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AN INVESTIGATION OF PHYTOPHTHORA CAPSICI ON VEGETABLE HOSTS IN MICHIGAN: SURVIVAL, SPREAD, AND RESPONSE TO THE PHENYLAMIDE FUNGICIDE MEFENOXAM

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AN INVESTIGATION OF *PHYTOPHTHORA CAPSICI* ON VEGETABLE HOSTS IN MICHIGAN: SURVIVAL, SPREAD, AND RESPONSE TO THE PHENYLAMIDE FUNGICIDE MEFENOXAM.

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ABSTRACT

AN INVESTIGATION OF *PHYTOPHTHORA CAPSICI* ON VEGETABLE HOSTS IN MICHIGAN: SURVIVAL, SPREAD, AND RESPONSE TO THE PHENYLAMIDE FUNGICIDE MEFENOXAM.

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The incidence of root, crown, and fruit rot of pumpkins, squash, tomatoes, and peppers caused by *Phytophthora capsici* Leonian has increased during the last two decades in Michigan vegetable production fields. Currently recommended control strategies include the use of well drained fields, crop rotation, and the use of preventative fungicides. Growers employing all available management strategies have sustained significant losses and, at the behest of the cucurbit industry, an intensive investigation of the life history of *P. capsici* throughout Michigan was initiated. The primary objective of this study was to ascertain whether or not the sexual stage is active in natural populations and, if so, to determine what effect this may have on the survival and evolution of naturally occurring populations. The sensitivity to mefenoxam and compatibility type were assayed in natural populations. In 1997 and 1998, 523 isolates of *P. capsici* were recovered from infested fields throughout the state. In vitro crosses between sensitive and insensitive isolates from diverse locations indicated that mefenoxam insensitivity is inherited as a single incompletely dominant gene unlinked to compatibility type. All six possible mefenoxam sensitivity/compatibility type combinations were recovered from a single field and both mating types were recovered from every field sampled. Oospores were observed in diseased host tissue from four locations and all six phenotypic combinations were found in 223 oospore progeny recovered from a single naturally infected cucumber fruit. In 1999 and 2000, a single field from which only intermediately or fully insensitive *P. capsici* isolates were recovered in 1998 was sampled intensively in the absence of mefenoxam selection pressure. Isolates from 1998 and 1999 were analyzed using fluorescently labeled amplified fragment length polymorphism (AFLP) markers. Clonal reproduction was significant within a single season but no clones were recovered between years. Approximately fifty percent of the AFLP markers were polymorphic and 199 of the 263 isolates analyzed had unique multi-locus AFLP genotypes. Furthermore, the frequency of individual AFLP markers remained stable between years and mefenoxam insensitivity did not decrease over time. AFLP markers were then used to characterize an additional 383 isolates from 6 populations of *P. capsici* at locations ranging from 1 to >200 km distant. A similarly high level of genotypic and gene diversity was recorded in every population investigated. Cluster analysis indicated discrete clusters based on location with no blurring of the groupings based on the year of sampling. The overall picture presented by these results suggests that outcrossing occurs frequently in populations of *P. capsici*, that populations are large enough to withstand dramatic effects of genetic drift, and that migration between locations appears to be rare. Sexual recombination appears to have played a significant role in the integration of mefenoxam insensitivity into populations under mefenoxam selection pressure and there is no evidence that the frequency of mefenoxam insensitivity will decrease once selection pressure is removed.

To my parents, Thomas and Sharon Lamour,

whose love, patience, and humor

I cherish deeply.

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for giving me a dynamic growing love

which words could never capture.

To Iris Gloria Lamour,

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who teaches me daily

to smile.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LITERATURE REVIEW

Phytophthora capsici was first described by Leon H. Leonian in 1922 (23). Leonian reported considerable damage to chili pepper plants by a novel species of *Phytophthora* in the fall of 1918 on the farm of the New Mexico Experiment Station. In 1919, the same disease reappeared in this and surrounding farms. The initial in vitro characterization describes a highly torturous hyphal growth morphology, papillate sporangia, and suggests that oospores are formed in single cultures. The modern description of *P. capsici* as a species falls into Waterhouses Group II (39) and is characterized by sporangia that are conspicuously papillate with amphigynous oospores generally forming only when A1 and A2 mating types are paired. The first literature citing of *P. capsici* on a cucurbit host was reported by W. A. Kreutzer in 1937 (21). Kreutzer reports that disease was confined to an 8-acre cucumber field with 100 percent of the cucumber fruits infected (21). By 1940, in addition to pepper, *P. capsici* had been described on eggplant, honeydew melon fruit, summer squash, and tomato fruit (22, 40). During the thirty years following these initial reports there are occasional entries into the literature describing *P. capsici* on additional cucurbit hosts as well as a range of more exotic hosts (12), but overall there is little new information published (6, 38).

Information concerning the different spore types produced by members of the genus *Phytophthora* accumulated slowly between 1940 and 1970. In 1970, Waterhouse provided a useful, and still used, key for identifying isolates to species based on the determination of whether or not an isolate could produce oospores in single culture and the morphology of sporangia and oospores (39). Research with other *Phytophthora* species established much of what is known about the three dominant spore types produced by *P. capsici* (12). The thallus is composed of coenocytic mycelium which may give rise to lemon-shaped sporangia born on long caducous pedicels (1). When sporangia are immersed in free water they differentiate to produce 20-40 bi-motile swimming zoospores (2). Zoospores exhibit negative geotropism and chemotactically follow nutrient gradients while swimming (12). Once zoospores contact the plant surface they encyst and germinate to produce germ tubes (17). Penetration of leaf surfaces by *P. capsici* has been shown to occur directly and through natural openings such as stomata (19). *Phytophthora capsici* produces an extracellular macerating enzyme which likely plays a significant role in breaching the host epidermis and ramifying through susceptible host tissue (41). Approximately half of the sixty recognized species of *Phytophthora* are self-fertile (homothallic) while the other half, including *P. capsici*, generally produce oospores only when both mating types are present (heterothallic) (16). Oospores are formed when A1 and A2 compatibility types come into close association (20). Each of the parent isolates make both male (antheridium) and female (oogonium) gametangia once the sexual stage has been initiated and self-fertilization is possible in obligately outcrossing species (20).

The asexual sporangia and zoospores proved to be much easier to manipulate and study than the oospore and it is not surprising that the salient features of these spore types were outlined relatively early (17, 25). The main impediment to detailed studies of oospores and the inherent genetics therein was due primarily to difficulties in separating and germinating the oospores (36). In 1968, Satour and Butler provide crucial information concerning the generation and germination of *P. capsici* oospores (34). They report that relatively young oospores produced in paired cultures of *P. capsici* germinated to produce recombinant progeny after 30 days incubation. Prior to this it was generally thought that 6-9 month incubation periods were necessary for oospore germination. The progeny from their crosses were shown to differ from the parental types in both morphology and pathogenicity. Significantly, one progeny isolate exhibited increased virulence on pepper compared to either of the parents. A number of important milestones were reached in this investigation. A simple method for the production, germination, and harvesting of oospore progeny for *P. capsici* was formally presented and the authors convincingly argued that proper media containing ample nutrients as well as genetically compatible parent isolates are required for successful matings. In addition, this work provided convincing evidence for the potential role of oospores in generating genetic variation (34). In 1971, Polach and Webster corroborated this finding using the oospore techniques recently described (28). They investigated 391 single oospore progeny from four mating reactions and report that the parent isolates differed in their pathogenicity to cucurbit and solanaceous hosts and that segregation and recombination were observed for all the characters studied.

Early investigators recognized that the genus *Phytophthora* exhibited some striking dissimilarities to many other fungal organisms, but a full resolution of its taxonomic and evolutionary standing would not be made until DNA sequence analysis was completed by Forster et al. in 1990. They found that oomycetes are more closely related to heterokont photosynthetic algae than to members of the kingdom Fungi (13). Significant investigations into the genetics of *P. capsici* do not appear in the literature until the late 1980's and early 1990's when isozyme and restriction fragment length polymorphism (RFLP) analysis of both mitochondreal and nuclear DNA were conducted on isolates from widely different geographical locations, years, and hosts located in a worldwide *Phytophthora* culture collection at the University of California at Riverside (14, 24, 26). An isozyme study involving 113 *P. capsici* isolates was interpreted as revealing two subgroups within the *P. capsici* species (24). Subgroups are defined as being significantly different based on sporangia morphology and ontogeny. RFLP investigation of mitochondreal DNA revealed no patterns of similarity based on host or geographical location (14). RFLP analysis of nuclear DNA's using low copy number probes of fifteen *P. capsici* isolates indicated nuclear DNA diversity was high (14). There are no published studies of isozyme or DNA level diversity within single populations of *P. capsici*.

In 1981, the presence of A1 and A2 isolates of *P. capsici* from single fields was reported in New Jersey (27). The investigators provide the only report of naturally occurring *P. capsici* oospores being observed in diseased host tissue in North America. This investigation focused on the oospore as a means for survival and does not address the potential role of the sexual stage in generating variability. In 1990, the presence of A1 and A2 isolates of *P. capsici* was described in single fields in North Carolina (29). The author characterized morphological variation in field isolates in an attempt to determine if there are significant differences between isolates infecting cucurbit or solanaceous hosts. The morphological characters studied exhibited continuous rather than discrete variation within populations based on host type and the overall conclusion was that a combination of molecular and classical taxonomic approaches may be necessary to further delimit the species. The author reports that oospores may be formed in the field but does not discuss the possibility that the sexual stage may have contributed to the observed variability (29).

Much of the published literature on *P. capsici* concerns the environmental conditions favorable for infection and spread. The most prominent and recurring finding is that excess moisture is the single most important component to the initial infection and subsequent spread of *P. capsici* (3, 30, 31, 33, 35, 37). This follows in the wake of similar findings for many species in the genus *Phytophthora* and is not surprising in light of this organisms evolutionary ties to the algae (10, 11). Recommended control strategies reflect our understanding of the importance of water in the epidemiology of *P. capsici* and include planting into well drained fields and into raised beds whenever possible (32). Rotation to non-susceptible hosts is recommended for at least 2-3 years and further recommendations suggest that the highly active phenylamide fungicide mefenoxam may be useful in preventing disease. Mefenoxam is a relatively new compound which has the same mode of action as metalaxyl (9). Metalaxyl has been shown to specifically inhibit the incorporation of uridine into RNA in sensitive oomycetes (9). Metalaxyl was first used on a large scale by potato growers to control epidemics caused by *P. infestans* in the early 1980's in Europe (8). As early as 1981 researchers working with *P. capsici* demonstrated that insensitivity to metalaxyl was readily selected for using sub-lethally amended media (4, 5). Insensitivity soon developed in natural populations of oomycetous organisms where metalaxyl was heavily relied upon (7-9, 15, 18).

