed by the same letter are not significantly different using Fischer's LSD ($\alpha = 0.05$).

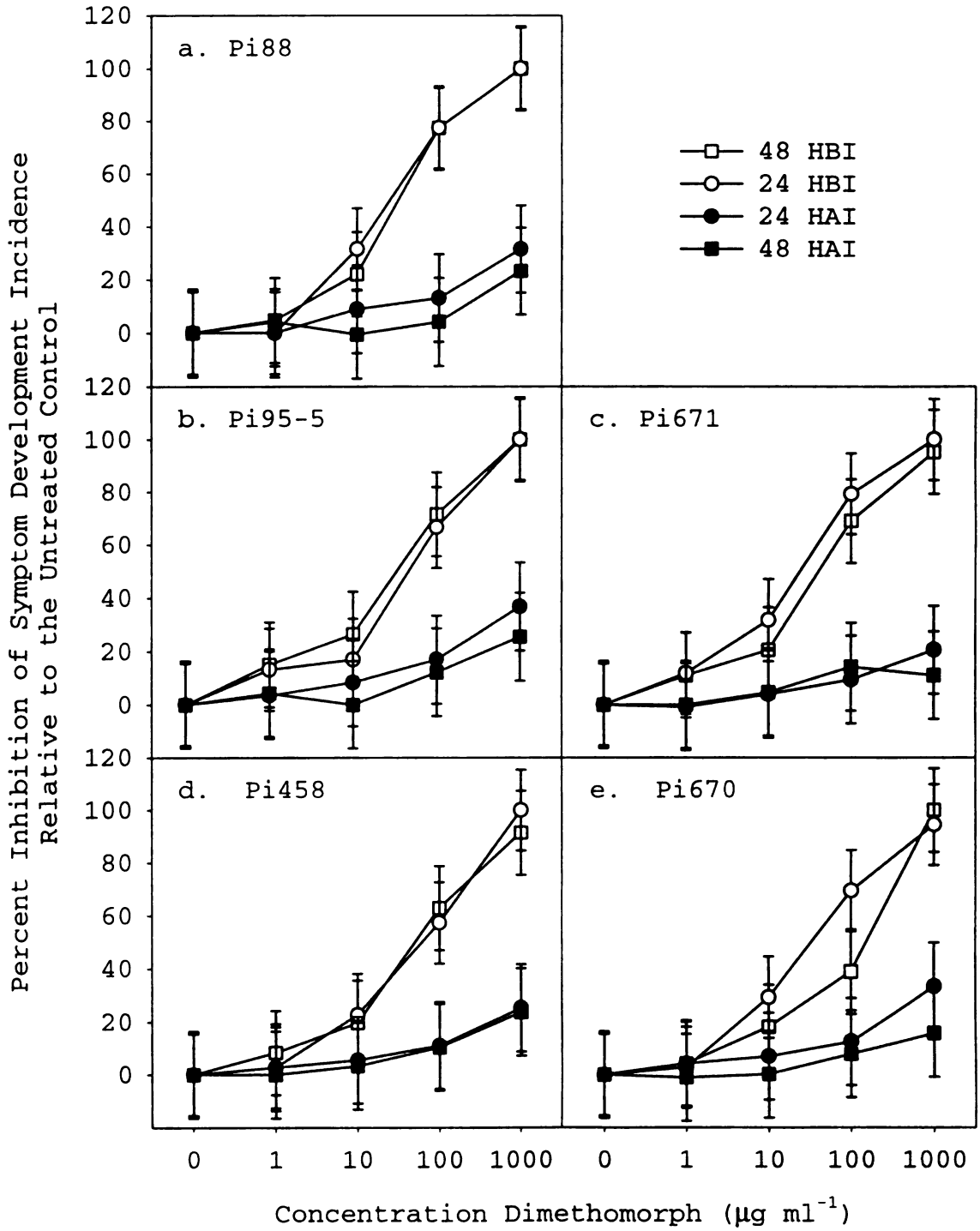
Table 5. Correlation of the stages of the asexual life cycle of *P. infestans* examined and the P-value for each, excluding zoosporogenesis, analyzed with Pearson's Product Moment Correlation.

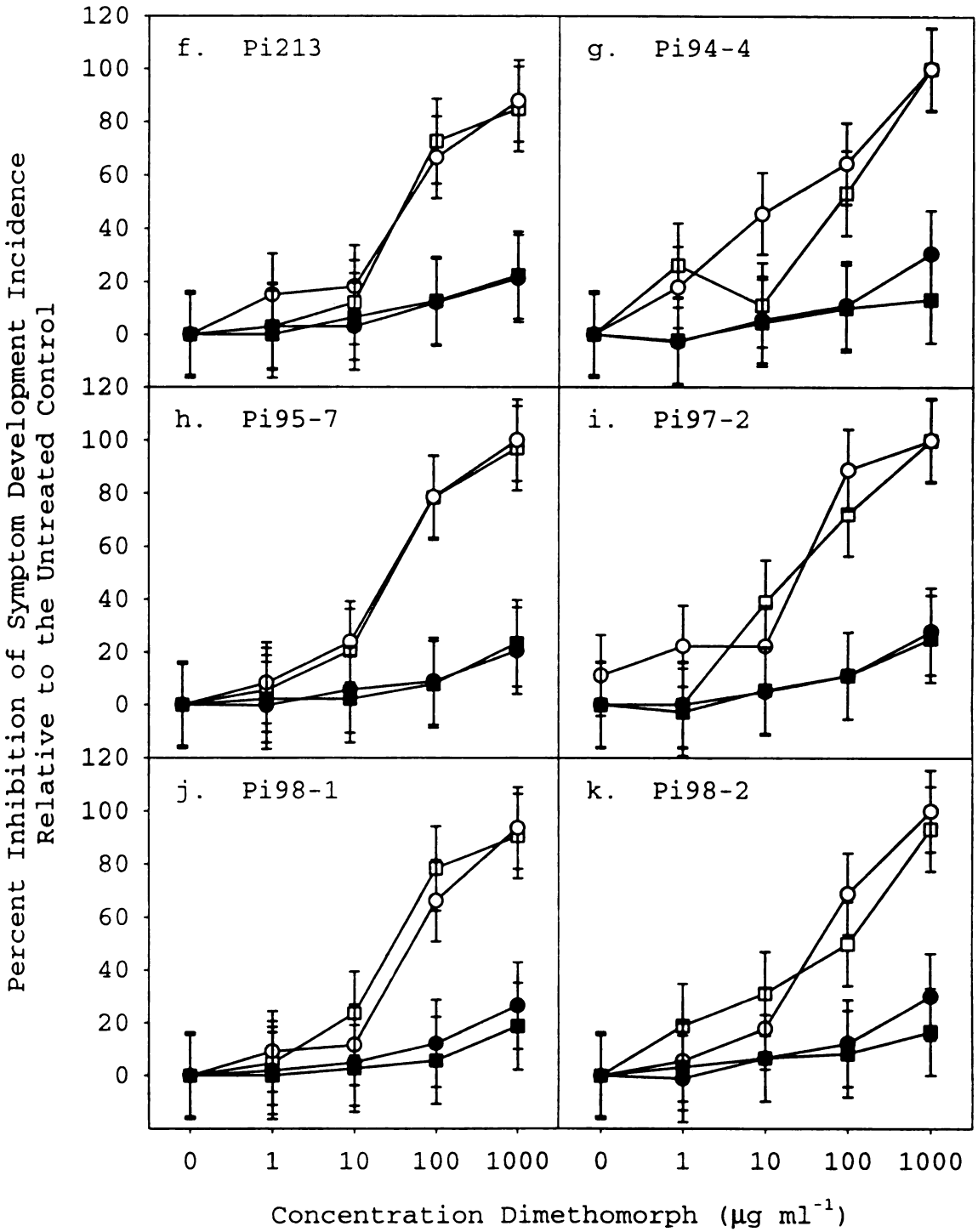
Table 5.

Variable	Correlation Coefficient[1] and (P-value) of EC_{50} Values			
	in vitro Sporulation	Sporangia Germination	Zoospore Encystment	Cystospore Germination
Hyphal Growth	0.811 (0.002)	0.779 (0.005)	0.847 (>0.001)	0.458 (0.157)
***in vitro* Sporulation**	n/a[2]	0.769 (0.006)	0.706 (0.015)	0.529 (0.094)
Sporangia Germination	n/a	n/a	0.815 (0.002)	0.314 (0.347)
Zoospore Encystment	n/a	n/a	n/a	0.320 (0.338)

1. Pearson Product Moment Correlation Coefficient[89], where >0.50 (P-value >0.05) indicates a significant positive correlation between variables, while a coefficient <0.50 (P-value >0.05) indicates a significant negative correlation.
2. A non-applicable self-comparison or displayed in another cell of the matrix.

Figure 3 (a-k). The percent inhibition, relative to the untreated control, of the incidence of leaf disk symptom development vs. dimethomorph concentration (0.0, 1.0, 10.0, 100.0, and 1000.0 μg ml^{-1}). Dimethomorph was applied as a commercial formulated product (Acrobat 50WP, BASF Corporation) at 48 or 24 hours before inoculation (HBI) or 24 or 48 hours after inoculation (HAI) by a sporangia / zoospore suspension of *P. infestans*. Error bars represent Fischer's LSD (α = 0.05).


a. Pi88
b. Pi95-5
c. Pi671
d. Pi458
e. Pi670
48 HBI
24 HBI
24 HAI
48 HAI
Percent Inhibition of Symptom Development Incidence Relative to the Untreated Control
Concentration Dimethomorph (µg ml-1)
0
20
40
60
80
100
120
0
1
10
100
1000

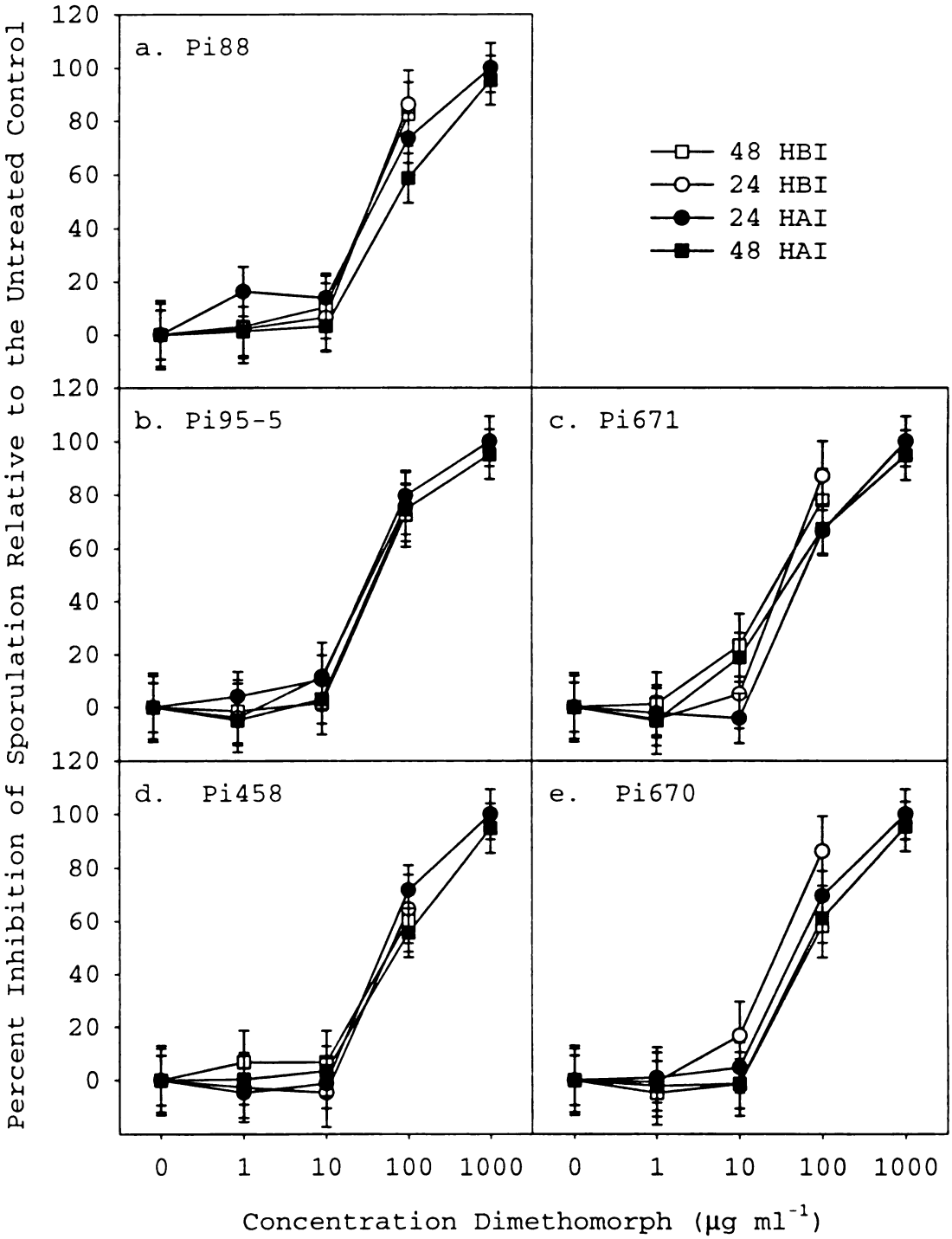

f. Pi213
g. Pi94-4
h. Pi95-7
i. Pi97-2
j. Pi98-1
k. Pi98-2
Percent Inhibition of Symptom Development Incidence Relative to the Untreated Control
120
100
80
60
40
20
0
0
1
10
100
1000
Concentration Dimethomorph (µg ml-1)