When the following research was initiated in 1997 our understanding of *P. capsici* was limited to a relatively good understanding of the asexual phase of disease development and did not include many studies specifically exploring the impact, or lack thereof, that the sexual stage may have on the life history of *P. capsici*. Mefenoxam was being applied by some growers as a part of a *Phytophthora* management strategy and the sensitivity of natural populations of *P. capsici* in Michigan to mefenoxam was unknown.

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Chapter 1: Mefenoxam insensitivity and the sexual stage of *Phytophthora capsici* in Michigan cucurbit fields.

ABSTRACT

Lamour, K. H., and Hausbeck, M. K. 2000. Mefenoxam insensitivity and the sexual stage of *Phytophthora capsici* in Michigan cucurbit fields. Phytopathology 90:396-400

The potential for outcrossing, occurrence of oospores, and inheritance of mefenoxam sensitivity was assessed in naturally occurring populations of *Phytopthora capsici*. Between 1997 and 1998 14 farms were sampled with 473 isolates recovered from cucurbit hosts and 30 from bell pepper. The A1 and A2 compatibility types were recovered in a roughly 1:1 ratio in 8 of 14 farms with sample sizes greater than 15. In 1997, one isolate was designated as insensitive and four as sensitive to mefenoxam. In 1998, 55% of the 498 isolates sampled were sensitive, 32% were intermediate, and 13% were fully insensitive to mefenoxam. *In vitro* characterization of mefenoxam sensitivity was conducted by crossing field isolates. Chi-square analysis of crosses between sensitive, intermediately sensitive, and insensitive isolates indicate that mefenoxam insensitivity segregate as an incompletely dominant trait unlinked to compatibility type ($P = 0.05$). Oospores were observed in diseased cucurbit fruit from four farms in 1998 and 223 oospore progeny were recovered from a single diseased cucumber. All six mefenoxam sensitivity/compatibility type combinations were present in these oospore progeny and within single fields. Based on these findings we conclude that oospores likely play a role in the survival of *P. capsici* and that sexual recombination may significantly influence population structure.

INTRODUCTION

Phytophthora capsici Leonian causes root, crown, and fruit rot on solanaceous and cucurbit hosts worldwide (6, 11, 16). In Michigan, this pathogen causes serious damage annually to peppers, cucumbers, pumpkins, and squash. The asexual phase of the life cycle includes a mycelial thallus which produces extracellular enzymes capable of macerating host tissue (31), papillate sporangia borne on long (>20um) caducous pedicels (29), and biflagellate, chemotactic, negatively geotropic zoospores which are liberated from sporangia under free water conditions (26). The sexual phase occurs when isolates of opposite compatibility type (designated A1 and A2) are in close proximity leading to the formation of thick-walled oospores. Oospores require an indeterminate time period (2 weeks to 3 months) for maturation, remain viable for extended periods of time (years), and are thought to be the primary survival structure (16). Because *P. capsici*' is heterothallic, oospores have the potential to represent new gene combinations in addition to their role as long-term dormant inoculum (11).

The principal methods of controlling *P. capsici* include cultural practices and the use of fungicides. Recommended cultural strategies are aimed at avoiding a build-up of inoculum by rotating to non-susceptible hosts and planting in well-drained fields (25). Oomycetes, although morphologically similar to true fungi, are genetically and biochemically dissimilar (11) and are not susceptible to most broad-spectrum fungicides (8). For this reason growers tend to rely on a limited number of fungicides. The phenylamide class of fungicides (PAF), specifically metalaxyl and the newest formulation mefenoxam (Novartis, Greensboro, NC), have been used on cucurbits in Michigan. Mefenoxam is the active enantiomer contained in the racemic fungicide metalaxyl (22). The mode of action of metalaxyl is postulated to be site specific and it was not surprising when resistance surfaced in populations of susceptible plant pathogens after the PAFs were introduced in the late 1970s (8). Researchers investigating a range of obligate and hemibiotrophic oomycetes suggest that tolerance to the PAFs is conditioned by a single locus with major effect exhibiting incomplete dominance subject to modification by genes of minor effect (2, 5, 7, 12, 28). Insensitivity to mefenoxam, which also conferred insensitivity to metalaxyl, has recently been reported from field populations of *P. capsici* on bell pepper (22).

The potential for outcrossing in epidemic populations of *P. capsici* has been reported based on the recovery of both the A1 and A2 mating types from soil samples and diseased plant material from single fields (20, 21, 24). Typical amphyginous oospores have been reported in the U.S. on a single occasion from diseased bell pepper tissue (21). *In vitro* analysis indicates that *P. capsici* oospore progeny are recombinant for pathogenicity to various hosts and mating type (3, 23).

This paper reports on the initial phase of an investigation into the biology and structure of *P. capsici* populations in Michigan vegetable production fields. The primary objective was to test the hypothesis that the sexual stage of *P. capsici* occurs in Michigan vegetable production fields. In addition, we tested the hypothesis that mefenoxam resistance is inherited as a single dominant gene (19).

MATERIALS AND METHODS

Sampling strategy and pathogen isolation. Diseased plant material was collected in a haphazard fashion from fields with limited disease (e.g., a single focus of infection). In fields with widespread disease, the spatial distribution of isolates was documented using permanent structures (e.g., telephone poles, houses, and barns) to construct field-specific grid patterns prior to sampling (Figure 1.1). Grid block areas varied from approximately 40 m^2 to 12 km^2 (Table 1.1). Infected plant material was selected at random from within blocks.

Diseased tissue was surface sterilized with 70% EtOH for approximately 10 s, and small pieces of actively expanding lesions were plated onto 100 x 15 mm BARP (Benomyl 25ppm, Ampicillin 100ppm, Rifampicin 30ppm, and Pentachloronitrobenzene 100ppm) amended UCV8 (840 ml distilled water, 163 ml un-clarified V8 juice, 3 g $CaCO_{3,}$ 16 g Bacto agar) plates (27). Plates were wrapped with Parafilm and incubated for 3-10 days in the dark at 23 - 25°C. Once an 8 cm diameter or larger colony had developed, the Parafilm was removed from the petri dish and the plates were incubated under lab lighting at 23 - 25°C for 2-3 days. This protocol stimulated ample sporangia production for zoospore release. Single-zoospore isolates were obtained by flooding the plates with sterile distilled water (SDW), placing plates into a 10°C refrigerator for 30 min, incubating the plates at 23-25°C for 30 min, placing four drops of the zoospore solution onto 100 x 15 cm water agar plates and tilting the plates to get streaks of zoospores on the water agar surface. Plates were incubated 45-90 min at 23-25°C and single germinated zoospores transferred to UCV8 using a dissecting microscope (25 X) (11). Single-zoospore cultures were stored on UCV8 plates at 15°C and transferred monthly or bi-monthly. In addition, two 7 mm plugs were placed into 20 ml screw-top vials with 2 sterilized hemp seeds and 10 ml SDW water, incubated at 23-25°C for 2 weeks under laboratory lighting, and stored long term at 15°C (11).

Determination of compatibility type. Agar plugs from the edge of an expanding single- zoospore derived colony were placed at the center of UCV8 plates approximately 2 cm from ATCC (American Type Culture Collection, Rockville, MD) isolate 15427 (A1 compatibility type) and ATCC 15399 (A2 compatibility type), incubated at 23-25°C in the dark for 3-6 days, and compatibility type determined. Thereafter, all compatibility type determinations were accomplished using the OP97 (A1) and SP98 (A2) field isolates.

In vitro response to mefenoxam. Agar plugs from the edge of actively expanding single-zoospore colonies were placed at the center of 100 x 15 cm UCV8 plates amended with 0 and 100 ppm mefenoxam (Ridomil Gold EC, Novartis, Greensboro, NC; 48% AI, suspended in SDW; added to UCV8 cooled to 49°C). Inoculated plates were incubated at 23-25°C for 3 days and colony diameters measured. Percent growth of an isolate on amended media was calculated by subtracting the inoculation plug diameter (7 mm) from the diameter of each colony and dividing the average diameter of the amended plates by the average diameter of the unamended control plates (9). All tests were conducted at least twice. Field isolates were assigned putative mefenoxam sensitivities based on the percent growth of the control as determined above. An isolate was scored as sensitive if growth at 100ppm was less than 30% of the control, intermediately sensitive if growth was between 30 and 90% of the control, and insensitive if growth was greater than 90% of the control. Preliminary cutoffs were determined by visual assessment of the frequency of mefenoxam sensitivity in field isolates (Figure 1.2A).

Segregation analysis. The validity of these mefenoxam sensitivity assignments was tested by crossing isolates representative of the three groups from within and between diverse geographical locations and comparing the ratios of progeny phenotypes to those expected under Mendelian inheritance using chi-square analysis. Isolate OP97 (A1, sensitive, farm NW2B) was crossed with isolates SSB98 (A2, insensitive, farm

SW1A), SLCC-6B (A2, sensitive, farm SC1B), and SFF-3 (A2, intermediate sensitivity, farm SC1A) and isolate 216 (A1, intermediate sensitivity, farm SC1A) was crossed with 244 (A2, intermediate sensitivity, farm SC1A). Crossing was performed as described in the compatibility type screen. Plates were incubated at 23-25°C in the dark for 3-4 months (15) and the surface of a 1cm square area was scraped from the zone containing oospores between the inoculation plugs. Scrapings were placed in 10 ml SDW and homogenized in a Sorvall mixer (Ivan Sorvall, Inc., Norwalk, CT) on the highest setting for 3 min. Novozyme (Sigma, St. Louis, MO) (5mg/ml) was added to the homogenate and the solutions incubated on a shaker (200 rpm) overnight (18 - 24 h). Solutions were then diluted 1 to 10 with SDW and incubated in 15 x 100 petri dishes under flourescent lighting (1). After approximately 24 h, germinated oospores were retrieved from the suspensions using a suction device constructed from a pasteur pippette (11). Germinated oospores are characterized by dissolution of the electron dense thick-wall and the presence of one to many germ tubes with or without terminal sporangia. Individual oospores, were transferred to water agar plates and after 1-2 days single hyphal tips were transferred to UCV8 plates. Single-oospore hyphal tip progeny were then screened for mating type and sensitivity to mefonoxam as described above.

Naturally occurring oospores. Selected infected fruits were observed for the presence or absence of oospores. Slides were prepared by excising a thin slice of suspect tissue, staining with crystal violet (300 ppm), and inspecting for typical amphigynous oospores under a light microscope (Leitz Laborlux S, Wetzlar, West Germany). Diseased plant material containing amphigynous oospores typical for *P. capsici* was surface sterilized with 70% EtOH for approximately 10 s and a 1 cm section embedded in BARP-

UCV8 media. Plates were incubated for 3-4 months in the dark. Following incubation the initial diseased plant material was removed and subjected to the germination procedure described in segregation analysis.

RESULTS

Mefenoxam sensitivity and compatibility type among isolates. Single isolates of *P. capsici* were recovered from five farms in 1997; 1 from bell pepper (A2, resistant) and 4 from cucurbit hosts (A1, sensitive). In 1998, 498 isolates were obtained from 11 farms; 468 from cucurbit hosts and 30 from bell pepper (Table 1.1). In 1998, 258 A1 and 240 A2 compatibility types were recovered with both compatibility types being found in every field sampled. Eight of ten fields with sample sizes greater than 15 had an approximate 1:1 ratio of A1 and A2 compatibility types (Table 1.1). When fields were sampled on a grid, both A1 and A2 compatibility types were located within and among geographically diverse quadrants (Figure 1.1).

In 1997, one isolate was designated as insensitive and four as sensitive to mefenoxam. In 1998, 55% (274) of the isolates were sensitive, 32% (161) were intermediate, and 13% (63) were fully insensitive to mefenoxam (Table 1.1). Of the 14 farms sampled during 1997 and 1998, 43% had fully insensitive isolates, and 79% had isolates of intermediate sensitivity (Table 1.1). Insensitive isolates were not recovered from 6 of the farms sampled. With one exception, fully sensitive isolates were recovered from all farms sampled (Figure 1.2A).

Three fields with sample sizes greater than 15 were of particular interest because they represent predominantly sensitive, intermediately sensitive, or fully insensitive populations of *P. capsici* (Figure 1.1). Within field SW1A, in the southwestern region, 87% of the isolates (56) were fully insensitive to mefenoxam with no sensitive isolates being recovered (Table 1.1). Both A1 and A2 isolates were recovered from 9 of the 13 quadrants sampled (Figure 1.1C). Whereas, in field SW1B,on the same farm, the majority (62%) of the isolates (56) had intermediate sensitivity and 20% were fully sensitive (Table 1.1). When four other fields in southwest Michigan were sampled in a limited manner (i.e., fewer than 16 samples), an insensitive isolate was recovered in one field (Table 1.1). In south central Michigan, intermediately sensitive strains dominated in all fields (Table 1.1). Isolates (145) collected on a single day from a field of pickling cucumbers in south central Michigan represent all six combinations of mefenoxam sensitivity and compatibility type with 17% sensitive A2, 20% sensitive A1, 28% intermediately sensitive A2, 32% intermediately sensitive A1, 1% insensitive A2, and 2% insensitive A1 isolates (Figure 1.1A). In northwest Michigan, completely sensitive strains dominated all fields sampled (Table 1.1). In one of the seven grids where two samples were collected both A1 and A2 compatibility types were present (Figure 1.1B).

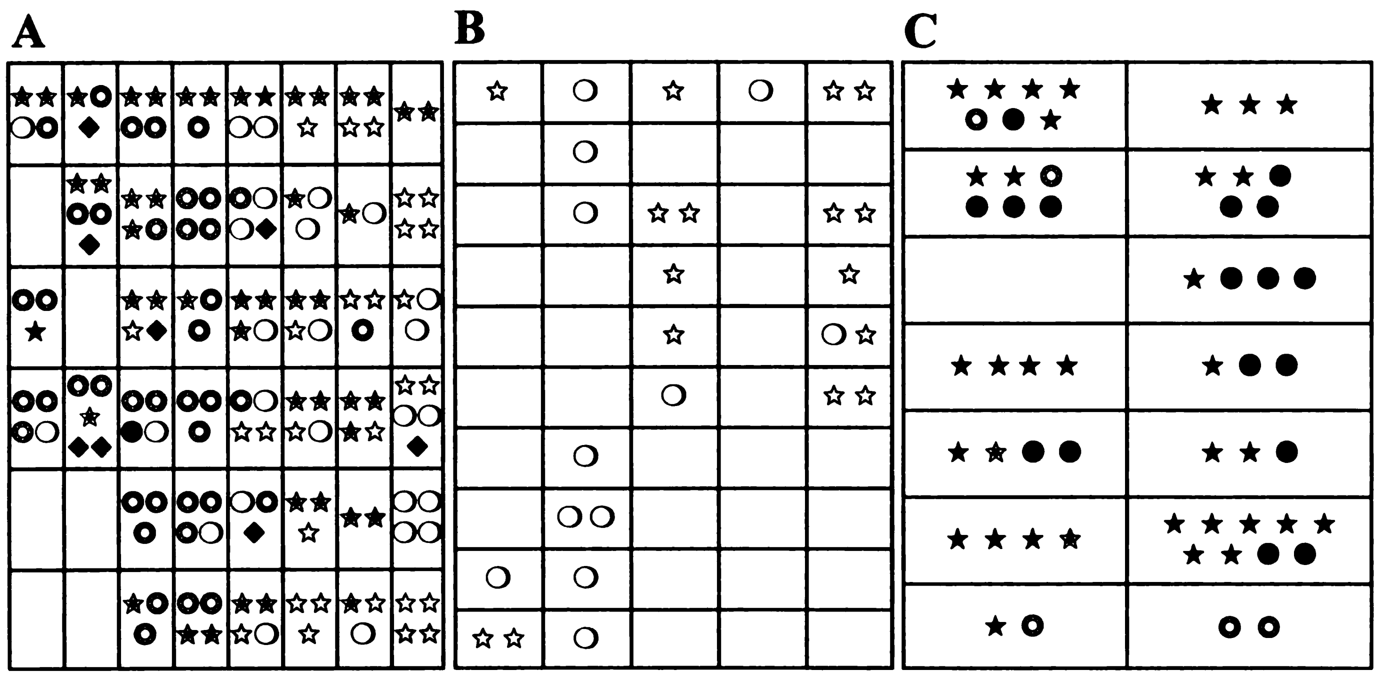

Figure 1.1. Spatial distribution of *Phytophthora capsici* isolates illustrating compatibility type (open circles = A2, open stars = A1), infections with oospores (black diamonds),and mefenoxam sensitivity (open circles/ open stars = sensitive, patterned circles/ patterned stars = intermediate sensitivity, and black circles/ black stars = insensitive) for cucurbit fields in **A**, south central (SC1A); **B**, northwestern (NW2B); and **C**, southwestern (SW1A) Michigan sampled during 1998. Each symbol represents a single isolate from a single infected plant or cucurbit fruit.

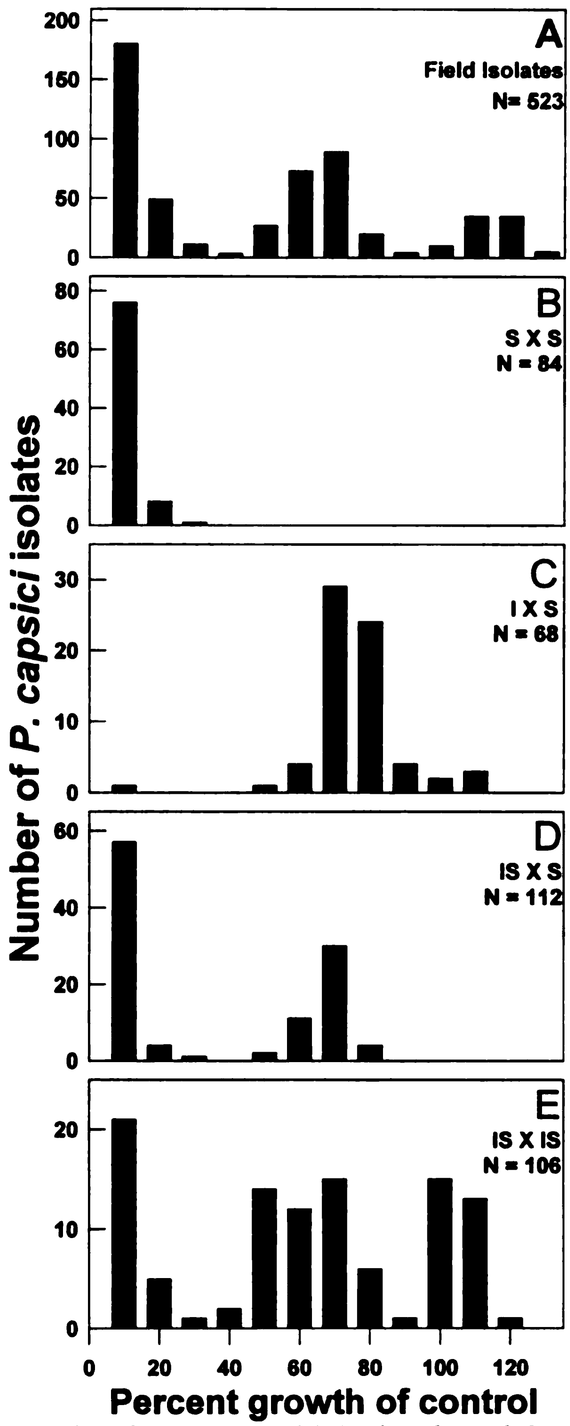

Figure 2. Frequency of mefenoxam sensitivity in *Phytophthora capsici* for **A**, 1997 to 1998 field isolates and in vitro progeny from crosses: **B**, SLCC-6B x OP9; **C**, SSB98 x OP97; **D**, SFF-3 x OP97; and **E**, 244 x 216. S = sensitive, <30% growth of control (GC); IS = intermediate, 30 to 90% GC; I = insensitive, > 90% GC; and N = number of isolates.

TABLE 1.1. *Phytophthora capsici* isolates described by collection in 1998

Location[a]	Host[b]	Sampling	Number of isolates	Compatibility type (%)		Mefenoxam sensitivity[g] (%)		
				A1	A2	S	IS	I
SW1A	S, P	[c, d]4 km^2 / (1-5)[e]	56	61	39	0	13	87
SW1B	S, C	40 m^2 / (0-3)	56	45	55	20	62	18
SW2	P, M, S	12 km^2 / (0-6)	16	56	44	81	19	0
SW3	P	H[f]	6	17	83	80	20	0
SW4	C	H	5	80	20	20	60	20
SW5	P	H	4	75	25	40	60	0
SC1A	C	1 km^2 / (0-4)	145	54	46	36	62	2
SC1B	C	6 km^2 / (0-6)	82	46	54	44	53	3
SC2	C	H	20	90	10	5	80	15
C1	P, S	H	16	19	81	88	12	0
NW1	BP	[c]4 km^2 / (0-5)	30	63	37	93	3	4
NW2A	S	H	29	52	48	90	7	3
NW2B	C, S	40 m^2 / (0-3)	28	57	43	100	0	0
NW3	S	H	5	20	80	80	20	0
Total			498	52	48	55	32	13

[a] First uppercase letters denote region, number denotes farm, and A or B represent fields on a single farm.
[b] P = pumpkin, C = cucumber, M = melon, S = squash, BP = bell pepper.
[c] Refers to samples collected from fungicide trial plots with mefenoxam as one of the treatments. [d] Approximate grid quadrant area in square meters.
[e] Number of samples recovered per quadrant.
[f] Haphazard acquisition of diseased material from fields.
[g] S = sensitive, IS = intermediate sensitivity, and I = insensitive.