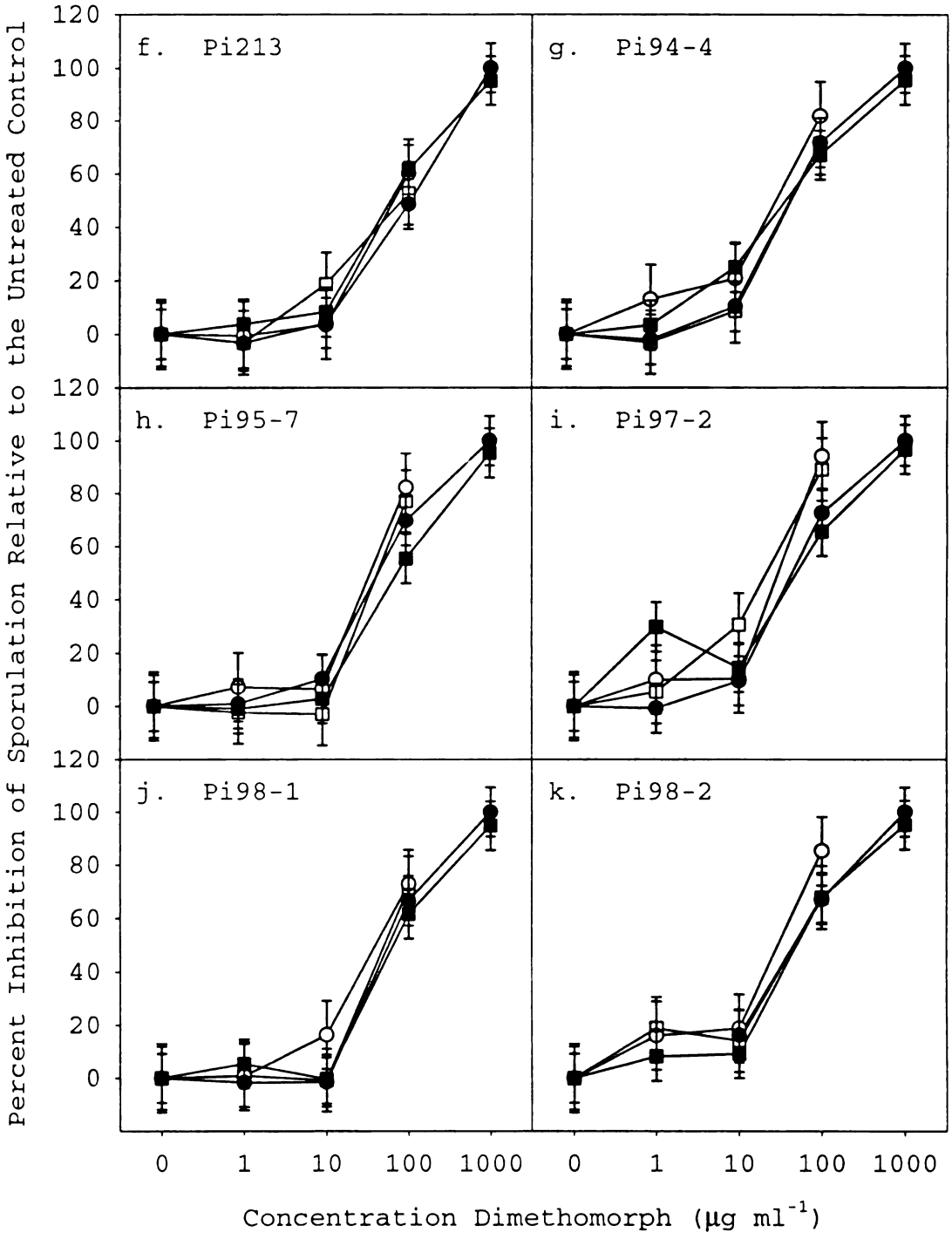
incidence of symptom development for most isolates was inhibited to approximately 20% at 1000.0 μg ml^{-1} dimethomorph.

Within each application timing, the EC_{50} values for the incidence of symptom development were not significantly different among isolates (data not shown). Mean EC_{50} values for symptom development were not significantly different between application timings before inoculation; nor for application timings after inoculation. The mean EC_{50} values for the inhibition of the incidence of symptom development for dimethomorph application before and after inoculation were 36.18 and >1000.0 μg ml^{-1} dimethomorph, respectively, with the latter being significantly larger.

For all isolates, no significant difference in the percent inhibition of sporulation was present between application timings at each concentration of dimethomorph (Figure 4). No significant inhibition of sporulation occurred at 1.0 or 10.0 μg ml^{-1} dimethomorph, while 100.0 and 1000.0 μg ml^{-1} dimethomorph significantly inhibited sporulation, relatively to the untreated control, to 70.7% and 98.4%, respectively. The lack of symptom development for leaf disks treated with 1000.0 μg ml^{-1} dimethomorph before inoculation did not allow for assessment of

Figure 4 (a-k). The percent inhibition, relative to the untreated control, for *P. infestans* sporulation from inoculated leaf disks vs. dimethomorph concentration (0.0, 1.0, 10.0, 100.0, and 1000.0 $\mu g\ ml^{-1}$). Dimethomorph was applied as a commercial formulated product (Acrobat 50WP, BASF Corporation) at 48 or 24 hours before inoculation (HBI) or 24 or 48 hours after inoculation (HAI) by a sporangia / zoospore suspension of *P. infestans*. Error bars represent Fischer's LSD ($\alpha = 0.05$).


a. Pi88
b. Pi95-5
c. Pi671
d. Pi458
e. Pi670
48 HBI
24 HBI
24 HAI
48 HAI
Percent Inhibition of Sporulation Relative to the Untreated Control
120
100
80
60
40
20
0
0
1
10
100
1000
Concentration Dimethomorph (µg ml-1)


f. Pi213
g. Pi94-4
h. Pi95-7
i. Pi97-2
j. Pi98-1
k. Pi98-2
Percent Inhibition of Sporulation Relative to the Untreated Control
120
100
80
60
40
20
0
0
1
10
100
1000
Concentration Dimethomorph (µg ml-1)

antisporulation. No significant differences between isolates, timings, or application relative to the inoculation event were present for the EC_{50} values of sporulation inhibition (data not shown). The mean EC_{50} value for the inhibition of sporulation was 49.44 μg ml^{-1} dimethomorph.

Discussion

The concentrations required for the inhibition of *P. infestans in vitro* hyphal growth, direct sporangia germination, zoospore encystment, cystospore germination, and inhibition of symptom development and sporulation *in vivo* were similar to those previously reported (1,2,29,90,97). Zoosporogenesis was not sensitive to the concentrations of dimethomorph examined, while *in vitro* hyphal growth and sporulation, and direct sporangia germination were moderately sensitive to inhibition by dimethomorph, with mean EC_{50} values of 0.45, 0.22, and 0.19. In contrast, zoospore encystment and cystospore germination were both highly sensitive to dimethomorph with EC_{50} values for most isolates <0.10 μg ml^{-1} dimethomorph.

The higher sensitivity of the zoospore encystment and cystospore stages of the asexual life cycle may be related to the physiology of *Phytophthora* at those stages.

Specifically, disrupting the extension of hyphal tips or sporangiophore formation will not be immediately lethal to a colony and it is possible that physiological changes in the cytoplasm could temporarily or partially off-set the disruption of cell wall formation. In contrast, disruption of the *de novo* synthesis of the cell wall, as in zoospore encystment, would be lethal in a much shorter time period as zoospores must actively offset osmotic pressure and have limited energy reserves (46). As with zoospores, cystospores have relatively limited energy reserves, saprophytic ability, and longevity [Coffey, 1991 #378]. Therefore, the inhibition of germ tube formation would be lethal to each cyst while a sporangium might be able to attempt multiple germinations. It is possible that direct sporangia germination was less susceptible because of the larger physical size and energy reserves of the sporangium in comparison to a zoospore or cystospore.

Direct sporangia germination and *in vitro* hyphal growth had the smallest ranges in sensitivity between isolates, with >4 and >6-fold differences of EC_{50} values between the most and least insensitive isolates, respectively. *In vitro* sporulation, zoospore encystment and cystospore germination all had larger ranges in sensitivity with >10-fold differences. The ranges of

dimethomorph sensitivity are much smaller than those reported for phenylamide insensitivity in *P. infestans* (128) and other *Phytophthora* species (49,103). However, these studies included isolates with field resistance to phenylamides, and exclusion of those isolates results in a similar sensitivity distribution. The sensitivity ranges determined from disease incidence for *Plasmopara viticola* to the strobilurin fungicide azoxystrobin were similar to those presented here (138).

Application of 1000.0 μg ml^{-1} dimethomorph at 24 or 48 hours before inoculation almost completely inhibited symptom development incidence in inoculated leaf disks, while application within 48 hours after inoculation failed to offer significant inhibition. When used to control *P. infestans* in the field, dimethomorph should be applied in a protectant fashion, reinforcing previously reported results (1,29,124). The EC_{50} values for the inhibition of *in vivo* symptom development were much larger than those at the specific stages in the asexual life cycle of *P. infestans*. While dimethomorph is considered to be slightly systemic, it is also known to have a high resistance to washing off with water following application (29). These results, and the fact that dimethomorph has low solubility in water, indicates that dimethomorph probably has a high affinity

for the cuticle of the leaf. Once applied, only a small percentage of dimethomorph is likely to dissolve into any free water on the leaf surface, therefore requiring larger concentrations of dimethomorph for *in vivo* efficacy to offset the low solubility.

Inhibition of sporulation of *P. infestans* occurred when dimethomorph was applied between 48 hours before and 48 hours after inoculation at 100.0 μg ml^{-1} or higher. These results are similar to those previously reported (29). Outside of the examined time frame and under field conditions, dimethomorph may not have the same level of antisporulation activity. A study examining antisporulation under field conditions failed to demonstrate any inhibition of sporulation following dimethomorph, or other fungicides, applications (123). Application of fungicides under field conditions is unlikely to confer the same homogeneity of leaf coverage as application under controlled conditions. Thus, the lack of antisporulation activity in the field is due to either 1) incomplete coverage leaving unprotected foliage, 2) sub-efficacious concentrations of dimethomorph on the leaf surface, or a combination of the two.

Dimethomorph at low concentrations is highly inhibitory to most stages of the asexual portion of the *P.*

infestans life cycle. Frequent protectant applications of dimethomorph at concentrations exceeding 1000.0 µg ml^{-1} would likely inhibit infection by *P. infestans* in the field and possibly reduce sporulation from previously established infections, but not cure them. The *P. infestans* isolates examined all had similar sensitivity to dimethomorph in the assays performed. However, low levels of resistance to dimethomorph have been generated *in vitro* for *P. infestans (141)* and other *Phytophthora* species (21,141), and therefore should be examined further in order to manage resistance development.

CHAPTER THREE

THE GENERATION, QUANTIFICATION, AND CHARACTERIZATION OF DIMETHOMORPH INSENSITIVITY IN *PHYTOPHTHORA INFESTANS* AND OTHER *PHYTOPHTHORA* SPECIES

Abstract

The generation of dimethomorph insensitive strains of *Phytophthora infestans* was attempted using ethidium bromide / ultra-violet light mutagenesis and repeated culturing on amended media. Ethidium bromide / UV mutagenesis created two strains of *P. infestans* with resistance factors >20, i.e. the ratio of the EC_{50} of the mutant strain to that of the wild-type. For most *P. infestans* isolates, the rate of growth on dimethomorph amended media increased until the fourth sub-culture. Resistance factors for strains generated from repeated culturing were <8. For most isolates of *P. infestans*, dimethomorph insensitive strains had reduced growth rates on un-amended media, regardless of the level of insensitivity. Additionally, virulence on leaf disks and in whole tubers was significantly reduced in >20% of the isolates examined. Insensitivity generation of other *Phytophthora* species was attempted with repeated culturing on amended media and one strain of *P. erythroseptica* occurred through colony sectoring with a

resistance factor >7.

Introduction

Resistance to fungicides in fungal and Oomycete plant pathogens has become increasingly more common following the release of systemic fungicides in the 1960's (45). Field resistance to every major systemic fungicide class has occurred in at least one species of plant pathogenic fungi or Oomycetes (109,122), leading to drastic shifts in fungicide programs for many pathosystems, such as potato late blight (61) and *Phytophthora* blight of bell pepper (103). The migration of phenylamide insensitive populations of *Phytophthora infestans*, the causal agent of potato and tomato late blight, from Mexico to the rest of North America (61), necessitated cultural control methods and crop protection strategies that relied primarily on protectant foliar fungicide applications (116). Concurrently, the agrichemical industry developed and released systemic fungicides in an attempt to replace the phenylamides.

Dimethomorph, one of the fungicides released in response to phenylamide resistance, is a cinnamic acid derivative with a high specificity to the genus *Phytophthora* and certain members of the Peronosporaceae (1). Cells of *P. infestans* lacking cell walls, such as

zoospores and artificially generated protoplasts, were not affected by dimethomorph (2) and it was concluded that dimethomorph disrupted cell wall formation by interfering with the molecular arrangement of wall components and not the inhibition of component synthesis (90).

Insensitivity to dimethomorph was generated with ultraviolet (UV) light mutagenesis in one isolate of *P. parasitica* (21) and with chemical mutagenesis in one isolate of *P. capsici* Leonian (141). In both cases, an approximate 20-fold decrease in sensitivity resulted. Virulence of dimethomorph insensitive *P. parasitica* strains was equal to, or less than, the wild-type. In addition, insensitivity in *P. parasitica* was stable in the absence of dimethomorph through several *in vitro* sub-cultures for three months. Conversely, attempts to generate insensitivity using mycelial adaptation in *P. infestans* and *P. capsici* resulted in small (<2-fold) decreases in sensitivity to dimethomorph (141). The use of only one culture of *P. infestans* and *P. capsici* in the latter experiment may have limited the results as genetic variability was absent.

The objectives of this study were to 1) generate strains of *P. infestans* insensitive to dimethomorph using ethidium bromide / UV mutagenesis or mycelial adaptation

and 2) determine the level of resistance generated and examine virulence of *P. infestans* insensitive strains on potato foliage and tubers.

Materials and Methods

Media Preparation

Experiments involving hyphal growth were performed on modified (CHAPTER TWO) rye B agar (2,19) because of the relatively rapid growth compared to synthetic media. All plates for each sub-culture on dimethomorph amended media or run of the baseline sensitivity assay were prepared from the same batch of media in order to reduce variability within each run of an experiment. Fungicide amended media was prepared as described (CHAPTER TWO).

Generation of Insensitivity: UV Mutagenesis

Cultures of *P. infestans* for mutagenesis were grown on rye B agar at 21°C until complete colonization of the media. Plates were flooded with 5.0 ml of a 1 µg ml^{-1} ethidium bromide solution (in sterile diH_2O), allowed to dry, and exposed to 254 nm ultraviolet (UV) light (2.03 x 10^3 ergs cm^{-2} s^{-1}) for five minutes. From each isolate, four 4.0 mm diameter colonized agar plugs were transferred to rye B media amended with 0.0 or 10.0 µg ml^{-1} dimethomorph. Non-

exposed cultures were also transferred onto control and amended rye B media. Cultures were incubated in the dark and examined on seven-day intervals for colony diameter and rapid growth or sectoring on the fungicide amended media. The fastest growing sector of each isolate from the fungicide amended media was used for further assays, and hereafter labeled strain "UV", e.g. Pi95-7_{UV}.

Generation of Insensitivity: Sub-Lethal Culturing

Using previously determined effective concentration for a 50% reduction in colony diameter (EC_{50}) values for inhibition of *in vitro* growth by dimethomorph (CHAPTER TWO), culturing of *Phytophthora sp.* on media amended with a highly inhibitory (1.0 μg ml^{-1}), sub-lethal concentration of dimethomorph was performed to select for insensitivity in cultures. Initial wild-type isolates were previously characterized (Table 6), two sub-cultures from re-isolation from infected host tissue, and labeled strain "WT", e.g. Pi95-7_{WT}. Colonized agar plugs, 4.0 mm in diameter, from the margin of an actively growing colony were transferred mycelium-side down onto modified rye B media amended with 0.0 or 1.0 μg ml^{-1} dimethomorph and incubated at 21°C in the dark with three replicate plates per concentration. Colony diameter was measured after 11 days for *P. infestans*, and

Table 6. Isolate identification code, *Phytophthora* species, mating type (if applicable), *P. infestans* genotype (if applicable), and State in which isolated (U.S.A.).