TABLE 1.2. Phenotypic diversity of 223 *Phytophthora capsici* oospores isolated from a single naturally infected cucumber

Compatibility type	Mefenoxam sensitivity (%)[a]			
	S	IS	I	Total
A1	16 (35)	23 (52)	10 (23)	49 (110)
A2	19 (41)	22 (49)	10 (23)	51 (113)
Total	35 (76)	45 (101)	20 (46)	...

[a] S = sensitive, IS = intermediate sensitivity, and I = insensitive. Percentage of total followed by number of isolates in parentheses.

Oospores in the field. In 1998, typical amphigynous oospores were observed in diseased cucurbit fruit collected from multiple locations on farms in south central (cucumber, Figure 1.1A) and southwest Michigan (butternut squash), and single locations on additional farms (pumpkin, cucumber) located in these regions. Single-zoospore isolates were cultured from each of the infected fruit. Two hundred and twenty-three single-oospore progeny were recovered from a single cucumber from the southwest region and identified as *P. capsici* based on the presence of typical papillate, caducous sporangia borne on long (>20um) pedicels (11). These progeny exhibited diversity for mating type and mefenoxam sensitivity (Table 1.2).

In vitro segregation analysis of mefenoxam insensitivity and compatibility type. Each of the *in vitro* crosses resulted in greater than 60% germination of oospores. A cross between sensitive isolates originating from the northern (OP97) and south central (SLCC-6B) regions of Michigan (Figure 1.2B, Table 3) resulted in 48 A1 and 37 A2 sensitive progeny (Figure 1.2B). Crossing OP97 (sensitive) with an insensitive isolate (SSB98) from the

southwestern region of Michigan resulted in 33 Al and 35 A2 isolates with 91% of the progeny designated as intermediately sensitive,2% as sensitive, and 7% as insensitive (Figure 1.2C). OP97 (sensitive) crossed with an intermediately sensitive isolate (SFF3) from southwestern Michigan resulted in 51 A1 and 61 A2 isolates with 53% of the A1 and 58% of the A2 designated as sensitive, and 47% of the A1 and 42% of the A2 designated as intermediately sensitive (Figure 1.2D). A cross between two intermediately sensitive isolates (244 x 216) from the same field in south central Michigan resulted in 10% A1 and 16% A2 sensitive isolates, 20% A1 and 28% A2 intermediately sensitive isolates, and 11% A1 and 15% A2 insensitive isolates (Figure 1.2E). None of the progeny sets deviated from the expected ratios for mefenoxam insensitivity segregating as an incompletely dominant trait or compatibility type segregating in a 1:1 ratio ($P = 0.05$). Chi-square tests for linkage between mefenoxam insensitivity and compatibility type in the progeny of the *in vitro* sexual crosses SFF3 x OP97 and 244 x 216 indicate that these phenotypes are unlinked ($P = 0.05$) (Table 1.3).

TABLE 1.3. Chi-square analysis of *Phytophthora capsici* crosses for segregation and linkage of compatibility type (CT) and mefenoxam sensitivity (MS)

Cross number	Parent isolates	Isolate[a] origin	CT	MS[b]	CT	MS	CT and MS linkage
1	SLCC-6B	SC1B	A2	S	*1:1[c]	...	...
					48:37[d]		
	OP97	NW2B	A1	S		...	...
2	SSB98	SW1A	A2	I	*1:1	...	...
					33:35		
	OP97	NW2B	A1	S		...	...
3	SFF3	SC1A	A2	IS	*1:1	*1:1	*1:1:1:1
					51:61	63:49[e]	27:35:25:25[g]
	OP97	NW2B	A1	S			
4	244	SC1A	A2	IS	*1:1	*1:2:1	*1:1:2:2:1:1
					43:63	27:51:28[f]	10:17:21:30:12:16[h]
	216	SC1A	A1	IS			

[a] First uppercase letters denote region, number denotes farm, and A or B indicate fields.
[b] S = sensitive, IS = intermediate sensitivity, and I = insensitive.
[c] Expected Mendelian ratio.
[d] A1:A2 progeny.
[e] S:IS progeny.
[f] S:IS:I progeny.
Table 1.3 (cont'd).
[g] S/A1:S/A2:IS/A1:IS/A2 progeny.
[h] S/A1:S/A2:IS/A1:IS/A2:I/A1:I/A2 progeny.
* Chi-square value not significant at $P = 0.05$.

DISCUSSION

In the past 10 years, Michigan has experienced a steady increase in the incidence of root, fruit, and crown rot on cucurbits caused by *P. capsici*. Rotation to non-susceptible hosts for up to four years, in conjunction with cultural and chemical control strategies, have not provided economic control. The polycyclic nature of asexual reproduction and the role of environmental factors such as free water in disease development are well understood (4), but the role of the sexual stage in natural populations has not been investigated. Due to the survival capabilities of the oospore and the genetic implications inherent in outcrossing and recombination, sexual reproduction has the potential to significantly affect naturally occurring populations (10, 14).

A hallmark of sexually active populations is the presence of both compatibility types in a one to one ratio (14, 17). This criterion was met in many of the fields sampled during 1998. Sample sets which reflect the spatial distribution of isolates indicate that A1 and A2 compatibility types occur throughout epidemic populations.

Once the sexual stage of heterothallic spp. of *Phytophthora* has been stimulated there is the potential for selfing, as well as outcrossing. This may explain the fully sensitive and fully insensitive progeny recovered from our SSB98 (insensitive) x OP97 (sensitive) cross. Our initial assignment of three mefenoxam sensitivity classes was confirmed by chi-square analysis of progeny phenotypes compared to those expected under Mendelian segregation for a single dominant gene. Our *in vitro* findings that mefenoxam sensitivity in *P. capsici* is conditioned by a single incompletely dominant gene and that this phenotype is unlinked to compatibility type provides useful information for assessing population structure. Although the frequency of mefenoxam sensitivity is influenced by the method, frequency, and rate of fungicide application and becomes less informative in situations where populations are either fully resistant or fully sensitive, it does provide useful information in fields with a mixture of phenotypes. The probability of finding all six mefenoxam sensitivity/compatibility type combinations in the same field in the absence of sex is unlikely (13, 18).

The recovery and germination of typical amphigynous oospores from naturally infected cucurbit fruit provides direct evidence for sexual reproduction. It is clear that these progeny have the potential to serve as a diverse inoculum representing every combination of compatibility type and mefenoxam sensitivity.

Based on the composite picture presented by these various lines of investigation we conclude that sexual reproduction plays an active role in the survival of *P. capsici* in Michigan and may play a significant role in shaping population structure. Overwintering and long-term viability of oospores represents an obvious advantage gained by sexually active populations of *P. capsici*, but there may be an additional gain when considering insensitivity to mefenoxam. A viable strategy for recovering the effectiveness of a fungicidel management tool in the advent of widespread resistance is to stop using the fungicide and allow populations to shift back to sensitivity. This strategy is based on the phenomenon of a fitness cost for resistance and hinges upon the idea that resistant isolates are less fit and will be out-competed by sensitive isolates with the removal of the selective pressure (30). If sexual reproduction has played a role in the overwintering and survival of *P. capsici,* concomitant with PAF usage, it is possible that outcrossing and recombination in the population may generate a genotypically diverse array of resistant isolates. In this scenario the negative impact of genetic hitchhiking which may occur when resistance occurs within a single clone would be avoided and it would be less likely that resistant isolates in total harbor fitness disadvantages linked to mefenoxam insensitivity. Even if the wild type sensitive allele is not pushed to complete extinction there may be no reason for sensitive isolates to increase disproportionally when the selection pressure is removed.

The findings reported in this paper may explain why rotational strategies (> 2 years) to a non-susceptible host have not provided control and may give some insight into the failure of mefenoxam in controlling blight caused by *P. capsici* on cucurbits in

Michigan.

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Chapter 2: The dynamics of mefenoxam insensitivity in a recombining population of *Phytophthora capsici* characterized with AFLP markers.

ABSTRACT

Lamour, K. H., and Hausbeck, M. K. 2000. The dynamics of mefenoxam insensitivity in a recombining population of *Phytophthora capsici* characterized with AFLP markers. Phytopathology (in press).

Recent findings from Michigan suggest that recombination may play a role in the survival and evolution of sensitivity to the fungicide mefenoxam in populations of *Phytophthora capsici* on cucurbit hosts. In 1998, 63 mefenoxam insensitive isolates were recovered from a squash field in which mefenoxam had been applied. Additional isolates were recovered from untreated squash fields planted at this location in 1999 (200 isolates) and the spring of 2000 (34 isolates). Isolates from 1998 and 1999 were characterized using fluorescent amplified fragment length polymorphism (AFLP) markers and all isolates were screened for compatibility type and mefenoxam sensitivity. In 1998 and 1999, 92% and 71% of the isolates, respectively, had unique multilocus AFLP genotypes with no identical isolates recovered between years. Seventy-two identical AFLP markers were clearly resolved in both the 1998 and 1999 sample sets and fixation indices for the 37 polymorphic AFLP loci indicates little differentiation between years. There was no decrease in the frequency of resistant isolates during the 2 years without mefenoxam selection. We conclude that oospores play a key role in overwintering and that the frequency of mefenoxam insensitivity may not decrease in an agriculturally significant time period (2 years) once mefenoxam selection pressure is removed.

INTRODUCTION

Crown, root, and fruit rot caused by *Phytophthora capsici* is increasing in

Michigan cucurbit production fields, and uninfested land suitable for rotation is becoming increasingly scarce, especially in areas undergoing rapid urban development. The phenylamide fungicide (PAF) mefenoxam is a systemic fungicide which appears to be acting at the level of DNA transcription, and is fungistatic to fully sensitive isolates of *P. capsici* (2, 13). While mefenoxam has been considered by some growers to be helpful, mefenoxam insensitive isolates were reported on bell peppers in North Carolina and New Jersey by Parra and Ristaino in 1998 (18) and have since been recovered from 10 of 11 farms sampled in Michigan (13), as well as, in Georgia (15) and southern Italy (19). Mefenoxam insensitivity in Michigan *P. capsici* isolates is inherited as a single gene exhibiting incomplete dominance (13) which is consistent with the reports for a variety of other oomycetous organisms (2). Investigations with *P. infestans* indicate that insensitivity may be conferred by genes at different chromosomal positions (5) suggesting that the basis of insensitivity in different populations may not be identical. Sexual recombination, in particular, has the potential to impact management strategies that employ PAF's because the fully insensitive (two copies of the insensitivity allele) phenotype may be directly generated. *Phytophthora capsici* is heterothallic and the sexual stage is initiated when isolates of opposite compatibility type, designated A1 and A2, come into close association to form thick-walled oospores (4). The asexual stage includes the production of caducous sporangia borne on long pedicels which may release motile zoospores if free water is present. Asexual spores are thought to be responsible for the polycyclic nature of disease development (20).