Isolate ID	*Phytophthora* species	Mating Type[1]	Genotype[2]	Source
Pi88	*P. infestans*	A1	US1	ND
Pi95-5	*P. infestans*	A1	US1	MI
Pi671	*P. infestans*	A1	US14	WA
Pi458	*P. infestans*	A2	US17	ID
Pi670	*P. infestans*	A2	US7	OR
Pi213	*P. infestans*	A2	US8	CO
Pi94-4	*P. infestans*	A2	US8	MI
Pi95-7	*P. infestans*	A2	US8	MI
Pi97-2	*P. infestans*	A2	US8	MI
Pi98-1	*P. infestans*	A2	US8	MI
Pi98-2	*P. infestans*	A2	US8	MI
Pcap	*P. capcisi*	A2	n/a	MI
Pcac X	*P. cactorum*	H	n/a	MI
Pcac Y	*P. cactorum*	H	n/a	MI
Pe96-2	*P. erythroseptica*	H	n/a	MI
Pe00-1	*P. erythroseptica*	H	n/a	ID

1. Homothallic species designated by "H".
2. Allozyme-based genotype[59].

for other *Phytophthora* species at 5 and 11 days for 0.0 and 1.0 μg ml^{-1}, respectively. Isolates grown on control (0.0 μg ml^{-1}) and sub-lethal dimethomorph (1.0 μg ml^{-1}) amended media were labeled strains "CT" and "SL", respectively, e.g. Pi95-7_{CT} and Pi95-7_{SL}.

After the colony diameter was measured, one plate from each concentration was used to sub-culture the strain onto media with the same concentration of dimethomorph, for ten sub-cultures total. Portions of the colony were chosen for sub-culturing in an attempt to retain the wild-type colony phenotype of each isolate, while selecting sectors with higher growth rates. In the absence of accelerated growth on dimethomorph, plugs were chosen randomly from the margin of the colony. Following the tenth sub-culture, the cultures were examined for baseline sensitivity and pathogenicity.

Dimethomorph Sensitivity Assays

Prior to performing baseline sensitivity assays, all cultures were grown on non-amended media. Baseline sensitivity of *in vitro* growth to dimethomorph was determined as previously described (CHAPTER TWO). Baseline sensitivity assays were conducted three times with three replication plates per run for each strain of every

isolate, e.g. Pi95-7_{WT}, Pi95-7_{CT}, Pi95-7_{SL}, and Pi95-7_{UV}. EC_{50} values were calculated as previously described (CHAPTER TWO). Resistance factors were calculated for all strains using the following formula:

$$RF = \frac{EC_{50}X}{EC_{50}WT}$$

where $EC_{50}X$ is the EC_{50} value of the strain being examined, and $EC_{50}WT$ is the EC_{50} value of the wild-type strain of that isolate. Analysis of variance was performed on the final colony diameter (FCD) measurement (mm) and the EC_{50} values calculated (Proc GLM - SAS/Stat, SAS Institute, Cary, NC, U.S.A.) at α = 0.05 via pair-wise comparisons using Fisher's LSD. Prior to the combined ANOVA, each run was analyzed separately and the homogeneity of variance was confirmed using the F Max test (68).

Virulence Assays

Leaf disks for inoculation, and sporangia/zoospore suspensions were prepared as previously described (CHAPTER TWO) for a final concentration of 1.0 x 10^4 sporangia ml^{-1}. The sporangia/zoospore suspension from each strain of every isolate was applied (50 µl) to four leaf disks per replicate, with three replicates. The experiment was repeated twice. Inoculated leaf disks were incubated at

21°C light / 18°C dark (12 hour cycles) and were examined with a dissecting microscope at 96 hours after inoculation for symptoms and signs of infection by *P. infestans*, such as sporulation. Pathogen identity was confirmed with a compound microscope. Leaf disk inoculations were not performed on ethidium bromide / UV generated strains as sporulation was disrupted for most isolates. An analysis of variance on the number of leaf disks infected was performed as described.

Pathogen free potato tubers (cv. Snowden), approximately 6.35 cm diameter, were surface sterilized as described (CHAPTER TWO). Colonized portions of rye B agar, 1.0 cm^2, were excised from the margin of an actively growing colony of *P. infestans* and placed into a sterile 1.0 ml syringe with an 18½ gauge needle. A wound approximately 1.0 cm from the apex of the tuber and 0.5 cm deep into the tissue was created by stabbing the tuber with a pair of sterile forceps. Three tubers per replicate were inoculated by extruding approximately 0.1 ml of macerated colonized agar through the needle into the wound and placed into a covered plastic container. There were three replicates per run and the experiment was repeated twice. The tubers were incubated in the dark for seven days at 18°C and tuber surface and cross-sections were evaluated for

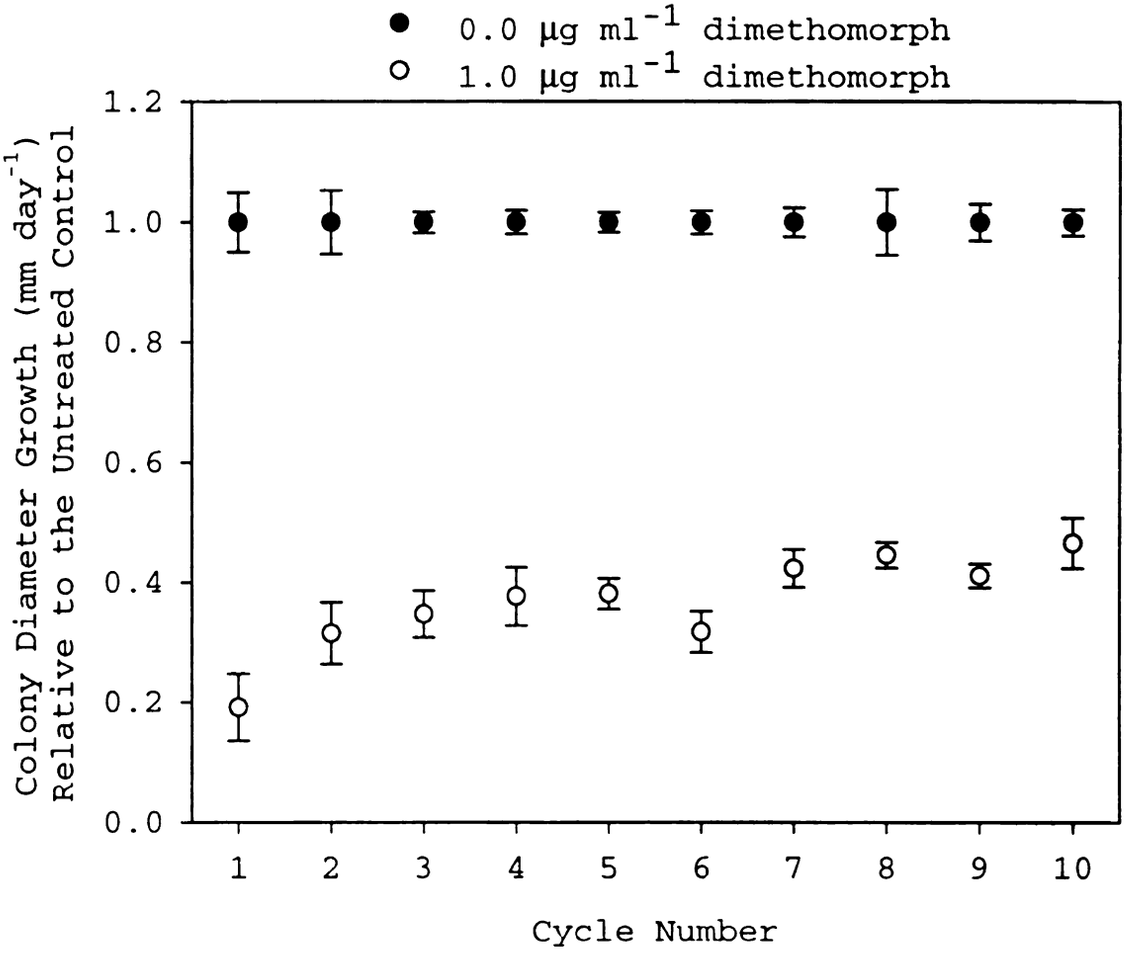
symptoms and signs of infection by *P. infestans*. Pathogen presence was verified with a compound microscope when applicable. Tuber inoculations were not performed on ethidium bromide / UV generated strains. An analysis of variance on the number of tubers infected was performed as described.

Results

Following ethidium bromide / UV light treatment, all isolates except Pi88 grew on non-amended media rye B media (data not shown). When cultured on non-amended media, treated cultures exhibited disrupted colony morphology and lower growth rates compared to untreated cultures. Of the isolates that grew following ethidium bromide / UV light treatment, only Pi458 and Pi98-2 failed to grow on media amended with 10.0 μg ml^{-1} dimethomorph. However, most isolates required more than 14 days to develop sufficient growth for sub-culturing. Sectors with increased growth rates on dimethomorph amended media were common, but not always present.

For all isolates, regardless of species, the rate of growth on media amended with 1.0 μg ml^{-1} dimethomorph (strain SL) increased with sub-culture number, e.g. Pi213 (Figure 5). With most isolates, the largest increase

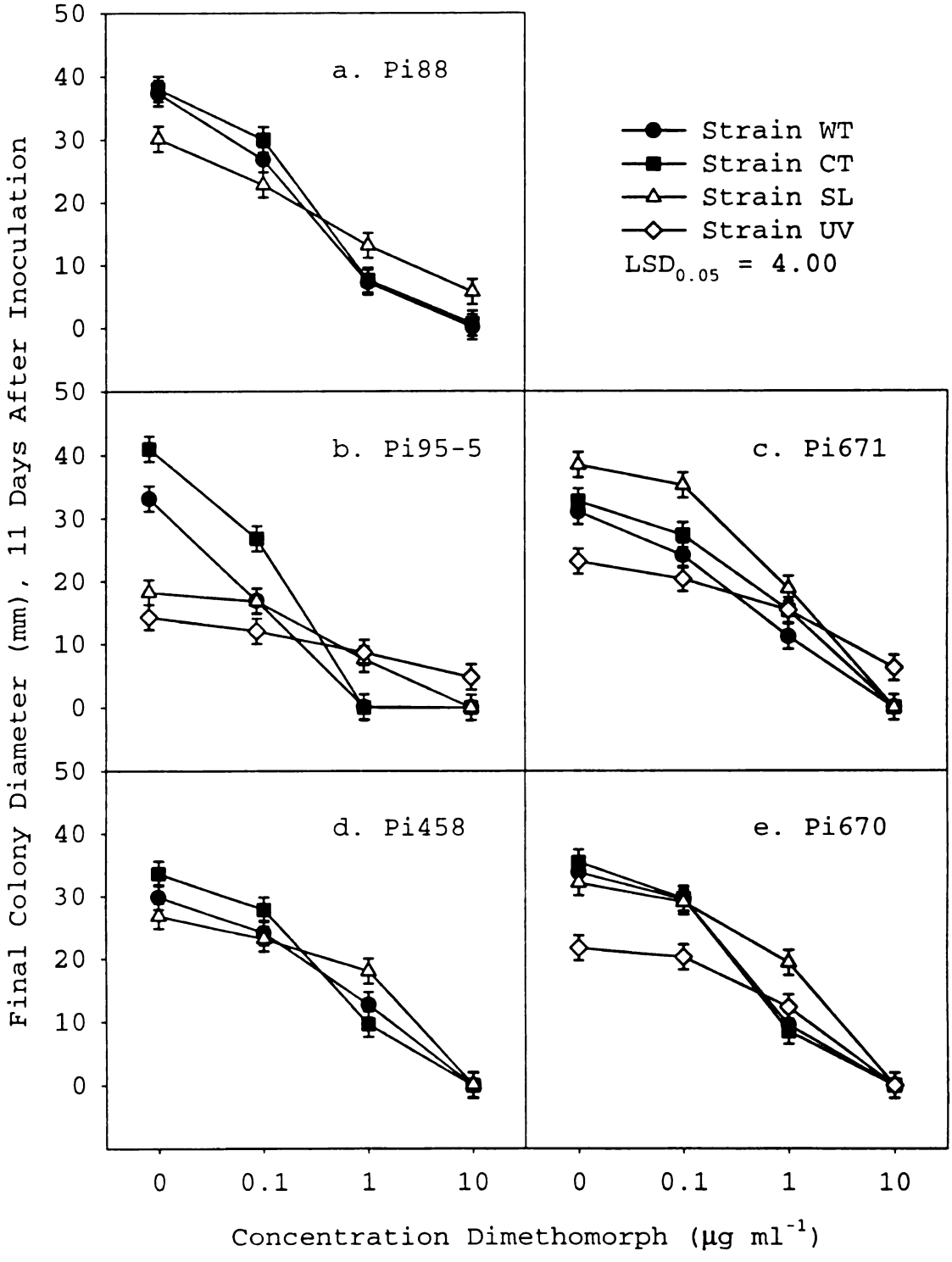
Figure 5. Representative colony growth, relative to the untreated control, in diameter (mm) day^{-1} vs. cycle number for isolate Pi213 when grown on rye B media amended with 0.0 or 1.0 µg ml^{-1} dimethomorph. Error bars represent one standard deviation for replicate plates.

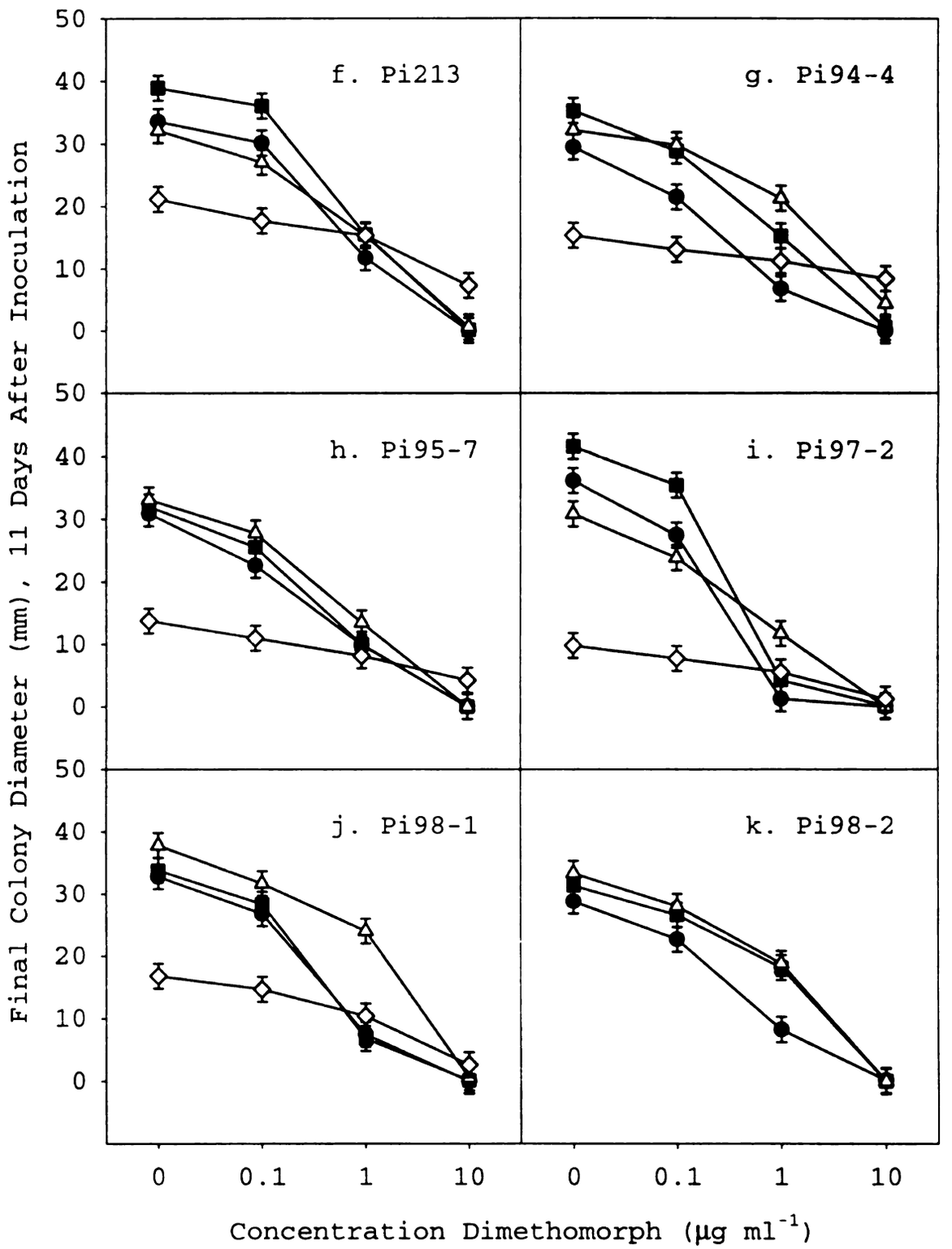


occurred between the initial and second sub-cultures, while changes at subsequent sub-cultures were smaller or absent. A fast growing sector appeared from one isolate of *P. erythroseptica*, Pe00-1, following sub-culture five on dimethomorph amended media, hereafter denoted strain "RS". Pe00-1_{RS} exhibited different colony morphology than all other strains of Pe00-1, and consistently died when cultured on non-amended media, or stored for more than 21 days at 18°C.

The final colony diameter (FCD) of the sub-cultured control strain of each *P. infestans* isolate was either similar to or significantly larger (Fischer's LSD, α = 0.05), than the corresponding wild-type strain on non-amended media, while the ethidium bromide / UV generated strain had the smallest FCD (Figure 6). The sub-cultured on dimethomorph amended media strains of Pi88, Pi95-5, and Pi97-2 had a significantly smaller FCD than both wild-type and control sub-cultured strains on non-amended media. On media amended with 0.1 µg ml^{-1} dimethomorph, differences in FCD between wild-type and strains sub-cultured on control or dimethomorph amended media became smaller, or non-significant, while the ethidium bromide / UV generated strains had the lowest FCD values. At 1.0 µg ml^{-1}

Figure 6 (a-k). Final colony diameter of *P. infestans* isolates for the wild-type strains (●), repeated culturing control strains (□), repeated culturing on 1.0 µg ml^{-1} dimethomorph amended media strains (▲), and UV / ethidium bromide generated strains (◊) at 11 days after inoculation. Isolates were grown on rye B media amended with 0.0, 0.1, 1.0, and 10.0 µg ml^{-1} dimethomorph. Error bars represent Fischer's LSD (α = 0.05).


a. Pi88
b. Pi95-5
c. Pi671
d. Pi458
e. Pi670
Strain WT
Strain CT
Strain SL
Strain UV
LSD0.05 = 4.00
Final Colony Diameter (mm), 11 Days After Inoculation
Concentration Dimethomorph (µg ml-1)
0
10
20
30
40
50
0
0.1
1
10


f. Pi213
g. Pi94-4
h. Pi95-7
i. Pi97-2
j. Pi98-1
k. Pi98-2
Final Colony Diameter (mm), 11 Days After Inoculation
Concentration Dimethomorph (µg ml-1)
0
10
20
30
40
50
0
0.1
1
10

dimethomorph, the sub-cultured on dimethomorph amended media strain of each isolate typically had a significantly larger FCD than all other strains. If growth occurred on media amended with 10.0 μg ml^{-1} dimethomorph, it was always a sub-cultured on dimethomorph amended media or ethidium bromide / UV generated strain, with the latter typically having the larger FCD.

Of the other *Phytophthora* species examined, FCD curves were similar to those of *P. infestans*, and wild-type and sub-cultured on non-amended media strains had similar FCD values at each concentration (Figure 7). The sub-cultured on dimethomorph amended media strains typically exhibited larger FCD values at 1.0 μg ml^{-1} dimethomorph, but not other concentrations. The only strains that grew at 10.0 μg ml^{-1} dimethomorph were the sub-cultured on dimethomorph amended media strains of Pcap A2 and Pe96-2, Pcap $A2_{SL}$ and Pe96-2_{SL}, and the strain that spontaneously sectored from Pe00-1_{SL}, Pe00-1_{RS}.

The *P. infestans* isolates Pi458, Pi94-4, and Pi98-1 had significantly larger EC_{50} values for the sub-cultured on dimethomorph amended media strain than both wild-type and sub-cultured on control media strains (Table 7). The calculated EC_{50} values for *in vitro* growth of sub-cultured

Figure 7 (a-e). Final colony diameter at 5 days after inoculation of the wild-type strains (●), repeated culturing control strains (□), repeated culturing on 1.0 μg ml^{-1} dimethomorph amended media strains (▲), and fast growing sector of *P. erythroseptica* that spontaneously appeared (◊) for *P. capcisi* (Pcap), *P. cactorum* (Pcac), or *P. erythroseptica* (Pe). Isolates were grown on rye B media amended with 0.0, 0.1, 1.0, and 10.0 μg ml^{-1} dimethomorph. Error bars represent Fischer's LSD (α = 0.05).

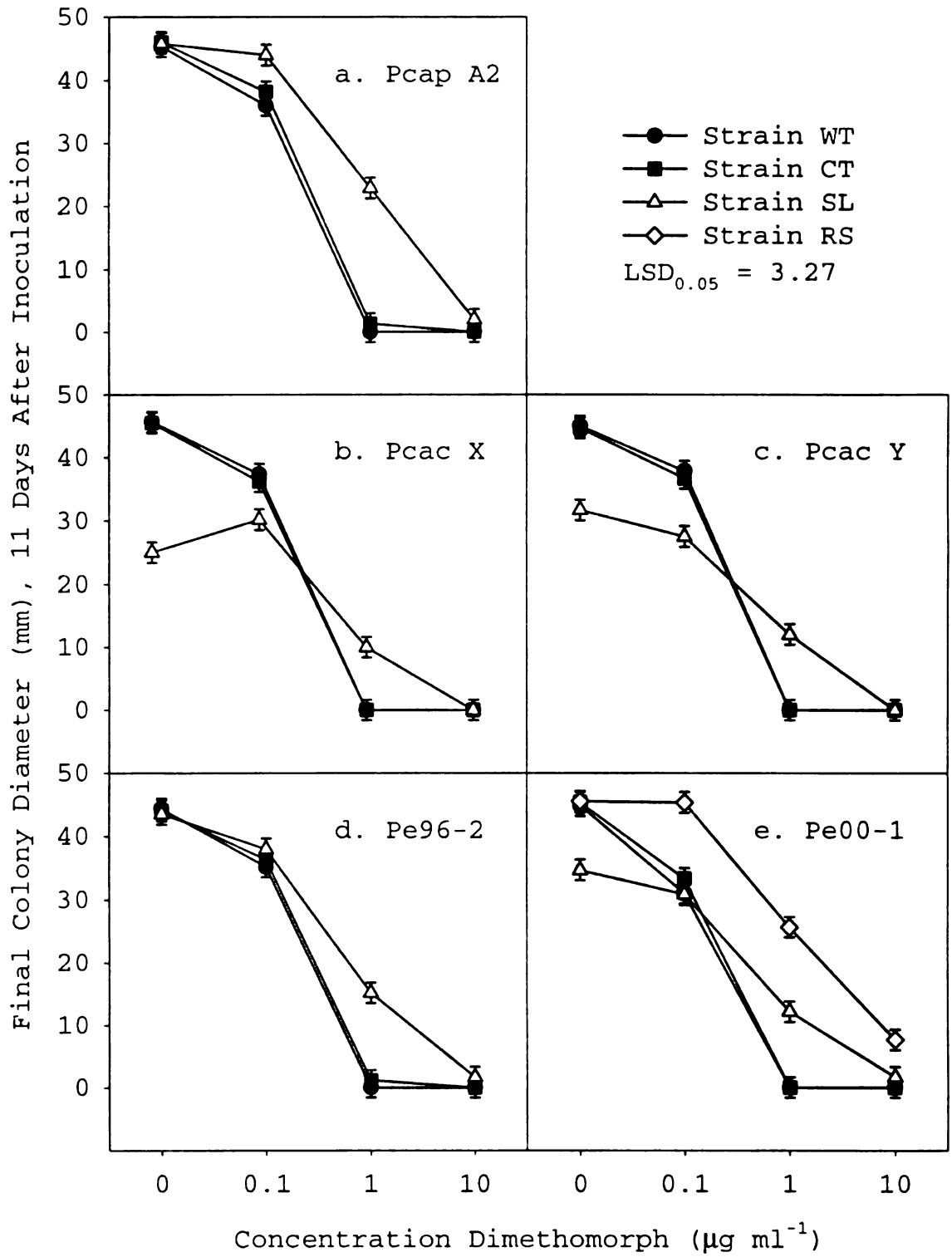

a. Pcap A2
b. Pcac X
c. Pcac Y
d. Pe96-2
e. Pe00-1
Strain WT
Strain CT
Strain SL
Strain RS
LSD0.05 = 3.27
Final Colony Diameter (mm), 11 Days After Inoculation
Concentration Dimethomorph (µg ml-1)
0
10
20
30
40
50
0
0.1
1
10

Table 7. Calculated EC_{50} for *in vitro* hyphal growth (diameter) and resistance factors for all strains of *P. infestans* isolates examined. Statistical comparisons shown within each isolate using Fischer's LDS (α = 0.05).

Isolate	Strain[1]	EC_{50}[2] ($\mu g\ ml^{-1}$)		Resistance Factor[3]
Pi88	WT	0.347	a[4]	-
	CT	0.373	a	1.075
	SL	0.770	a	2.219
	UV	*	[5]	*
Pi95-5	WT	0.107	b	-
	CT	0.186	b	1.138
	SL	0.800	b	7.447
	UV	2.479	a	23.168
Pi671	WT	0.703	b	-
	CT	1.049	b	1.492
	SL	1.083	b	1.541
	UV	2.290	a	3.257
Pi458	WT	0.843	b	-
	CT	0.555	b	0.658
	SL	1.818	a	2.157
	UV	*		*
Pi670	WT	0.617	ab	-
	CT	0.544	b	0.882
	SL	1.494	a	2.422
	UV	1.213	ab	1.966
Pi213	WT	0.680	b	-
	CT	0.869	b	1.278
	SL	0.979	b	1.439
	UV	4.368	a	6.424

Table 7 (cont'd).

Isolate	Strain[1]	EC_{50}[2] ($\mu g\ ml^{-1}$)		Resistance Factor[3]
Pi94-4	WT	0.442	c[4]	-
	CT	0.865	c	1.958
	SL	2.851	b	6.452
	UV	9.921	a	22.446
Pi95-7	WT	0.613	b	-
	CT	0.617	b	1.007
	SL	0.742	b	1.210
	UV	4.430	a	7.227
Pi97-2	WT	0.237	b	-
	CT	0.370	ab	1.561
	SL	0.850	ab	3.586
	UV	1.237	a	5.219
Pi98-1	WT	0.558	b	-
	CT	0.535	b	0.958
	SL	1.653	a	2.960
	UV	1.984	a	3.556
Pi98-2	WT	0.587	a	-
	CT	1.259	a	2.145
	SL	1.253	a	2.135
	UV	*[5]		*

1. WT = wild-type, or CT and SL = sub-cultured 10 times on media amended with 0.0 or 1.0 $\mu g\ ml^{-1}$ dimethomorph, respectively, or UV = mutated with ethidium bromide and UV light.
2. Effective Concentration to reduce colony diameter to 50% of fungicide un-amended media.
3. Resistance factor = EC_{50} of manipulated strain / EC_{50} of WT strain.
4. Means followed by the same letter are not significantly different within each isolate using Fischer's LSD (α = 0.05). Comparisons between strains of different isolates are not shown.
5. Attempted mutagenesis was lethal with this isolate or no growth occurred on 10.0 $\mu g\ ml^{-1}$ dimethomorph.

on dimethomorph amended media strains were numerically larger than those of the wild-type and sub-cultured on control media strains for nine of 11 isolates of *P. infestans*, indicating a decrease in sensitivity to dimethomorph through sub-culturing. The isolates Pi94-4 and Pi98-2 showed large, but not significant, increases in EC_{50} of the sub-cultured on control media strain in comparison to the wild-type strain. The ethidium bromide / UV generated strains from five of the eight isolates had significantly higher EC_{50} values than the other strains within each isolate. For all isolates of *P. infestans*, except Pi98-2, the calculated resistance factor (RF) was larger for the sub-lethal generated strain than the control strain. The RF values for the ethidium bromide / UV generated strains were larger than all other strains for seven of the eight isolates with such strains. The isolates Pi95-5 and Pi94-4 had the largest RF factors for sub-lethal and ethidium bromide / UV generated strains with 7.447 and 23.168, and 6.452 and 22.446, respectively.

The sub-cultured on dimethomorph amended media strains of the other *Phytophthora* species examined had significantly larger EC_{50} values compared to both the wild-type and the sub-cultured on control media strains within isolates (Table 8). Within the isolate Pe00-1, the RS

Table 8. Calculated EC_{50} for *in vitro* hyphal growth (diameter) and resistance factor, for all strains of *P. capcisi* (Pcap), *P. cactorum* (Pcac), and *P. erythroseptica* (Pe) isolates examined. Statistical comparisons shown within each isolate using Fischer's LDS (α = 0.05).