Phenylamide fungicide resistance in the genus *Phytophthora* and, in particular, the *Phytophthora infestans*/potato pathosystem, is well documented (2, 4, 8). Until recently, the population structure of *P. infestans* appeared to be largely clonal outside of *P. infestans* putative center of origin (6). The recent detection of both *P. infestans* compatibility types along with increased genotypic diversity in some potato growing regions indicates that the sexual stage is likely active and may significantly impact control strategies which have proved useful in the past (3, 9). When PAF resistance in European *P. infestans* populations increased significantly in the early 1980's, the efficacy of the PAF metalaxyl was only regained after the product was not made available to growers for a period of time (2). This strategy apparently allowed the resistant populations to decline or become extinct and depends on ephemeral populations or, in the case of resident populations, upon a significant cost for resistance outside of selection pressure. A recent study of sensitive versus PAF resistant *P. nicotianae* isolates from citrus suggests negligible fitness costs for PAF resistance and reports that two years without PAF use did not reduce the proportion of resistant isolates in groves (22). Kadish and Cohen report that PAF resistant *P. infestans* isolates in Israel were more aggressive in colonizing tuber tissue than sensitive isolates (12).

Novel techniques have been developed recently that allow characterization of DNA level polymorphism in organisms for which little is known about the genome. An example is the amplified fragment length polymorphism (AFLP) technique introduced by Vos et al. in 1995 (24). This technique relies on restriction enzyme fragmentation of genomic DNA with the concomitant ligation of synthetic adaptors to the DNA fragment ends. Stringent PCR amplification using adaptor-complementary primers with additional selective nucleotides allow for the amplification of fragment subsets. DNA fragment subsets are termed fingerprints and may be resolved using a range of techniques (1).

AFLP markers have been used on a variety of organisms (14, 23) and the procedure has been shown to generate a large number of reproducible markers (1, 23). The limitation that these markers are generally scored as dominant markers (eg, either present or absent) for diploid organisms requires the use of relatively large sample sets (11, 26).

Our null hypotheses are that sexual recombination has a significant impact on the population structure of *P.capsici* in Michigan and that mefenoxam insensitivity may not decrease in the time frame of a typical 2 year rotation outside of mefenoxam selection pressure.

Materials and Methods

Field plot: Research was conducted on a commercial farm in southwest Michigan with a history (> 11 years) of *P. capsici* on bell peppers and squash and intensive use of PAF. The 4.05 hectare field sampled had previously been cropped to soybeans and corn with no known record of *P. capsici* susceptible crops (e.g.; tomatoes, peppers, or cucurbits) prior to 1997. During 1997 and 1998, yellow squash and zucchini grown in this field became diseased with *Phytophthora* crown, root, and fruit rot and the grower applied mefenoxam as part of a disease management strategy (Novartis, Greensboro, NC). In 1998, all isolates recovered were either intermediately or fully insensitive to mefenoxam. Both A1 and A2 compatibility types were present, and oospores were detected in diseased fruit. In 1999 and 2000, yellow squash was established in an 1,124 m^2 experimental plot in this field, and mefenoxam was not applied. Diseased plants and/or fruit were sampled on 20 August 1998 (63 isolates from entire field), June through August 1999 (200 isolates from experimental plot), and 13 July 2000 (34 isolates from experimental plot). All isolates were recovered from single diseased plants or fruit.

Isolate collection and maintenance: Isolation from diseased plant material was made onto BARP (Benomyl 25ppm, Ampicillin 100ppm, Rifampicin 30ppm, and Pentachloronitrobenzene 100ppm) amended UCV8 (840 ml distilled water, 163 ml unclarified V8 juice, 3 g $CaCO_3$, and 16 g Bacto agar) plates. Procedures for obtaining single zoospore isolates were as previously described (13). Single zoospore cultures were maintained on RA (Rifampicin 30ppm, Ampicillin 100ppm)-UCV8 plates and transferred bi-monthly. Long term storage consisted of a single 7 mm plug of expanding mycelium from each single zoospore culture being placed into a 1.5ml microfuge tube with one sterilized hemp seed and 1 ml of sterile distilled water, incubated for 2-3 weeks at 23 to 25°C, and stored at 15°C long term.

Phenotypic characterization: Isolates were screened for compatibility type as previously described (13). Mefenoxam sensitivity was characterized using the in vitro screening technique described by Lamour and Hausbeck (LH technique) for *P. capsici* isolates in Michigan (13). Isolates are scored as sensitive (S) if growth on UC-V8 agar amended with 100 ppm mefenoxam is less than 30% compared to a control, as intermediately sensitive (IS) if between 30 and 90%, and fully insensitive (I) if greater than 90% compared to the unamended control. These mefenoxam sensitivity categories are based on a tri-modal distribution of 523 field isolates of *P. capsici.* Clear modal distributions were only attained when screening was conducted with a single high rate of mefenoxam (100 ppm) amended media (K. Lamour, *unpublished data*). These putative mefenoxam sensitivity categories were tested by in vitro crosses (I x S, IS x IS, IS x S, and S x S) and chi-square analysis confirmed that the observed progeny numbers were not significantly different than expected for Mendelian inheritance of an incompletely dominant trait (13).

The LH technique differs from a commonly used method described by Goodwin, Sujkowski, and Fry (GSF technique) (8) for *P. infestans* which uses two levels of amended media (5 ppm and 100 ppm) to differentiate the three mefenoxam sensitivity phenotypes and which has been used to characterize *P. capsici* isolates (15, 18, 19). Unfortunately, analysis of our *in vitro* crosses and field isolates using the GSF technique did not resolve a clear modal distribution (K. Lamour, *unpublished data*). Assignment of Michigan *P. capsici* isolates to the sensitive (S) category is the same whether using the LH or GSF technique. The only difference is that some *P. capsici* isolates from Michigan rated as fully insensitive using the GSF technique are rated as intermediately sensitive using the LH technique.

DNA extraction and AFLP fingerprinting: A technique for avoiding bacterial contamination prior to growing isolates for DNA extraction was implemented using a modified Van Teigham cell (4). The uppermost portion of a 7 mm plug of mycelium was placed onto the surface of RA-WA plates (Rifampicin 30ppm, Ampicillin 100ppm, 1000 ml distilled water, and 16 g Bacto agar) and an autoclaved cap from a 1.5ml microfuge tube was placed over the plug which forced the isolate to grow through the amended media. Isolates were incubated in the dark for 2-3 days before two 7 mm plugs were transferred to approximately 15ml of RA-UCV8 broth in 100 x 15mm Petri dishes and incubated in the dark for three days at 23 to 25°C. Mycelial mats were washed with distilled water and dried briefly under vacuum before being frozen to -20°C and lyophilized.

Lyophilized mats were ground with a sterile mortar and pestle. Whole genomic

DNA from approximately 50mg of ground mycelium was extracted using a QIAGEN Dneasy Plant Mini Kit (QIAGEN Inc., Valencia, CA) according to the manufacturers directions. DNA was quantified using Nucleic Acid QuickSticks (CLONTECH, Palo Alto, CA) according to the manufacturers directions and approximately 100 ng of DNA was then subjected to a restriction / ligation reaction, pre-selective amplification, and selective amplifications using the PCR core mix, adaptor sequences, core primer sequences and fluorescent labeled primers available in the Perkin-Elmer Applied Biosystems AFLP™ Microbial Fingerprinting Kit (The Perkin-Elmer Corp., Foster City, CA henceforth referred to as PE/ABI) and performed exactly as described in the PE/ABI AFLP Microbial Fingerprinting protocol part # 402977 Rev A (24). All PCR reactions were performed using an MJ Research Minicycler (MJ Research Inc., Waltham, MA) in 0.2 ml tubes according to the cycling parameters outlined in the Microbial Fingerprinting protocol.

An initial optimization set of reactions was performed using pre-selective products from *P. capsici* isolate OP97 which was isolated from a cucumber fruit in 1997 (13). Selective amplifications with the selective primers EcoRI-AA, AC, AG and AT were performed in all 16 combinations with the MseI-CA, CC, CG and CT selective primers. EcoRI selective primers available from PE/ABI are labeled at the 5' end with either carboxyfluorescein (FAM), carboxytetramethyrhodamine (TAMRA), or carboxy-4',5'-dichloro-2',7'-dimethoxyfluorescein (JOE) fluorescent dyes. The fluorescent dyes are excited by laser radiation and visualized by their characteristic absorption-emission frequencies. Only the fragments containing an EcoRI restriction site are resolved.

Products from three reactions labeled with different colored dyes and a carboxy-

X-rhodamine (ROX) size standard were loaded into each lane on a denaturing polyacrylamide gel and the fragments resolved in an ABI Prism 377 DNA Sequencer. Results were prepared for analysis in the form of electropherograms using GeneScan Analysis software (PE/ABI). AFLP fragments were scored manually as present (1) or absent (0) using Genotyper (PE/ABI). Only DNA bands which consistently exhibited unambiguous presence/absence profiles were scored.

A single isolate, OP97, was subjected to the aforementioned protocol using three primer pair combinations which were chosen as optimal on 3 separate occasions approximately 3 months apart to test for reproducibility of AFLP profiles.

Clone detection and cluster analysis: AFLP fragments were considered polymorphic if the most common allele was present in less than 95% of the isolates from a given sample set and scored for presence (1) or absence (0) (10). AFLP fragments present in more than 95% of the isolates from a given sample set were considered monomorphic. Analysis of the resulting binary data matrix was performed using NTSYSpc version 2.02k (21). Unweighted pair group method with arithmetic averages (UPGMA) cluster analysis was performed on the matrix of similarity coefficients calculated from all possible pairwise comparisons of individuals within and among the 1998 and 1999 populations and a tree generated. Isolates showing complete homology at all loci were considered to be clones and except for a single representative isolate were excluded from frequency calculations.

Allele frequency and fixation indices: Allele frequencies for AFLP markers were estimated utilizing the expected relationship between gene and genotype frequencies in a randomly mating population (i.e. Hardy-Weinberg proportions). The frequency of the recessive (absent) allele (q) was calculated from the observed number of recessive homozygote individuals (X) in a sample of n individuals using the formula for dominant markers described by Jorde (11):

$$\hat{q} = \sqrt{x + \frac{1-x}{4n}}$$

where $x = X/n$ is the observed proportion of individuals that do not display the dominant (present) marker phenotype. In order to test whether the composite genetic profiles from 1998 and 1999 were consistent with a single randomly mating population, the fixation index was calculated for each AFLP loci from the variance in allele frequencies according to the following formula: $F_{ST} = ((p_1\text{-}p_2)^2/4)/(\text{Avg } p \text{ x Avg } q)$, where p is the allele frequency for the present state with p_1 and p_2 indicating the two sample populations and q is the allele frequency for the absent state (10). Fixation indices for individual loci were interpreted according to the qualitative guidelines suggested by Wright (25) where the range 0 to 0.05 may be considered as indicating little genetic differentiation, the range 0.05 to 0.15 indicates moderate genetic differentiation, and greater than 0.25 indicates great genetic differentiation (10).

Results

AFLP band characterization: Evaluation of the 16 EcoRI + 2/MseI + 2 selective primer pair combinations indicated that EcoRI + AC/ MseI + CA gave the most clearly resolved fragment profile and was used to amplify genomic DNA from all isolates in both the 1998 and 1999 sample sets. This primer combination resulted in 72 clearly resolved fragments of which 37 (51%) fragments were polymorphic in both 1998 and 1999 (Table 2.1). All 72 fragments were present in both 1998 and 1999 and no novel fragments were detected between years. The following 35 fragments (size in basepairs) were monomorphic in both the 1998 and 1999 sample sets: 41, 43, 47, 49, 58, 66, 70, 82, 85, 114, 118, 123, 133, 135, 140, 159, 174, 235, 247, 249, 272, 278, 295, 298, 300, 341, 351, 355, 367, 402, 474, 488, 502, 519, and 527. AFLP profiles for isolate OP97, generated from separate DNA extractions on three separate occasions over a one year period, resulted in identical banding patterns with the only difference being minor changes in the intensity of the electropherogram signal. Occasionally individual reactions resulted in poorly resolved fingerprint profiles (eg, low intensity of signal) and were repeated until signals were deemed optimal.

Phenotypic, genotypic and gene diversity: No isolates sensitive to mefenoxam were recovered in 1998 or 2000 and single A1 sensitive and A2 sensitive isolates were recovered in 1999 (Table 2.2). In 1998, 18% of the isolates were intermediately sensitive and 82% were insensitive, in 1999, 2% were sensitive, 28% were intermediately sensitive and 70% were insensitive, and in 2000, 15% of the isolates were intermediately sensitive and 85% were insensitive to mefenoxam (Table 2.2).

Table 2.1. Fixation indices (F_{ST}) for 37 AFLP loci from unique *Phytophthora capsici* isolates collected from a single Michigan cucurbit field during 1998 (N = 57) and 1999 (N = 141).

Fragment[a]	1998 f(aa)[b]	1999 f(aa)	F_{ST}[c]
45	0.02	0.06	0.018
54	0.29	0.29	0.000
64	0.82	0.55	0.048
104	0.11	0.06	0.007
106	0.11	0.04	0.025
110	0.41	0.36	0.002
130	0.41	0.30	0.009
146	0.47	0.24	0.038
149	0.12	0.27	0.029
154	0.39	0.31	0.004
156	0.53	0.83	0.054
172	0.56	0.33	0.034
189	0.16	0.56	0.121
192	0.16	0.37	0.044
193	0.35	0.20	0.022
211	0.47	0.15	0.088
241	0.48	0.32	0.018
256	0.04	0.01	0.022
258	0.43	0.49	0.002
261	0.55	0.54	0.000
270	0.57	0.41	0.015
282	0.35	0.40	0.002
285	0.51	0.73	0.030
314	0.51	0.34	0.019
320	0.41	0.51	0.006
333	0.16	0.20	0.002
346	0.36	0.33	0.001
361	0.33	0.49	0.017
383	0.21	0.15	0.005
418	0.40	0.34	0.002
431	0.34	0.32	0.001
438	0.67	0.45	0.028
454	0.65	0.49	0.015
492	0.29	0.40	0.009
504	0.51	0.47	0.001
511	0.38	0.28	0.007
548	0.78	0.78	0.000

[a] EcoR1-AC/Mse1-CA selectively amplified fragment size in base-pairs.
[b] Observed frequency of the absent state where 'a' represents the absence of a fragment.
[c] F_{ST} calculated from estimated allele frequencies. According to Wright's qualitative guidelines values between 0-0.05 indicate little genetic differentiation and values between

Table 2.1 (cont'd).
0.05-0.15 indicate moderate genetic differentiation.

Table 2.2. Phenotypic diversity of *Phytophthora capsici* isolates recovered from the same cucurbit field in 1998, 1999, and 2000.

Year[a]	Number[b] of isolates	Compatibility type and mefenoxam sensitivity[c,d]					
		A1/S	A1/IS	A1/I	A2/S	A2/IS	A2/I
1998	57	-	4	31	-	6	16
1999	141	1 (2)	17 (20)	57 (53)	1 (1)	23 (18)	42 (47)
2000	34	-	2	8	-	3	21

[a] Mefenoxam was applied in 1998 but not in 1999 or 2000.
[b] Sample sets from 1998 and 1999 consist of unique multilocus genotypes as determined with AFLP fingerprinting. The 2000 sample set was recovered at the beginning of the growing season and was not fingerprinted.
[c] MS = mefenoxam sensitivity where S = sensitive, IS = intermediately sensitive and I = insensitive as determined by in vitro screening on 100 ppm mefenoxam amended agar.
[d] Numbers in parentheses indicate the expected number of isolates if mefenoxam insensitivity is assumed to be controlled by a single incompletely dominant gene in Hardy-Weinberg equilibrium unlinked to compatibility type.

Fifty seven of the 63 isolates recovered in 1998, and 141 of the 200 isolates recovered in 1999 were unique based on multilocus AFLP profiles. No identical multilocus genotypes were recovered between 1998 and 1999. Five isolates (2 A2/I, 2 A2/IS, and 1 A1/I) of *P. capsici* collected in 1998 had one clonal representative. Fourteen isolates collected in 1999 had between 2 and 4 clones (Table 2.3). A single A1 compatibility type insensitive isolate had 40 clones recovered over the course of the 1999 season and comprised 3% of the early, 15% of the mid, and 43% of the late sampling intervals (Table 2.3). The 1999 sampling intervals (early, mid, and late) are based on the dates of sampling and are not intended to reflect stages of plant growth or the epidemiology of *P. capsici*. Cluster analysis of AFLP fingerprint variation indicated no significant clustering of isolates between 1998 and 1999.

Table 2.3. Clone contribution of fifteen *Phytophthora capsici* isolates to the total number of isolates collected in 1999 (N = 200).

Isolate	No. of clones[a]	CT and MS[b]	No. of clones in early, mid and late season sample intervals[c]		
			6/22 - 7/16 N = 60	7/20 - 8/3 N = 80	8/5 - 8/18 N = 60
JP571	2	A1/I	2	-	-
JP583	2	A1/I	2	-	-
JP944	3	A1/I	2	1	-
JP999	3	A1/I	2	1	-
JP1007	2	A1/I	1	1	-
JP1042	2	A2/I	1	1	-
JP1096	2	A1/I	-	1	1
JP1102	2	A2/I	-	2	-
JP1215	3	A2/I	3	-	-
JP1342	2	A2/IS	-	2	-
JP1369	2	A1/I	1	1	-
JP1384	4	A2/I	3	1	-
JP1512	2	A1/I	1	-	1
JP1555	3	A1/I	-	-	3
JP1632	40	A1/I	2	12	26

[a] Total number of isolates with identical multilocus AFLP profiles.
[b] CT = compatibility type and MS = mefenoxam sensitivity where S = sensitive, IS = intermediately sensitive and I = insensitive as determined by in vitro screening on 100 ppm mefenoxam amended agar.
[c] Sample intervals based on sampling dates only.

The majority (98%) of the 37 polymorphic AFLP fragments showed little genetic differentiation ($F_{ST} < 0.05$) between 1998 and 1999 according to Wrights qualitative criterion (Table 2.1) (25).

Discussion

Phytophthora capsici causes significant damage to cucurbit hosts in Michigan each year. In an effort to prevent or control epidemics, many growers have used either metalaxyl or the newer, but similarly acting compound, mefenoxam as a part of their disease management strategy. This study was initiated in an effort to address the concerns of growers who have high levels of mefenoxam insensitivity.

Phenotypic data (mefenoxam sensitivity and compatibility type) from a 1998 survey suggested that insensitivity to mefenoxam was common and that some level of recombination is occurring in the field (13) but without the application of additional polymorphic markers our ability to assess population structure was severely restricted. AFLP analysis proved to be a powerful tool for resolving the population dynamics of *P. capsici*. A single selective primer combination, EcorRI-AC/MseI-CA, generated 72 bands of which 37 were polymorphic in our 1998 and 1999 sample sets. AFLP fingerprinting, in conjunction with temporal sampling, provided a useful characterization of *P. capsici* from one season to the next and allowed us to track asexual disease development over the course of a single season.

Our data suggests that sexual recombination significantly impacts the structure of this *P. capsici* population. The finding that 198 of the 262 isolates recovered between 1998 and 1999 had unique multilocus AFLP genotypes is consistent with the high level of genotypic diversity expected in an outcrossing population (7, 16, 17). Even though clonal reproduction occurred in 1998 and 1999, no identical genotypes were recovered between years, suggesting that oospores are important for overwintering. The finding that 35 of the 37 polymorphic fragments exhibited very little differentiation (ie: change in allele frequency) based on the estimated fixation indices between 1998 and 1999 is consistent with the expectations for a recombining population large enough to avoid dramatic changes due to genetic drift.

In 1999 and 2000, sensitive and intermediately sensitive isolates (42 of 175) did not increase in a manner suggesting selection in favor of mefenoxam sensitivity outside of mefenoxam selection pressure. The fact that 14 of the 15 isolates with clonal reproduction in 1999 were fully insensitive may be another indication that mefenoxam insensitivity does not have significant costs outside of mefenoxam selection pressure. If we assume that there is only a single mefenoxam insensitivity gene in this population unlinked to compatibility type, designated *I*, and that this population is effectively free from the effects of migration and genetic drift, some interesting speculations can be made. For instance, in 1999, if the mefenoxam sensitivity phenotypes are assumed to represent genotypes (eg; a fully insensitive isolate has two copies of the *I* allele) then the frequency of *I* can be estimated and the observed number of unique isolates that fall into each of the six mefenoxam sensitivity/compatibility type categories can be compared to the expectations under Hardy-Weinberg equilibrium. In 1999, the estimated frequency of *I* is 0.84 and chi-square analysis using the data in Table 2 indicates that the observed numbers do not differ from those expected under Hardy-Weinberg equilibria at $P = 0.50$ (X^2 of 3.09, df = 4). Although this is not a particularly powerful test due to the large number of assumptions (10), it does lend support to the hypothesis that this population meets the criterion for panmixia.