Isolate	Strain[1]	EC_{50}[2] ($\mu g\ ml^{-1}$)		Resistance Factor[3]
Pcap A2	WT	0.234	b[4]	-
	CT	0.269	b	1.150
	SL	1.004	a	4.291
Pcac X	WT	0.247	b	-
	CT	0.235	b	0.951
	SL	0.662	a	2.680
Pcac Y	WT	0.259	b	-
	CT	0.249	b	0.961
	SL	0.700	a	2.703
Pe96-2	WT	0.234	b	-
	CT	0.264	b	1.128
	SL	0.648	a	2.769
Pe00-1	WT	0.176	c	-
	CT	0.201	c	1.142
	SL	0.662	b	3.761
	RS	1.253	a	7.119

1. WT = wild-type, or CT and SL = sub-cultured 10 times on media amended with 0.0 or 1.0 $\mu g\ ml^{-1}$ dimethomorph, respectively or R = fast growing sector of *P. erythroseptica* that spontaneously appeared.
2. Effective Concentration to reduce colony diameter to 50% of fungicide un-amended media.
3. Resistance factor = EC_{50} of manipulated strain / EC_{50} of WT strain.
4. Means followed by the same letter are not significantly different within each isolate using Fischer's LSD (α = 0.05). Comparisons between strains of different isolates are not shown.

strain had a significantly larger EC_{50} than the other strains. $Pe00\text{-}1_{RS}$ also had the largest resistance factor.

The ability to infect and cause symptoms on potato leaf disks (cv. Snowden) was limited for most isolates, regardless of strain, as only four of 11 wild-type strains resulted in 75% of more leaf disks infected (Table 9). The wild-type strains of isolates, Pi95-5, Pi671, Pi670, Pi94-4, Pi97-2, and Pi98-2 successfully infected 50% or less of leaf disks, indicating low virulence of the wild-type strain, while only the wild-type strain of Pi458 infected all leaf disks. In comparison to the wild-type strains, the sub-cultured on dimethomorph amended media strains had significantly reduced percent leaf disks infected for five of 11 isolates of *P. infestans*. However, the sub-cultured on control media strains of isolates Pi458 and Pi95-7 infected significantly fewer leaf disks than the wild-type strain.

Infection and symptom development of tubers following inoculation of mycelium was more efficient than leaf disk inoculation with the wild-type strains of eight of 11 isolates causing symptoms in all tubers inoculated. The sub-cultured on dimethomorph amended media strains of seven isolates infected significantly less tubers compared to the wild-type strain of each isolate. Some of the sub-cultured

Table 9. Percent of infection by *P. infestans* isolates and strains, expressed as percent incidence, when inoculated onto both excised leaf disks and into whole tubers (>4.0 cm, <6.5 cm). Statistical comparisons shown within each isolate using Fischer's LSD (α = 0.05).

Table 9.

Isolate	Strain[1]	Percent Incidence of Infection[2]	
		Leaf Disks	Tubers
Pi88	WT	58 a[3]	100 a
	CT	50 a	33 b
	SL	58 a	22 b
Pi95-5	WT	25 a	33 a
	CT	8 a	22 ab
	SL	25 a	0 b
Pi671	WT	50 a	100 a
	CT	50 a	100 a
	SL	17 b	100 a
Pi458	WT	100 a	100 a
	CT	8 b	89 a
	SL	25 b	44 b
Pi670	WT	50 a	100 a
	CT	50 a	67 b
	SL	33 a	22 c
Pi213	WT	92 a	100 a
	CT	75 a	100 a
	SL	25 b	100 a
Pi94-4	WT	42 a	56 a
	CT	42 a	56 a
	SL	8 a	44 a
Pi95-7	WT	92 a	100 a
	CT	58 b	78 a
	SL	42 b	44 b
Pi97-2	WT	17 a	67 a
	CT	17 a	67 a
	SL	25 a	22 b
Pi98-1	WT	92 a	100 a
	CT	75 a	56 b
	SL	8 b	33 b
Pi98-2	WT	42 a	100 a
	CT	33 a	100 a
	SL	33 a	89 a

1. WT = wild-type, or CT and SL = sub-cultured 10 times on media amended with 0.0 or 1.0 $\mu g\ ml^{-1}$ dimethomorph, respectively.
2. Percent of leaf disks (out of four) or tubers (out of three) with symptoms and signs of infection by *P. infestans* 96 or 168 hours after inoculation with a sporangia / zoospore suspension or mycelial homogenate, respectively.
3. Means followed by the same letter are not significantly different within each isolate using Fischer's LSD ($\alpha = 0.05$). Comparisons between strains of different isolates are not shown.

on control media strains infected significantly lower numbers of tubers when compared to the corresponding wild-type strains.

Discussion

The development of insensitivity to dimethomorph was demonstrated in *P. infestans* following ethidium bromide / UV light exposure of mycelium. Mutagenesis created strains of two *P. infestans* isolates with resistance factor (RF) values >20. These values are similar to previously reported RF values generated for *P. parasitica* using UV irradiation (21), and *P. capsici* using chemical mutagenesis (141). In comparison, the resulting RF of *P. capsici* for the phenylamide fungicide metalaxyl was >100 in the latter study, and a RF value of 20 for dimethomorph was considered moderate (141). All isolates of *P. infestans* that survived the mutagenesis treatment had reduced growth rates on non-amended media and disrupted colony morphology compared to the wild-type. On non-amended media, both factors could be attributable to 1) mutations unrelated to dimethomorph insensitivity negatively affecting growth or 2) that the dimethomorph insensitivity mechanism(s) themselves disrupted growth. Particularly in the former situation, one might have expected additional fitness reductions,

including virulence (99), as mutations generally reduce the fitness of an organism. The loss of the ability to sporulate supports this idea. Excluding the composition of the cell wall, little is known about the biochemistry involved with cell wall formation in *Phytophthora* (7) and therefore the genetic basis for insensitivity to dimethomorph may be difficult to discern.

The development of insensitivity to dimethomorph was demonstrated in *P. infestans* and other *Phytophthora* species following repeated sub-culturing on media amended with a sub-lethal concentration of dimethomorph. A significant decrease in sensitivity (*in vitro* growth) occurred in three of 11 isolates of *P. infestans* and all isolates of the other *Phytophthora* species examined. Almost all isolates had numerically decreased sensitivity compared to the wild-type. However, the resistance factor for most isolates was less than 3.0. The only strain generated from a distinct sectoring of the colony was formed from the *P. erythroseptica* isolate Pe00-1.

The rapid increase in final colony diameter by the third sub-culture cycle on amended media indicates 1) a physiological adaptation to *in vitro* growth on dimethomorph or 2) selection of the genetic factors responsible for insensitivity following the *de novo* development of nuclear

or cytoplasmic insensitivity. Culturing the isolates on non-amended media prior to the *in vitro* growth sensitivity assessments was used to reduce the effects of physiological adaptation, but without detailed investigation of cell wall composition and formation it is difficult to discern the true cause and should be further investigated.

The EC_{50} metric of *in vitro* colony growth, while commonly used in fungicide sensitivity studies (49,81,141), is sensitive to changes in the growth rate of the fungus because of the percentage inhibition transformation relative to the control colonies. A mutant strain with a reduced growth rate may have the same colony diameter as the wild-type at all concentrations of fungicide and yet the smaller colony diameter on non-amended media results in a lower percent inhibition. Therefore, when calculating the EC_{50} value using percent inhibition versus concentration, a larger EC_{50} is calculated. Similarly, the RF value, being calculated using the EC_{50}, is dependent on both reduced growth rates on non-amended media and to extremely sensitive wild-type isolates. For example, the isolate Pi95-5 had reduced growth on non-amended media for both repeated culturing on dimethomorph amended media and ethidium bromide / UV generated strains and the lowest wild-type EC_{50} of any isolate. The former strain of this

isolate had an EC_{50} that was not significantly different from either the wild-type or repeated culturing on control media strains, and yet had the largest RF value of that strain type for all *P. infestans* isolates. As an alternative to EC_{50}, the growth of an isolate at a pre-determined (discriminating) concentration or inoculation of fungicide treated plant tissue would likely be better indicators of sensitivity, as previously described (120).

The low virulence of several wild-type strains of *P. infestans* on leaf disks was not surprising as variable virulence (85) and pathogenic specialization (94) have been documented, even on potato cultivars with no known R-genes, such as Snowden. Possible reductions in virulence of the strains generated by repeated sub-culturing, regardless of fungicide amendments, was expected as *P. infestans* is known to lose virulence following long-term *in vitro* culturing (46). However, since most isolates sub-cultured on non-amended media retained near wild-type virulence, any reduction in virulence of the strains sub-cultured on dimethomorph amended media was probably caused by the mechanism(s) responsible for dimethomorph insensitivity. *Phytophthora infestans* requires the formation of viable sporangia for pathogenicity on foliar tissue (107), and any putative changes in cell wall formation resulting from the

development of dimethomorph insensitivity might be disruptive to sporangia formation. Additionally, cell wall components are known to induce resistance in potato (50) and it is possible that any changes which occurred as a result of dimethomorph insensitivity allowed for the release of such compounds.

The tuber inoculation test, while not biologically accurate (91), was performed because differences in foliar and tuber susceptibility have been noted (41,43) and may be important. The lack of correlation between the foliar and tuber inoculations is probably related to the differences in the assays in that the tuber inoculation did not require the development of viable sporangia and/or zoospores for infection. Instead, it only required the pathogen to overcome any tuber defenses, and since the periderm was eliminated, the major tuber defense mechanism was removed. *In situ* foliar and tuber infection studies would be required to more fully evaluate this relationship.

The generation of insensitivity to dimethomorph in *Phytophthora* is possible through both repeated selection on sub-lethal amended media and chemical or UV induced mutation. The low amount of insensitivity that developed indicates that resistance may be quantitative and possibly multigenic, as with the dimethylation inhibitor fungicides

commonly used to control true fungi (111). If this hypothesis is true, one would expect resistance in the field to develop through directional selection (112) and occur in small increments. Also, resistance management techniques such as block treatments and co-application of dimethomorph with protectant fungicides would likely be effective. Currently, the development of insensitivity to dimethomorph in *P. infestans* is unlikely for most potato growing regions of the United States because growers rely primarily on protectant fungicide applications and the use of systemic fungicides, including dimethomorph, typically is limited. However, as use of protectant fungicides is restricted because of their larger environmental impact and higher rates required for control in comparison to many systemic fungicides, dimethomorph usage may increase and active resistance management strategies may require implementation.

CHAPTER FOUR

FIELD OPTIMIZATION OF DIMETHOMORPH FOR THE CONTROL OF *PHYTOPHTHORA INFESTANS* ON POTATO: APPLICATION RATE, INTERVAL, AND MIXTURE PARTNERS

Abstract

Aspects concerning the field use of dimethomorph for the control of foliar and tuber potato late blight were examined. When application rate and interval manipulation for a season-long dimethomorph / mancozeb mixture was performed, the rate increasing through the season and 80% of full rate programs had equal final foliar blight control as the full rate program, regardless of interval. The minimum application rate for control equivalent to the full rate program was 1.34 kg ha^{-1} $week^{-1}$. When dimethomorph was tank-mixed with one of three protectant fungicides and integrated into a chlorothalonil-based late blight control program, all programs were as effective as the season-long chlorothalonil program. None of the mixture partners were more effective than the others. When tank-mixed with pyraclostrobin and alternated with chlorothalonil applications, rate reduction to 50% of full rate gave foliar blight control equivalent to a full rate, for a dimethomorph / pyraclostrobin mixture and pyraclostrobin

alone.

Introduction

Chemical control strategies for potato late blight changed with the migration of phenylamide insensitive strains of *Phytophthora infestans* into the United States (61,79). In the early 1990's, several new chemistries were released, including dimethomorph, a cinnamic acid derivative. Dimethomorph was highly selective towards certain members of the Peronosporales (2), had a moderate level of apoplastic systemicity (29), and inhibited many stages of the life cycle of *P. infestans* via the disruption of cell wall formation (90). Dimethomorph was considered to disrupt one or a few biological processes within the target organisms because it fit the characteristics of other systemic fungicides in that it was biologically active at low concentrations (2) and moderate insensitivity could be generated *in vitro* relatively easy (21). To reduce the risk of resistance development, dimethomorph was initially released in the United States for emergency use on potatoes to control *P. infestans* as a pre-mixed wettable powder consisting of 60% mancozeb and 9% dimethomorph by weight. The optimal usage protocols of the dimethomorph / mancozeb mixture for controlling late blight of potatoes on a chipping variety under Michigan growing conditions had

not been completely elucidated. In 2001, dimethomorph was released as a single active ingredient (a.i.) product with a requirement that it must be applied with a fungicide having a different biological mode of action, excluding the phenylamides.

The most common potato late blight fungicide program used by Michigan growers is a chlorothalonil-based program with applications occurring on a five to ten day interval, depending on weather conditions and epidemic development (88). The incorporation of systemic fungicides into the fungicide program is common. Systemic fungicide applications are often timed to correspond to weather-based disease thresholds (6) and specific points within the growing season, such as at tuber initiation and immediately prior to foliar desiccation. The dominance of chlorothalonil over other protectant fungicides in Michigan and the higher cost of the pre-mixed dimethomorph / mancozeb product encouraged the investigation of dimethomorph applications with other mixture partners and at multiple rates within a chlorothalonil program.

Protectant fungicides such as chlorothalonil and mancozeb, two potential mixtures partners for dimethomorph, are considered probable carcinogens by the Environmental Protection Agency (EPA) and their use may eventually be

restricted (62). Such restrictions have expedited the development of alternative chemistries with lower environmental impact. The introduction of fungicides that inhibit the Qo site of the cytochrome bc_1 complex, including the strobilurins (54), offered growers one such alternative. Strobilurins have both high field efficacy when used in a protectant fashion (24) and a high resistance development risk (119). Strict anti-resistance programs have been mandated to eliminate or delay the onset of resistance including: co-application and alternating applications with fungicides having different modes of action, and limiting the rate and number of applications within a season (13). Dimethomorph has a different mode of action than Qo inhibitors (97). Therefore, there is potential to use dimethomorph as a mixture partner for the recently released strobilurin fungicide pyraclostrobin (F500). Rate reduction of a dimethomorph / pyraclostrobin mixture within a protectant-based late blight control program offers an economically feasible putative resistance management strategy by reducing the Qo resistance selective pressure placed on the population. However, efficacious rates are undetermined.

The objectives of this study were to 1) examine application rate and interval manipulation of a

dimethomorph / mancozeb mixture in order to optimize efficacy, 2) compare three non-systemic fungicide mixture partners for dimethomorph when applied at three points within a chlorothalonil based late blight control program, and 3) examine rate reductions of a dimethomorph / pyraclostrobin mixture when alternated with chlorothalonil in order to determine the minimal required for effective late blight control.