Our results do not allow us to reject the null hypothesis that sexual recombination significantly impacts the structure of this population. It appears that sexual recombination plays a significant role in maintaining genotypic and gene diversity while concomitantly producing overwintering inoculum. Our data also suggests that sexual recombination may serve as a potent force for integrating a beneficial allele based on the finding that there were a total of 133 unique multi-locus genotypes fully insensitive to mefenoxam between 1998 and 1999. An interesting question that can only be answered by following a fully sensitive population as it shifts to insensitivity is how much genetic diversity is lost, if any, during the PAF selection process? The question of how long mefenoxam resistance will remain in a population of *P. capsici* when selection pressure is removed can only be answered in a tentative way. It appears that in this population insensitivity will not decrease within the time frame of a typical 2 year rotation and , once resistance to mefenoxam is established, the future usefulness of this fungicide may be extremely limited.

Comparison of the population structure reported at this single location is currently being compared to other locations in Michigan and the United States and should provide useful insight into the amount of genetic diversity in sensitive vs. insensitive populations as well as the contribution of migration to *P. capsici* population structure.

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Chapter 3: The spatiotemporal genetic structure of *Phytophthora capsici* in Michigan and implications for disease management.

ABSTRACT

Lamour, K. H. and Hausbeck, M. K. 2001. The spatiotemporal genetic structure of *Phytophthora capsici* in Michigan and implications for disease management. Phytopathology (submitted).

Phytophthora capsici isolates were recovered from pepper and cucurbit hosts at seven locations in Michigan from 1998 to 2000. Isolates were characterized for compatibility type (CT), mefenoxam sensitivity (MS), and AFLP marker profiles. In total, 94 AFLP bands were resolved. Individual populations were highly variable. Within populations, 39 to 49% of the AFLP bands were polymorphic and estimated heterozygosities ranged from 0.16 to 0.19. Of the 646 isolates fingerprinted, 70% (454) had unique AFLP profiles. No clones were recovered between years or locations. Pairwise F-statistics (Φ_{ST}) between populations ranged from 0.18 to 0.40. There was no correlation between Φ_{ST} and geographical distance. A tree based on UPGMA cluster analysis indicates discrete clusters based on location with no clustering based on year of sampling. Analysis of molecular variance (AMOVA) partitioned variability among (40%) and within populations (60%). The overall estimated Φ_{ST} was 0.34 (sd = 0.03). A1:A2 CT ratio's were ≈ 1:1and MS frequencies were similar between years. These data suggest that long distance dispersal is rare, that the sexual stage plays a significant role in survival and maintaining high levels of genetic diversity, and that control strategies aimed at preventing the introduction of *P. capsici* need to be investigated.

Introduction

Phytophthora capsici Leonian causes significant damage to a variety of plant hosts worldwide and in the United States seriously impacts the production of cucurbits and peppers (10, 17, 23). In Michigan, *P. capsici*'s life history is divided between an active growth phase in the presence of susceptible host tissue and a state of dormancy over the winter. Overwintering survival is thought to be accomplished by thick walled oospores which are produced during sexual reproduction (10, 11). *Phytophthora capsici* is heterothallic and completion of the sexual stage requires both the A1 and A2 compatibility types. Sexual reproduction is mediated by extracellular hormonal signals and there is the potential for both self and cross-fertilization (9). Oospores generally require a dormancy period prior to germination. Germinating oospores produce coenocytic mycelium which can directly infect or, under suitable conditions, differentiate into caducous sporangia. Sporangia can be dislodged and cause infection directly, or, in the presence of free water, release 20 to 40 motile zoospores. Polycyclic asexual spread of *P. capsici* between and down rows has been clearly documented in the pepper/*P. capsici* pathosystem (24) but, to our knowledge, there have been no reports suggesting that *P. capsici* has a significant long distance mode of dispersal.

Ristaino has recently summarized management strategies useful for disease control (23). The primary strategy is to manage soil water dynamics by providing the best possible drainage for the host plants rhizosphere and the field in general. Growers are advised to rotate fields to non-susceptible hosts and when appropriate to apply fungicides. The phenylamide fungicide (PAF) mefenoxam has been shown to be fungistatic to sensitive isolates of *P. capsici* (19), but, as has occurred with many oomycetes, insensitivity has developed in field populations (10, 20, 21). Research using *P. capsici* isolates from Michigan indicates that insensitivity is controlled by an incompletely dominant gene of major effect (10) which is consistent with the findings for a number of other oomycetes (3).

In Michigan, fruit, stem and root rots caused by *P. capsici* on cucurbit hosts have increased in recent years and growers employing available management strategies have experienced significant losses. Over the last 4 years an investigation of *P. capsici* populations in Michigan vegetable production fields has been conducted (10, 11). The initial phase of this study was based on the distribution and frequency of compatibility type (CT) and mefenoxam sensitivity (MS) phenotypes within and between fields. In 1998, an approximate 1:1 ratio of A1:A2 isolates was discovered in the majority of fields sampled and oospores were detected in diseased cucurbit fruit on 4 separate farms. All six CT/MS phenotypes were recovered as oospore progeny from a single diseased cucumber fruit as well as from a single diseased cucumber field (10). These initial findings suggested that the sexual stage is active in populations of *P. capsici* in Michigan and, based on the MS findings, that sexual recombination may play an important role in directly generating the fully insensitive MS phenotype.

The ability to assess population dynamics using only CT and MS is limited by the fact that only 6 phenotypic combinations are able to be resolved and is further limited because some populations appear to have only sensitive or insensitive isolates (10). Amplified fragment length polymorphism (AFLP) markers are increasingly being used as a tool to investigate population genetic structure in a wide variety of living organisms including plants (27, 29), animals (22), insects (4), and microorganisms (12). A molecular map of the *P. infestans* genome was constructed based on AFLP and RFLP markers and corroborates the finding of other researchers that AFLP markers span the genome (28). No prior sequencing or cloning of fragments is needed to utilize this marker system and it has been shown to be highly reproducible between labs (1). AFLP markers are generally scored as present or absent (eg; dominant markers) and the confidence with which population level inferences can be made is greatly increased by sample sets that are approximately twice the size used for co-dominant markers (8, 13, 32).

A single field (SW1-A) from which only intermediately or fully insensitive MS phenotypes were collected in 1998 was sampled in the absence of mefenoxam selection pressure over 1999 and 2000 (11). Characterization of the 1998 and 1999 samples with AFLP markers revealed that genotypic and genic diversity were high, that clonal reproduction was significant within a single season but that members of the same clone were not detected between years, that AFLP marker frequencies did not change significantly between years, and that the frequency of mefenoxam insensitivity did not appear to decrease in the absence of mefenoxam selection pressure (11). These results suggested that this population was large enough to withstand dramatic effects of genetic drift and that there may not be a significant cost for mefenoxam insensitivity. At the time this information was reported it was not known what effects, if any, migration of *P. capsici* from outside sources might have had in determining the observed population structure.

In this paper we report on the genetic structure of *P. capsici* populations throughout Michigan. Our objectives were to determine if the level of genetic diversity found in the population at SW1-A is representative of *P. capsici* populations at different geographical locations and to determine if long distance dispersal is a significant component in determining the genetic structure of populations in Michigan. A related objective was to investigate how long *P. capsici* can survive in the absence of known susceptible host material. We also report on the frequency of self-fertilized vs. hybrid progeny in a sexual cross between isolates from different geographical locations and the inheritance of AFLP markers in this cross. Portions of the information in this paper have been reported previously (10, 11).

Materials and Methods

Isolate collection and maintenance: Pepper, cucumber, pumpkin, tomato and squash plant material (root, crown and fruit) with typical signs and symptoms of infection by *P. capsici* were collected from 6 farms throughout Michigan between 1998 and 2000. Sampling was conducted using field specific grids with grid quadrants varying from $40m^2$ to $12km^2$ depending on the size of the field. Sample sets are labeled according to the following criterion; location (SW = southwest, SC = south central, C = central, and NW = northwest) followed by a farm designator (1, 2,...n) with a hyphen separating a field designator (A, B, ...n) and the year sampling was conducted (1998 = 98, 1999 = 99, and 2000 = 00). Diseased plant material was collected from quadrants in a haphazard fashion. Isolation from diseased plant material was made onto BARP (Benomyl 25ppm, Ampicillin 100ppm, Rifampicin 30ppm, and Pentachloronitrobenzene 100ppm) amended UCV8 (840 ml distilled water, 163 ml unclarified V8 juice, 3 g $CaCO_3$, and 16 g Bacto agar) plates. Procedures for obtaining single zoospore isolates were as previously described (10). Single zoospore cultures were maintained on RA (Rifampicin 30ppm, Ampicillin 100ppm)-UCV8 plates and transferred bi-monthly. Long term storage consisted of a single 7 mm plug of expanding mycelium from each single zoospore culture being placed into a 1.5ml microfuge tube with one sterilized hemp seed and 1 ml of sterile distilled water, incubated for 2-3 weeks at 23 to 25°C, and stored at 15°C.

Compatibility type and mefenoxam sensitivity determination: Agar plugs from the edge of an expanding single- zoospore colony were placed at the center of UCV8 plates approximately 2 cm from ATCC (American Type Culture Collection, Rockville, MD) isolate 15427 (A1 compatibility type) and ATCC 15399 (A2 compatibility type), incubated at 23-25°C in the dark for 3-6 days, and compatibility type determined. Thereafter, all compatibility type determinations were accomplished using the OP97 (A1) and SP98 (A2) field isolates.

Agar plugs from the edge of actively expanding single-zoospore colonies were placed at the center of 100 x 15 cm UCV8 plates amended with 0 and 100 ppm mefenoxam (Ridomil Gold EC, Novartis, Greensboro, NC; 48% AI, suspended in SDW; added to UCV8 cooled to 49°C). Inoculated plates were incubated at 23-25°C for 3 days and colony diameters measured. Percent growth of an isolate on amended media was calculated by subtracting the inoculation plug diameter (7 mm) from the diameter of each colony and dividing the average diameter of the amended plates by the average diameter of the unamended control plates. All tests were conducted at least twice. An isolate was scored as sensitive (S) if growth at 100ppm was less than 30% of the control, intermediately sensitive (IS) if growth was between 30 and 90% of the control, and insensitive (I) if growth was greater than 90% of the control (10).