Materials and Methods

Experimental Design and Agronomic Management

Cut potato seed (cv. Snowden) was planted at the Michigan State University Muck Soils Experimental Station, Bath, MI, between 3 - 10 June, 1999, 2000, and 2001. Treatments were replicated four times in a randomized complete block design. Inoculation, assessment of foliar late blight, maintenance pesticide application, and harvest dates were similar for all years. Treatments were applied to two-row by 6.10 m plots (90 cm row and 23 cm plant spacing). The two-row plots were separated by a 1.5 m unplanted row. Fungicides were applied with an ATV rear-mounted spray boom (R&D Sprayers, Opelousas, LA, U.S.A.) which traveled at 1 m s^{-1} and delivered 230 l ha^{-1} (3.5 kg cm^{-2} pressure) with three XR11003VS nozzles (Spray Systems,

Pomona, CA, U.S.A.) per row positioned 45 cm above the canopy. Fungicide applications commenced approximately 30 days after planting (DAP) and were applied on a seven-day application interval unless otherwise noted. Plots were irrigated as necessary to maintain canopy and soil moisture conditions conducive to the development of foliar (136) and tuber (91) late blight using turbine rotary garden sprinklers (Gilmour Group, Somerset, PA, U.S.A.) at 1055 l (H_2O) ha^{-1} hr^{-1}. Plots were managed under standard potato agronomic practices for the region (88,124). At 85 and 92 DAP diquat bromide was applied at 0.56 a.i. kg ha^{-1} to desiccate foliage and ease harvesting. Plots were harvested approximately 30 days after the initial desiccant application.

Inoculation

Inoculum for a mixed isolate zoospore suspension of phenylamide-insensitive *P. infestans* US8/A2 genotype (59) was produced by growing previously isolated and characterized isolates on modified rye B agar as described (CHAPTER TWO). Plots were inoculated by injecting the zoospore suspension into the irrigation system 1 x 10^6 zoospores ha^{-1}, 45 - 57 DAP.

Data Collection and Metric Generation

Emergence was evaluated after the majority of tubers had appeared and typically 80% of the seed pieces planted had successfully emerged 21 DAP. It was assumed that missing plants were due to skips during planting, rotted seed tubers, or those lacking eyes. In plots where less than 17 plants had emerged, pre-sprouted tubers were hand planted into the sections lacking sprouts so that all rows had a minimum of 17 plants per row.

Subjective visual evaluations of percentage foliar late blight were made four to five days after inoculation and repeated on a five to seven day interval until almost complete defoliation in untreated plots. The Relative Area Under the Disease Progress Curve (RAUDPC) metric was calculated for all plots using the following formula:

$$RAUDPC = \frac{\sum_{i=0}^{final} (T_i - T_{i-1})(P_{i-1}) + \frac{(T_i - T_{i-1})(P_i - P_{i-1})}{2}}{(T_{final} - T_0)(100)}$$

where T_0 was the day of inoculation (zero point for calculation), T_i was the i^{th} day after inoculation when an estimation of percent foliar late blight was made, T_{final} was the number of days after inoculation at which the final foliar assessment was taken, and P_i was the estimated percent foliar late blight at T_i.

About 115 DAP, one row of each plot was mechanically harvested and sorted into three size classes: culled tubers were <4.0 cm in any plane and dropped back onto the ground, B grade tubers were 4.0 - 6.5, and A grade tubers were >6.5 cm. Tubers from A and B size classes were placed into separate mesh bags and incubated for 14 - 21 days at 15°C / 80% relative humidity to encourage the development of symptoms. Tubers within each size class were weighed, counted, and visually assessed for infection by *P. infestans* by examining the tuber surface and internal tissue for symptoms and/or signs. The number of tubers per plant for each size class was calculated and used for all calculations involving tuber number or yield in order to reduce variability due to differences in the number of plants per plot.

Data Analysis

Analysis of variance (ANOVA) was calculated (Proc GLM - SAS/Stat, SAS Institute, Cary, NC, U.S.A.) at $\alpha = 0.05$ via pair-wise comparisons using Fisher's LSD and treatments were then arranged into statistical groupings. The F Max test (68) was used to determine if data from the repeated trials could be combined.

Fungicide Trials

Four separate trials were conducted: 1) dimethomorph / mancozeb rate manipulation (DMR), 2) dimethomorph / mancozeb rate and interval manipulation (DMRI), 3) dimethomorph mixture partner and rate trial (DMP), and 4) dimethomorph / pyraclostrobin rate trial (DSR). Because of the complexity of fungicide programs, abbreviations were created for treatments to ease discussion. The DMR trial was conducted in 1999 and 2001 and examined season-long rate manipulation for a dimethomorph / mancozeb pre-mixed commercial product. The DMRI trial was conducted in 2000 and 2001 and was a modified version of the DMR trial that incorporates both five and seven day application intervals in addition to different rate manipulations. For both the DMR and DMRI trials, actives were applied using a commercial product consisting of 60% mancozeb and 9% dimethomorph by weight, pre-mixed wettable powder fungicide. The maximum label rate (U.S.A.) for a single application is 1.74 kg ha^{-1} of total actives (2.52 kg ha^{-1} formulated product). The DMP trial was conducted in 2000 and 2001 and examined dimethomorph at two rates when tank-mixed with three different protectant fungicide partners; mancozeb, chlorothalonil, and metiram, within a chlorothalonil season-long late blight control program.

Applications of the dimethomorph / protectant fungicide mixtures occurred corresponding to the primary inoculation event and immediately prior to desiccation. The DSR trial was conducted only in 2001, and examines dimethomorph / pyraclostrobin mixtures at varying rates when alternated with chlorothalonil. Details of all combinations, application frequencies and rates are included in Tables 1-4.

Results

Dimethomorph/Mancozeb Rate Manipulation (DMR)

All experimental factors passed the F Max test and data from different years were combined (data not shown). All fungicide programs, regardless of application rate or schedule had significantly lower (Fischer's LSD, $\alpha = 0.05$) FFLB and RAUDPC values than the untreated control (Table 10a). The FR program had the numerically lowest final percentage of the foliage with late blight lesions (FFLB), but was only significantly lower than the HF and RI programs. All programs had significantly lower RAUDPC than the RI program, but were not different from each other. All programs had significantly higher class A and total yield (metric tons ha^{-1}) than the untreated control, but were not different from each other. Only the FR program

Table 10 (a-b). The effect of increasing, decreasing and maintaining a constant rate of active ingredient of mancozeb / dimethomorph fungicide programs on (a) final foliar late blight, disease progress, yield, (b) infected marketable tubers plant^{-1}, marketable tubers plant^{-1}, and mean mass (g tuber^{-1}) for 1999 and 2001 combined. Statistical comparisons performed using Fischer's LSD (α = 0.05).

Table 10a.

Program Abbreviation[1], Actives[2]: Application Rate (kg ha^{-1}) and Schedule[3]		FFLB[4]		RAUDPC[5]		Yield (metric tons ha^{-1})			
						A[6]		Total	
RD	Man / Dmm: 1.74 (A,B), 1.17 (C,D), 0.57, (E,F), 0.00 (G,H)	57.5	b[7]	0.135	b	25.4	a	28.0	a
RI	Man / Dmm: 0.00 (A,B), 0.57 (C,D), 1.17, (E,F), 1.74 (G,H)	31.5	cd	0.061	c	24.0	a	26.9	a
RP	Man / Dmm: 0.00 (A,H), 0.57 (B,G), 1.17, (C,F), 1.74 (D,E)	27.4	cd	0.048	c	25.3	a	27.7	a
HF	Man / Dmm: 0.87 (A-I)	39.0	c	0.079	c	25.1	a	27.9	a
FR	Man / Dmm: 1.74 (A-I)	25.5	d	0.051	c	25.9	a	28.0	a
UC	H_2O control: 0.00 (A-I)	80.6	a	0.209	a	16.2	b	19.5	b

Table 10b.

Program Abbreviation[1], Actives: Application Rate (kg ha^{-1}) and Schedule		Infected[8] A Tubers Plant^{-1}		Tubers Plant^{-1}				Mean Mass (g Tuber^{-1})			
				B		A		B		A	
RD	Man / Dmm: 1.74 (A,B), 1.17 (C,D), 0.57, (E,F), 0.00 (G,H)	0.05	ab	1.8	abc	6.1	a	42.8	b	121.6	a
RI	Man / Dmm: 0.00 (A,B), 0.57 (C,D), 1.17, (E,F), 1.74 (G,H)	0.04	ab	1.9	ab	5.5	a	42.9	b	129.4	a
RP	Man / Dmm: 0.00 (A,H), 0.57 (B,G), 1.17, (C,F), 1.74 (D,E)	0.04	ab	1.6	bc	6.0	a	45.0	a	127.5	a
HF	Man / Dmm: 0.87 (A-I)	0.02	ab	1.9	ab	6.1	a	44.3	ab	121.3	a
FR	Man / Dmm: 1.74 (A-I)	0.06	a	1.4	c	6.0	a	43.9	ab	128.9	a
UC	H_2O control: 0.00 (A-I)	0.01	b	2.2	a	4.4	b	44.1	ab	109.6	b

1. RD - rate decreasing through season, RI - rate increasing through season, RP - rate peak at mid-season, HF - half of full application rate, FR - full application rate, UC - H_2O control.
2. Man = mancozeb, Dmm = dimethomorph, pre-mixed commercial fungicide indicated by "/". Full application rate was 1.74 kg ha^{-1} combined a.i. of components, mancozeb 60% / dimethomorph 9% (w/w).
3. Application dates: A=3 Jul, B=10 Jul, C=17 Jul, D=24 Jul, E=31 Jul, F=7 Aug, G=14 Aug, H=21 Aug.
4. Final percentage of the foliage with late blight lesions.
5. Relative area under the disease progress curve.
6. A and B grade tubers were >6.5 cm and 4.0 - 6.5 cm in any dimension, respectively.
7. Means followed by the same letter are not significantly different using Fischer's LSD (α = 0.05).
8. Marketable grade tubers showing symptoms of infection by *P. infestans*.

had a significantly higher number of infected A tubers plant^{-1} than the untreated control, while remaining programs were not different (Table 10b). A shift in the number of tubers plant^{-1} within each size class and mean mass (g tuber^{-1}) occurred with fungicide application, as the untreated control had the numerically highest number of B tubers plant^{-1}, and a significantly lower number of A tubers plant^{-1} and mean mass A (g tuber^{-1}), while fungicide programs had less B tubers plant^{-1} and more A tubers plant^{-1}. No significant change occurred in the mean mass of B tubers.

Dimethomorph/Mancozeb Rate and Interval Manipulation (DMRI)

All experimental factors passed the F Max test and data from different years were combined (data not shown). All fungicide programs, regardless of application rate or interval had significantly lower FFLB and RAUDPC values than the untreated control (Table 11a). Programs with a five-day application interval generally had numerically lower FFLB and RAUDPC values than the corresponding seven-day interval programs, but only the L5 program had significantly less FFLB than the corresponding seven-day program (L7).

The untreated control had the numerically lowest class A and total yield, and was significantly lower than all of

Table 11 (a-b). The effect of increasing, decreasing and maintaining a constant rate of active ingredient of mancozeb / dimethomorph fungicide programs with a five or seven day application interval on (a) final foliar late blight, disease progress, yield, (b) infected marketable tubers plant^{-1}, marketable tubers plant^{-1}, and mean mass (g tuber^{-1}) for 2000 and 2001 combined. Statistical comparisons performed using Fischer's LSD (α = 0.05).

Table 11a.

Program Abbreviation[1], Actives[2]: Application Rate (kg ha^{-1}) and Schedule[3]		FFLB[4]		RAUDPC[5]		Yield (metric tons ha^{-1})			
						A[6]		Total	
L5	Man / Dmm: 0.96 (A,B,D,F,G,I,K,L,M,O,Q,R)	24.9	cde[7]	0.055	c	25.8	bcd	27.9	cd
M5	Man / Dmm: 1.36 (A,B,D,F,G,I,K,L,M,O,Q,R)	17.2	e	0.028	de	27.8	abc	30.2	abc
F5	Man / Dmm: 1.74 (A,B,D,F,G,I,K,L,M,O,Q,R)	20.3	de	0.041	cde	32.8	a	35.6	a
U5	Man / Dmm: 0.96 (A,B,D,F), 1.36 (G,I,K,L), 1.74 (M,O,Q,R)	17.8	e	0.042	cde	31.6	ab	34.1	a
D5	Man / Dmm: 1.74 (A,B,D,F), 1.36 (G,I,K,L), 0.96 (M,O,Q,R)	30.2	c	0.054	c	27.5	bc	29.4	bcd
L7	Man / Dmm: 0.96 (A,C,E,H,J,L,N,P,S)	40.1	b	0.082	b	24.2	cd	26.8	cd
M7	Man / Dmm: 1.36 (A,C,E,H,J,L,N,P,S)	23.4	de	0.046	cd	24.1	cd	26.6	cd
F7	Man / Dmm: 1.74 (A,C,E,H,J,L,N,P,S)	18.8	e	0.025	e	23.2	cd	25.6	cd
U7	Man / Dmm: 0.96 (A,C,E), 1.36 (H,J,L), 1.74 (N,P,S)	24.6	cde	0.047	c	28.6	abc	31.3	abc
D7	Man / Dmm: 1.74 (A,C,E), 1.36 (H,J,L), 0.96 (N,P,S)	28.1	cd	0.045	cde	31.3	ab	33.8	ab
C	H_2O control: 0.00 (A,B,D,F,G,I,K,L,M,O,Q,R)	88.5	a	0.278	a	22.1	d	24.8	d

1. L - lowest rate, M - mid-rate, F - full rate, U - rate increasing through season, D - rate decreasing through season, C - H_2O control. Abbreviations followed by a "5" and "7", represent a five or seven day application interval, respectively.
2. Man = mancozeb, Dmm = dimethomorph, pre-mixed commercial fungicide indicated by "/". Full application rate was 1.74 kg ha^{-1} combined a.i. of components, mancozeb 60% / dimethomorph 9% (w/w).
3. Application dates: A=3 Jul, B=8 Jul, C=10 Jul, D=13 Jul, E=17 Jul, F=18 Jul, G=23 Jul, H=24 Jul, I=28 Jul, J=31 Jul, K=2 Aug, L=7 Aug, M=12 Aug, N=14 Aug, O=17 Aug, P=21 Aug, Q=22 Aug, R=27 Aug, S=28 Aug.
4. Final percentage of the foliage with late blight lesions.
5. Relative area under the disease progress curve.
6. A and B grade tubers were >6.5 cm and 4.0 - 6.5 cm in any dimension, respectively.
7. Means followed by the same letter are not significantly different using Fischer's LSD (α = 0.05).

Table 11b.

Program Abbreviation[1], Actives[2]: Application Rate (kg ha^{-1}) and Schedule[3]	Infected[4] A Tubers Plant^{-1}		Tubers Plant^{-1} B[5]		Tubers Plant^{-1} A		Mean Mass (g Tuber^{-1}) B		Mean Mass (g Tuber^{-1}) A	
L5 Man / Dmm: 0.96 (A,B,D,F,G,I,K,L,M,O,Q,R)	0.04	ab[6]	1.5	bc	5.7	c	43.2	b	131.6	bc
M5 Man / Dmm: 1.36 (A,B,D,F,G,I,K,L,M,O,Q,R)	0.03	ab	1.6	abc	6.7	ab	43.7	b	123.3	cd
F5 Man / Dmm: 1.74 (A,B,D,F,G,I,K,L,M,O,Q,R)	0.05	a	2.0	a	7.0	a	41.6	b	136.5	ab
U5 Man / Dmm: 0.96 (A,B,D,F), 1.36 (G,I,K,L), 1.74 (M,O,Q,R)	0.00	b	1.9	ab	6.6	abc	39.7	b	139.6	a
D5 Man / Dmm: 1.74 (A,B,D,F), 1.36 (G,I,K,L), 0.96 (M,O,Q,R)	0.04	ab	1.3	c	5.9	bc	54.8	a	140.1	a
L7 Man / Dmm: 0.96 (A,C,E,H,J,L,N,P,S)	0.04	ab	1.9	a	5.8	bc	40.4	b	124.9	cd
M7 Man / Dmm: 1.36 (A,C,E,H,J,L,N,P,S)	0.05	a	1.5	abc	5.6	c	56.9	a	133.5	ab
F7 Man / Dmm: 1.74 (A,C,E,H,J,L,N,P,S)	0.00	b	1.7	abc	5.5	c	40.0	b	128.4	bc d
U7 Man / Dmm: 0.96 (A,C,E), 1.36 (H,J,L), 1.74 (N,P,S)	0.00	b	1.9	a	6.4	abc	41.5	b	132.1	bc
D7 Man / Dmm: 1.74 (A,C,E), 1.36 (H,J,L), 0.96 (N,P,S)	0.00	b	1.8	abc	6.8	ab	41.4	b	134.4	ab
C H_2O control: 0.00 (A,B,D,F,G,I,K,L,M,O,Q,R)	0.06	a	1.9	a	5.6	c	41.5	b	117.6	d

1. L - lowest rate, M - mid-rate, F - full rate, U - rate increasing through season, D - rate decreasing through season, C - H_2O control. Abbreviations followed by a "5" and "7", represent a five or seven day application interval, respectively.
2. Man = mancozeb, Dmm = dimethomorph, pre-mixed commercial fungicide indicated by "/". Full application rate was 1.74 kg ha^{-1} combined a.i. of both components, mancozeb 60% / dimethomorph 9% (w/w).
3. Application dates: A=3 Jul, B=8 Jul, C=10 Jul, D=13 Jul, E=17 Jul, F=18 Jul, G=23 Jul, H=24 Jul, I=28 Jul, J=31 Jul, K=2 Aug, L=7 Aug, M=12 Aug, N=14 Aug, O=17 Aug, P=21 Aug, Q=22 Aug, R=27 Aug, S=28 Aug.
4. Marketable grade tubers showing symptoms of infection by *P. infestans*.
5. A and B grade tubers were >6.5 cm and 4.0 - 6.5 cm in any dimension, respectively.
6. Means followed by the same letter are not significantly different using Fischer's LSD (α = 0.05).

the five-day interval programs except L5 (Table 11a). The F5 program had the numerically highest class A and total yield, and was significantly higher than most seven-day interval programs. The five-day interval programs generally had significantly higher infected class A tubers plant^{-1} than the corresponding seven-day programs (Table 11b). The F5 program had the numerically highest class A and B tubers plant^{-1}, while remaining programs were typically not different from the untreated control for both size classes. None of the programs had significantly higher mean mass B (g tuber^{-1}) than untreated control, except the D5 and M7 programs. All programs had significantly higher mean mass A (g tuber^{-1}) than the untreated control except the M5, L7, and F7 programs.

Dimethomorph Mixture Partner and Rate Manipulation (DMP)

All experimental factors passed the F Max test and data from different years were combined (data not shown). All fungicide programs, regardless of fungicide combination had significantly lower FFLB and RAUDPC values than the untreated control (Table 12a). Fungicide programs did not differ significantly from each other for FFLB. Numerically, the fungicide programs consisting of three chlorothalonil + dimethomorph applications within a season-

Table 12 (a-b). The effect of dimethomorph at two rates when tank-mixed with chlorothalonil, mancozeb, or metiram and incorporated into a season-long chlorothalonil program on (a) final foliar late blight, disease progress, yield, (b) infected marketable tubers plant^{-1}, marketable tubers plant^{-1}, and mean mass (g tuber^{-1}) for 2000 and 2001 combined. Statistical comparisons performed using Fischer's LSD (α = 0.05).

Table 12a.

Program Abbreviation[1], Actives: Application Rate (kg ha^{-1}) and Schedule[2]		FFLB[3]		RAUDPC[4]		Yield (metric tons ha^{-1})			
						A[5]		Total	
CHL	Chl: 0.63 (A,B), Chl: 1.13 (C-I).....	38.8	b[6]	0.068	bc	22.4	ab	26.8	ab
MED1	Chl: 0.63 (A,B), Chl: 1.13 (C,F,G,H), Met: 1.68 + Dmm: 0.15 (D,E,I).....	38.6	b	0.064	bc	22.0	ab	26.0	abc
MED2	Chl: 0.63 (A,B), Chl: 1.13 (C,F,G,H), Met: 1.68 + Dmm: 0.22 (D,E,I).....	50.5	b	0.079	bc	20.5	bc	24.8	bcd
CHLD1	Chl: 0.63 (A,B), Chl: 1.13 (C,F,G,H), Chl: 1.13 + Dmm: 0.15 (D,E,I).....	38.0	b	0.059	c	20.2	bc	24.6	bcd
CHLD2	Chl: 0.63 (A,B), Chl: 1.13 (C,F,G,H), Chl: 1.13 + Dmm: 0.22 (D,E,I).....	40.0	b	0.059	bc	21.9	b	25.7	bc
MAD1	Chl: 0.63 (A,B), Chl: 1.13 (C,F,G,H), Man: 1.68 + Dmm: 0.15 (D,E,I).....	48.8	b	0.076	bc	23.0	a	26.8	a
MAD2	Chl: 0.63 (A,B), Chl: 1.13 (C,F,G,H), Man: 1.68 + Dmm: 0.22 (D,E,I).....	43.5	b	0.082	b	19.8	bc	23.2	cd
CTL	H_2O control: 0.00 (A-I).....	89.6	a	0.212	a	18.1	c	21.8	d

1. CHL - Chlorothalonil season-long, CTL - H_2O control, remaining programs are incorporated into a season-long chlorothalonil program and include; ME - Metiram, MA - Mancozeb, or CHL with D1 or D2 - Dimethomorph at low or full rate, respectively. Actives separated with a "+" are tank mixed.
2. Application dates: A=3 Jul, B=10 Jul, C=17 Jul, D=24 Jul, E=31 Jul, F=7 Aug, G=14 Aug, H=21 Aug, I=28 Aug.
3. Final percentage of the Foliage with Late Blight lesions.
4. Relative Area Under the Disease Progress Curve.
5. A and B grade tubers were >6.5 cm and 4.0 - 6.5 cm in any dimension, respectively.
6. Means followed by the same letter are not significantly different using Fischer's LSD (α = 0.05).

Table 12b.

Program Abbreviation[1], Actives: Application Rate (kg ha^{-1}) and Schedule[2]		Infected[3] A Tubers Plant^{-1}		Tubers Plant^{-1}				Mean Mass (g Tuber^{-1})			
				B[4]		A		B		A	
CHL	Chl: 0.63 (A,B), Chl: 1.13 (C-I).....	0.21	a[5]	3.0	a	6.6	a	43.4	a	100.0	b
MED1	Chl: 0.63 (A,B), Chl: 1.13 (C,F,G,H), Met: 1.68 + Dmm: 0.15 (D,E,I).....	0.19	ab	2.8	ab	5.9	ab	43.9	a	109.5	a
MED2	Chl: 0.63 (A,B), Chl: 1.13 (C,F,G,H), Met: 1.68 + Dmm: 0.22 (D,E,I).....	0.15	ab	2.9	ab	5.8	ab	43.5	a	107.3	b
CHLD1	Chl: 0.63 (A,B), Chl: 1.13 (C,F,G,H), Chl: 1.13 + Dmm: 0.15 (D,E,I).....	0.09	b	2.4	b	5.9	ab	43.7	a	101.2	b
CHLD2	Chl: 0.63 (A,B), Chl: 1.13 (C,F,G,H), Chl: 1.13 + Dmm: 0.22 (D,E,I).....	0.14	ab	2.7	ab	5.8	ab	42.7	ab	117.1	a
MAD1	Chl: 0.63 (A,B), Chl: 1.13 (C,F,G,H), Man: 1.68 + Dmm: 0.15 (D,E,I).....	0.17	ab	2.7	ab	6.2	a	41.7	ab	109.4	ab
MAD2	Chl: 0.63 (A,B), Chl: 1.13 (C,F,G,H), Man: 1.68 + Dmm: 0.22 (D,E,I).....	0.09	b	2.4	b	5.8	ab	42.0	ab	102.3	b
CTL	H_2O control: 0.00 (A-I).....	0.12	ab	2.7	ab	5.4	b	41.0	b	102.3	b

1. CHL - Chlorothalonil season-long, CTL - H_2O control, remaining programs are incorporated into a season-long chlorothalonil program and include; ME - Metiram, MA - Mancozeb, or CHL with D1 or D2 - Dimethomorph at low or full rate, respectively. Actives separated with a "+" are tank mixed.
2. Application dates: A=3 Jul, B=10 Jul, C=17 Jul, D=24 Jul, E=31 Jul, F=7 Aug, G=14 Aug, H=21 Aug, I=28 Aug.
3. Marketable grade tubers showing symptoms of infection by *P. infestans*.
4. A and B grade tubers were >6.5 cm and 4.0 - 6.5 cm in any dimension, respectively.
5. Means followed by the same letter are not significantly different using Fischer's LSD (α = 0.05).

long chlorothalonil program (CLD1 and CLD2) had the numerically lowest RAUDPC values, regardless of dimethomorph rate. The untreated control had the numerically lowest class A and total yield (metric tons ha^{-1}) and was significantly lower than all programs except the MED1, CLD1, and MAD2. The CHL program had the numerically highest number of infected A tubers $plant^{-1}$, but was not significantly higher than any treatment except the CLD1 and MAD2 programs (Table 12b). No distinct trends were apparent in the number of A and B tubers $plant^{-1}$ or mean mass A and B (g $tuber^{-1}$). None of the replacement programs were more effective at controlling foliar late blight or resulted in higher yield than the season-long chlorothalonil program (CHL). Of the dimethomorph mixture partners, chlorothalonil was only numerically superior to mancozeb and metiram at controlling foliar late blight but no yield differences were apparent. No distinct trends were present in terms of foliar late blight or yield between the two rates of dimethomorph.

Dimethomorph/Pyraclostrobin Rate Manipulation (DSR)

All fungicide programs had significantly lower FFLB and RAUDPC than the untreated control (Table 13a). Numerical rate response trends in FFLB were measured for

Table 13 (a-b). The effect of dimethomorph and pyraclostrobin at varying rates when incorporated into a season-long chlorothalonil program on (a) final foliar late blight, disease progress, yield, (b) infected marketable tubers plant^{-1}, marketable tubers plant^{-1}, and mean mass (g tuber^{-1}) for 2001. Statistical comparisons performed using Fischer's LSD (α = 0.05).

Table 13a.

Program Abbreviation[1], Actives: Application Rate (kg ha^{-1}) and Schedule[2]		FFLB[3]		RAUDPC[4]		Yield (metric tons ha^{-1})			
						A[5]		Total	
CHL	Chl: 0.63 (A), Chl: 1.13 (B-I).....	31.3	d[6]	0.057	b	30.1	a	31.3	a
PD25	Chl: 0.63 (A), Chl: 1.13 (C,E,G,I) P: 0.04 + D: 0.06 (B,D,F,H).....	55.0	b	0.101	b	26.5	a	28.7	ab
PD50	Chl: 0.63 (A), Chl: 1.13 (C,E,G,I) P: 0.08 + D: 0.11 (B,D,F,H).....	50.0	bc	0.086	b	28.2	a	30.3	ab
PD75	Chl: 0.63 (A), Chl: 1.13 (C,E,G,I) P: 0.12 + D: 0.17 (B,D,F,H).....	40.5	bcd	0.080	b	23.3	ab	26.3	ab
PD100	Chl: 0.63 (A), Chl: 1.13 (C,E,G,I) P: 0.17 + D: 0.22 (B,D,F,H).....	38.8	bcd	0.062	b	29.0	a	31.0	a
P25	Chl: 0.63 (A), Chl: 1.13 (C,E,G,I) P: 0.04 (B,D,F,H).....	55.0	b	0.093	b	24.9	ab	27.2	ab
P50	Chl: 0.63 (A), Chl: 1.13 (C,E,G,I) P: 0.08 (B,D,F,H).....	50.0	bc	0.098	b	26.3	a	29.1	ab
P75	Chl: 0.63 (A), Chl: 1.13 (C,E,G,I) P: 0.12 (B,D,F,H).....	45.0	bcd	0.096	b	25.7	ab	27.4	ab
P100	Chl: 0.63 (A), Chl: 1.13 (C,E,G,I) P: 0.17 (B,D,F,H).....	33.8	cd	0.063	b	26.5	a	29.2	ab
AZO	Chl: 0.63 (A), Chl: 1.13 (C,E,G,I) Azo: 0.28 (B,D,F,H).....	32.5	cd	0.061	b	25.8	ab	28.0	ab
CTL	H_2O control: 0.00 (A-I).....	85.8	a	0.220	a	18.0	b	21.8	b

1. CHL - Chlorothalonil season-long, CTL - H_2O control, remaining programs are alternated with CHL; PD25, PD50, PD75, and PD100 - Pyraclostrobin + Dimethomorph at 25%, 50%, 75%, and 100% of the full label rates, P25, P50, P75, and P100 - Pyraclostrobin at 25%, 50%, 75%, and 100% of the full label rate, and AZO - Azoxystrobin. Actives separated with a "+" are tank mixed.
2. Application dates: A=3 Jul, B=10 Jul, C=17 Jul, D=24 Jul, E=31 Jul, F=7 Aug, G=14 Aug, H=21 Aug, I=28 Aug.
3. Final percentage of the Foliage with Late Blight lesions.
4. Relative Area Under the Disease Progress Curve.
5. A and B grade tubers were >6.5 cm and 4.0 - 6.5 cm in any dimension, respectively.
6. Means followed by the same letter are not significantly different using Fischer's LSD (α = 0.05).

Table 13b.

Program Abbreviation[1], Actives: Application Rate (kg ha^{-1}) and Schedule[2]		Infected[3] A Tubers Plant^{-1}		Tubers Plant^{-1}				Mean Mass (g Tuber^{-1})			
				B[4]		A		B		A	
CHL	Chl: 0.63 (A), Chl: 1.13 (B-I).....	0.03	ab[5]	2.0	ab	6.9	ab	41.2	ab	133.6	a
PD25	Chl: 0.63 (A), Chl: 1.13 (C,E,G,I) P: 0.04 + D: 0.06 (B,D,F,H).....	0.04	a	1.6	b	7.2	a	42.4	ab	111.2	ab
PD50	Chl: 0.63 (A), Chl: 1.13 (C,E,G,I) P: 0.08 + D: 0.11 (B,D,F,H).....	0.00	b	1.3	b	6.3	ab	46.9	a	133.4	ab
PD75	Chl: 0.63 (A), Chl: 1.13 (C,E,G,I) P: 0.12 + D: 0.17 (B,D,F,H).....	0.01	ab	2.0	ab	6.1	ab	45.1	a	113.5	ab
PD100	Chl: 0.63 (A), Chl: 1.13 (C,E,G,I) P: 0.17 + D: 0.22 (B,D,F,H).....	0.03	ab	1.4	b	7.0	a	43.3	ab	122.7	ab
P25	Chl: 0.63 (A), Chl: 1.13 (C,E,G,I) P: 0.04 (B,D,F,H).....	0.01	ab	1.6	b	6.0	ab	42.2	ab	122.1	ab
P50	Chl: 0.63 (A), Chl: 1.13 (C,E,G,I) P: 0.08 (B,D,F,H).....	0.00	b	1.9	ab	6.9	ab	42.6	ab	113.3	ab
P75	Chl: 0.63 (A), Chl: 1.13 (C,E,G,I) P: 0.12 (B,D,F,H).....	0.00	b	1.3	b	6.6	ab	39.1	b	115.6	ab
P100	Chl: 0.63 (A), Chl: 1.13 (C,E,G,I) P: 0.17 (B,D,F,H).....	0.00	b	1.9	ab	6.8	ab	44.8	a	114.9	ab
AZO	Chl: 0.63 (A), Chl: 1.13 (C,E,G,I) Azo: 0.28 (B,D,F,H).....	0.01	ab	1.6	b	6.6	ab	42.2	ab	116.1	ab
CTL	H_2O control: 0.00 (A-I).....	0.00	b	2.6	a	5.1	b	43.1	ab	105.4	b

1. CHL - Chlorothalonil season-long, CTL - H_2O control, remaining programs are alternated with CHL; PD25, PD50, PD75, and PD100 - Pyraclostrobin + Dimethomorph at 25%, 50%, 75%, and 100% of the full label rates, P25, P50, P75, and P100 - Pyraclostrobin at 25%, 50%, 75%, and 100% of the full label rate, and AZO - Azoxystrobin. Actives separated with a "+" are tank mixed.
2. Application dates: A=3 Jul, B=10 Jul, C=17 Jul, D=24 Jul, E=31 Jul, F=7 Aug, G=14 Aug, H=21 Aug, I=28 Aug.
3. Marketable grade tubers showing symptoms of infection by *P. infestans*.
4. A and B grade tubers were >6.5 cm and 4.0 - 6.5 cm in any dimension, respectively.
5. Means followed by the same letter are not significantly different using Fischer's LSD (α = 0.05).

both the pyraclostrobin alone and pyraclostrobin + dimethomorph mixture alternated with chlorothalonil; such that increasing the fungicide rate decreased the level of FFLB, but few significant differences existed between programs. The addition of dimethomorph to pyraclostrobin in a chlorothalonil alternating program did not result in increased foliar late blight control in comparison to pyraclostrobin alone, regardless of rate. The untreated control and season-long chlorothalonil programs had the numerically lowest and highest yields, respectively but other yield trends were not measured. The PD25 program had the numerically highest number of infected A tubers plant^{-1}, but no distinct trends were apparent (Table 13b). A shift in the number of tubers per plant in each size class was measured, with the untreated control having the numerically highest and lowest number of B and A tubers plant^{-1}, respectively. The untreated control also had the numerically lowest mean mass A (g tuber^{-1}) value, and was significantly lower than the CHL program. Significant differences between treatments for the number of A and B tubers plant^{-1}, and the mean mass (g tuber^{-1}), in both size classes, were not indicative of any trends.

Discussion

The optimal dimethomorph / mancozeb pre-mixed fungicide application rate and interval program for controlling foliar potato late blight was a minimum rate of 1.36 kg ha^{-1} product applied on either a five or seven-day application interval. With a constant application rate, control of foliar late blight with this mixture was dependent on the amount of fungicide applied per week and not the application interval. Thus, the 0.96 kg ha^{-1} five-day interval program (1.34 kg ha^{-1} $week^{-1}$) had equal efficacy as the 1.36 kg ha^{-1} $week^{-1}$ constant rate program on a seven-day interval. Either application interval would be sufficient at controlling foliar late blight at this rate. Results for application intervals beyond seven days should not be extrapolated from these results.

Rate manipulation may be used to reduce fungicide costs with the dimethomorph / mancozeb product. Initiating the fungicide program at a low rate and increasing the rate as the epidemic developed, would be preferred over trying the halt the epidemic prior to initiation with a high rate. The rate reducing dimethomorph / mancozeb programs were typically less effective than constant or increasing rate programs with an equal mean application rate active $week^{-1}$. This is slightly counter to conventional protectant

application practice where a high rate of fungicide is applied to the foliage prior to infection in an attempt to control the epidemic by limiting initial infections. In an isolated situation in which additional inoculum from outside sources was absent, it is possible that the initial full rate of application (1.74 kg ha^{-1} a.i.) could have successfully hindered the initiation of the epidemic. Such an epidemic would possibly be controlled later in the season with reduced rates. However, since additional inoculum from neighboring plots was present, it is impossible to determine if this was the case. Alternatively, in polycyclic epidemics, such as potato late blight, small numbers of successful infections could be sufficient to establish an epidemic later in the season when fungicide application rates were declining, resulting in more severe foliar blight.

The co-application of dimethomorph and one of three protectant fungicides or pyraclostrobin within a chlorothalonil-based late blight foliar fungicide program did not alter foliar efficacy, yield, or tuber infection in comparison to a chlorothalonil only program. In the dimethomorph mixture partner and rate trial, the use of replacement fungicide mixtures represented only 33% of the total applications. Such a small proportion may have not

had a large enough affect on the epidemic to discern any significant increase in foliar late blight control over a chlorothalonil only program. Alternatively, as with the dimethomorph / pyraclostrobin trial, dimethomorph may not have added to measurable foliar or tuber late blight control.

The lack of additional field efficacy with the co-application of dimethomorph is contrary to much of the results obtained from controlled environment studies, but had been noted a previous field study (124). Substantial inhibition of infection by *P. infestans* and symptom development on potato occurred at concentrations of 250 µg ml^{-1} (29). This concentration is lower than the standard full dimethomorph rate (U.S.) of 0.22 kg ha^{-1} in a carrier volume of 94.63 l ha^{-1} (958.7 µg ml^{-1}). Several possible contributory factors exist: incomplete foliar coverage, high affinity for the cuticle and low solubility in H_2O, environmental degradation, washing-off, metabolism by the plant, and dilution through foliar expansion or systemic movement could reduce fungicide concentration below levels that results in measurable biological activity. Regardless of the mechanism, increasing the application rate of dimethomorph and/or the distribution efficiency could possibly offer additional foliar blight control.

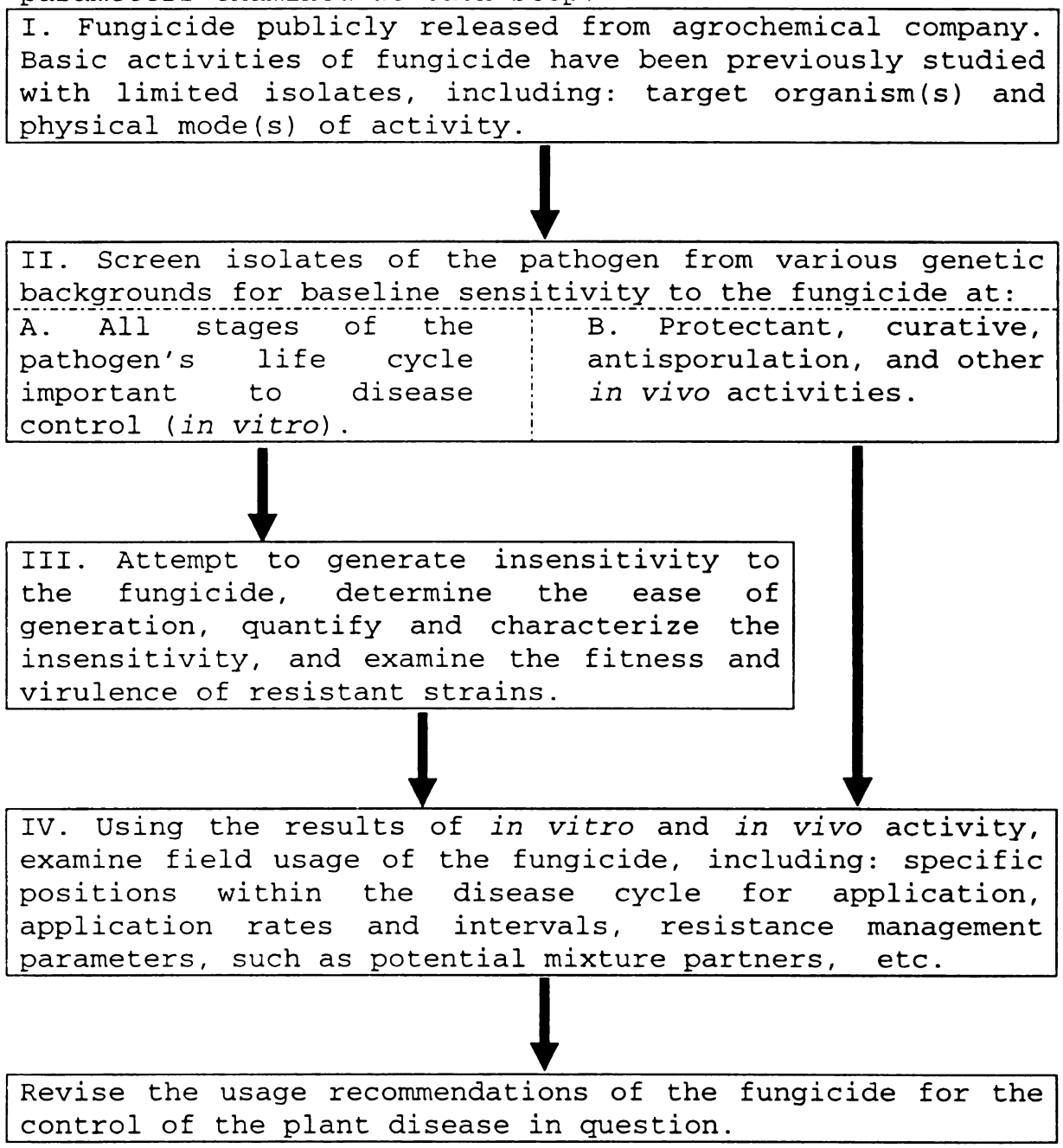
CHAPTER FIVE

SUMMARY AND CONCLUSIONS

The fungicide dimethomorph was released in response to the migration of phenylamide insensitive genotypes of *Phytophthora infestans* and to control other Oomycete plant pathogens. However, at the initiation of this research project, relatively little had been published concerning the efficacy of dimethomorph on potato late blight. When limited research is available for a fungicide, a series of steps can be followed (Figure 8) to optimize field efficacy.

Previous research examining the effects of dimethomorph on *P. infestans* concluded that dimethomorph was highly active under controlled environment conditions and inhibitory to all stages of the asexual life cycle except zoosporogenesis (1,2,29). However, the range of sensitivity of *P. infestans* isolates from various genetic backgrounds was essentially unknown. Also, the generation of moderate insensitivity in another *Phytophthora* species to dimethomorph had been demonstrated (21), but information for *P. infestans* was not available at the time. Finally, dimethomorph was released in the United States with mancozeb as a pre-formulated mixture partner at a specific

Figure 8. Diagram of the general steps required from the public release of a novel fungicide to the optimization of the commercial product under field conditions, and the parameters examined at each step.



rate and alternative mixture partners and rate reduction information was not available.

This research project initially examined the baseline sensitivity of isolates from various genetic backgrounds to dimethomorph at all stages of the *P. infestans* asexual life cycle and the protectant, curative, and antisporulation activity when applied to potato leaf disks. The EC_{50} values calculated for each stage of the asexual life cycle were similar to those previously reported and the variability of the *P. infestans* isolates examined was similar to those reported for other Oomycete fungicides. Under *in vitro* conditions, the stages responsible for infection by *Phytophthora* species were all highly sensitive to dimethomorph. However, when applied to leaf disks, much higher concentrations of dimethomorph were required for inhibition of symptom development, and little curative activity was found. The difference between the required concentrations is probably a result of the affinity of dimethomorph to the leaf cuticle and the low water solubility and systemicity of dimethomorph in the leaf. Antisporulation activity was present, but lower than that previously reported. In general, concentrations higher than 1000.0 $\mu g\ ml^{-1}$ should be used under field conditions to control potato late blight.

Next, the generation of insensitivity of dimethomorph in *P. infestans* isolates from various genetic backgrounds was attempted. Ethidium bromide / UV mutagenesis was more successful in generating strains with moderate levels of insensitivity than repeated culturing on sub-lethal media. In both cases, the saprophytic fitness of the insensitive strains was generally less than those of the wild-type. Additionally, the virulence of the most insensitive strains generated from repeated culturing was reduced from that of the wild-type on both leaf disks and in whole tubers. The generation of dimethomorph insensitive strains was possible, but the level of insensitivity was low and the resulting strains typically were less virulent. With the currently limited use of dimethomorph for the control of potato late blight in most potato growing regions of the United States and the negative aspects associated with insensitivity, it is unlikely that wide-spread field insensitivity will occur.

Finally, the pre-mixed dimethomorph / mancozeb commercial product and alternative mixture partners for dimethomorph were examined. Application rate and interval manipulation was possible with the pre-mixed dimethomorph / mancozeb product. The examination of such a fungicide program in conjunction with weather-based late blight

forecasting systems could allow for a reduction in overall fungicide use. In general, dimethomorph at the full label rate (U.S.A.) of 0.22 kg ha^{-1} did not measurably increase the efficacy of the mixture partners examined, when applied in the absence of dimethomorph.

The high activity of dimethomorph under controlled conditions supports the use for potato late blight control. However, until the field efficacy of dimethomorph can be increased, use will remain limited. Increasing the application rate and possibly the efficiency of crop coverage, will likely increase field efficacy. Dimethomorph has potential for use in resistance management schemes with other single mode of action fungicides, such as the strobilurins. Future restrictions on the use of multi-site fungicides with high environmental impact may encourage the changes in the label rate of dimethomorph and allow for the fungicide to be more effective in the control of potato late blight.

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