DNA extraction and AFLP fingerprinting: A technique for avoiding bacterial contamination prior to growing isolates for DNA extraction was implemented using a modified Van Teigham cell (5). The uppermost portion of a 7 mm plug of mycelium was placed onto the surface of RA-WA plates (Rifampicin 30ppm, Ampicillin 100ppm, 1000 ml distilled water, and 16 g Bacto agar) and an autoclaved cap from a 1.5ml microfuge tube was placed over the plug which forced the isolate to grow through the amended media. Isolates were incubated in the dark for 2-3 days before two 7 mm plugs were transferred to approximately 15ml of RA-UCV8 broth in 100 x 15mm Petri dishes and incubated in the dark for three days at 23 to 25°C. Mycelial mats were washed with distilled water and dried briefly under vacuum before being frozen to -20°C and lyophilized.

Lyophilized mats were ground with a sterile mortar and pestle. Whole genomic DNA from approximately 50mg of ground mycelium was extracted using a QIAGEN Dneasy Plant Mini Kit (QIAGEN Inc., Valencia, CA) according to the manufacturers directions or using a CTAB procedure in conjunction with an automated DNA extractor. DNA was quantified using Nucleic Acid QuickSticks (CLONTECH, Palo Alto, CA) according to the manufacturers directions or on 1.5% agarose gels and approximately 100 ng of DNA was then subjected to a restriction / ligation reaction, pre-selective amplification, and selective amplifications using the PCR core mix, adaptor sequences, core primer sequences and fluorescent labeled primers available in the Perkin-Elmer Applied Biosystems AFLP™ Microbial Fingerprinting Kit (The Perkin-Elmer Corp., Foster City, CA henceforth referred to as PE/ABI) and performed exactly as described in the PE/ABI AFLP Microbial Fingerprinting protocol part # 402977 Rev A (30). All PCR reactions were performed using an MJ Research Minicycler (MJ Research Inc., Waltham, MA) in 0.2 ml tubes according to the cycling parameters outlined in the Microbial Fingerprinting protocol.

An initial optimization set of reactions was performed using pre-selective products from *P. capsici* isolate OP97 which was isolated from a cucumber fruit in 1997 (10). Selective amplifications with the selective primers EcoRI-AA, AC, AG and AT were performed in all 16 combinations with the MseI-CA, CC, CG and CT selective primers. EcoRI selective primers available from PE/ABI are labeled at the 5' end with either carboxyfluorescein (FAM), carboxytetramethyrhodamine (TAMRA), or carboxy-4',5'-dichloro-2',7'-dimethoxyfluorescein (JOE) fluorescent dyes. The fluorescent dyes are excited by laser radiation and visualized by their characteristic absorption-emission frequencies. Only the fragments containing an EcoRI restriction site are resolved.

Selective amplification AFLP products and a carboxy-X-rhodamine (ROX) size standard were loaded into each lane on a denaturing polyacrylamide gel and the fragments resolved in an ABI Prism 377 DNA Sequencer. Results were prepared for analysis in the form of electropherograms using GeneScan Analysis software (PE/ABI). AFLP fragments were scored manually as present (1) or absent (0) using Genotyper (PE/ABI). Only DNA bands which consistently exhibited unambiguous presence/absence profiles were scored.

A single isolate, OP97, was subjected to the aforementioned protocol using three primer pair combinations which were chosen as optimal on 3 separate occasions approximately 3 months apart to test for reproducibility of AFLP profiles.

Marker inheritance: Oospore progeny (N = 107) resulting from a cross between isolate OP97 (A1/IS) x SFF3 (A2/S) were subjected to AFLP analysis as described above. Protocols for the generation, germination, and phenotypic characterization of the F1 oospores from this cross have been reported previously (10). The inheritance of AFLP bands present in one parent and absent in the other which were inherited consistent with one parent being heterozygous were analyzed using chi-square analysis to compare observed numbers to those expected under simple Mendelian inheritance (28). Individual oospore isolates were checked for the co-presence of AFLP markers present in single copies in each parent to determine if they were the products of self-fertilization or hybridization between the parent isolates. Bands present in both parents or homozygous present in one parent and absent in the other are not reported on in this study.

Clone detection: AFLP fragments were scored for presence or absence and the binary data matrix was converted to a similiarity matrix using a simple matching coefficient of resemblance with the program NTSYSpc version 2.02k (25). Unweighted pair group method with arithmetic averages (UPGMA) cluster analysis was performed on the similarity matrix and a tree generated. Isolates showing complete homology at all loci were considered to be members of the same clone and except for a single representative isolate were excluded from population genetic analysis (15).

Population genetic analysis: Sample sets collected from single fields during a single year were considered a population. Populations were assumed to be in Hardy-Weinberg equilibrium and each AFLP locus was assumed to be unambiguously di-allelic. The program 'Tools for population genetic analysis' (TFPGA) (16) was used to (i) assess genetic diversity within each population on the basis of estimated average heterozygosity (18) and the proportion of polymorphic loci at the 95% level (6), (ii) calculate pair-wise and overall F-statistics according to the methods of Weir and Cockerham (31) and (iii), to test the effect of spatial separation on genetic structure by performing a Mantel test (14) on the pairwise F-statistic matrix and the matrix of geographical distance between populations. The significance of the correlation between the two matrices was tested by 1000 random permutations to generate a null distribution of correlation coefficients (Z values) and a significant result inferred if ≥95% of the randomly generated statistics were greater than the observed value. Confidence intervals for F-statistics at the 95% confidence level were generated by boot-strapping using 1000 iterations.

Using the program NTSYS-pc (25) the combined 0/1 data matrix for isolates from all populations was used to construct a genetic similarity matrix of all possible pairwise comparisons of individuals within and among populations using Jaccard's similarity coefficient: $GS(ij) = a/(a + b + c)$. Where $GS(ij)$ is the measure of genetic similarity between individuals *i* and *j*, a is the number of polymorphic bands shared by *i* and *j*, *b* is the number of bands present in *i* and absent in *j*, and *c* is the number of bands present in *j* but absent in *i*. Trees were constructed using UPGMA cluster analysis to provide a graphic representation of the relationships among isolates. A cophenetic correlation coefficient was computed to assess the goodness of fit of the tree to the similarity matrix.

Genetic structure was also examined by analysis of molecular variance (AMOVA) using the ARLEQUIN software package (26). The AMOVA analysis was used to partition the variance in banding patterns within and among populations from the same geographical site over consecutive years, between sites on the same farm separated by approximately 1 km, and between all the locations sampled in Michigan. Significance values are assigned to variance components on the basis of a set of null distributions generated by a permutation process which randomly assigns individuals to populations and draws 1000 independent samples. AMOVA results were compared to the patterns and degrees of similarity revealed by fixation indices and cluster analysis.

Results

AFLP band characterization: Evaluation of 16 EcoRI + 2/MseI + 2 selective primer pair combinations indicated that EcoRI + AC/ MseI + CA (EAC/MCA) gave the most clearly resolved fragment profile and was used for AFLP analysis. AFLP profiles for isolate OP97, generated from separate DNA extractions on three separate occasions over a one year period, resulted in identical banding patterns with the only difference being minor changes in the intensity of the electropherogram signal. Occasionally individual reactions resulted in poorly resolved fingerprint profiles (eg, low intensity of signal) and were repeated until signals were deemed optimal. The EAC/MCA primer combination resulted in 94 clearly resolved fragments between 40 and 550 bps when considering the combined data from all the isolates recovered from Michigan. AFLP analysis of oospore progeny from cross OP97 x SFF3 revealed that all 107 progeny had the co-presence of bands which were present in only one of the parents indicating that each is a product of hybridization between the parent isolates. A comparison of the observed ratios to the 1:1 expected under Mendelian inheritance for 17 bands which were present in only one parent indicates that only one band segregated in a manner significantly different than expected at $P = 0.05$ (Table 3.1). Chi-square analysis also indicated that the observed ratios of A1:A2 compatibility types and S:IS mefenoxam sensitivities were not significantly different than expected under Mendelian inheritance (Table 3.1).

Table 3.1: Inheritance of 17 AFLP markers, compatibility type (CT), and mefenoxam sensitivity (MS) in 107 progeny of a cross between *Phytophthora capsici* isolates OP97 (A1/IS) and SFF3 (A2/S).

Marker[a]	Progeny ratio[b]	X^{2c}	P^{d}
E+AC/M+CA-66	47:60	1.58	0.20
E+AC/M+CA-97	51:56	0.23	0.70
E+AC/M+CA-146	53:54	0.01	0.90
E+AC/M+CA-149	60:47	1.58	0.20
E+AC/M+CA-156	64:43	4.12	0.04
E+AC/M+CA-159	56:51	0.23	0.70
E+AC/M+CA-244	46:61	2.10	0.17
E+AC/M+CA-258	52:55	0.08	0.80
E+AC/M+CA-270	53:54	0.01	0.98
E+AC/M+CA-282	56:51	0.23	0.70
E+AC/M+CA-290	62:45	2.70	0.13
E+AC/M+CA-328	55:52	0.08	0.80
E+AC/M+CA-351	61:46	2.10	0.15
E+AC/M+CA-398	55:52	0.08	0.80
E+AC/M+CA-431	55:52	0.08	0.80
E+AC/M+CA-435	57:50	0.46	0.90
E+AC/M+CA-444	49:58	0.76	0.85
CT	53:54	0.01	0.98
MS	47:60	1.58	0.20

[a] AFLP marker labels indicate the restriction enzymes (E = EcoR1, M=Mse1), the two selective nucleotides, and the size of the DNA fragment in basepairs.
[b] Presence:absence ratio's for AFLP markers, A1:A2 for CT, and sensitive (S): intermediately sensitive (IS) for mefenoxam sensitivity as determined by screening on 100 ppm AI mefenoxam amended media.
Table 3.1 (cont'd).
[c] X^2 value for testing 1:1 segregation (1 d.f.).
[d] Probability of the observed ratio occurring by chance under the null hypothesis of 1:1 segregation.

Gene and genotypic diversity: All 94 AFLP bands were scored for presence or absence in every isolate. The number of AFLP bands present in each population ranged from 68 to 80 with an average of 72, the number of polymorphic bands ranged from 39 to 49 with an average of 43, and the estimated average heterozygosity ranged from 0.16 to 0.19 with an average of 0.17 (Table 3.2).

Table 3.2: Population, number of isolates, total number of AFLP bands, number of polymorphic bands, and estimated heterozygosity for populations of *Phytophthora capsici* in Michigan.

Population[a]	No. of isolates[b]	No. of AFLP bands	No. and percent polymorphic bands (N = 94)	Estimated average Heterozygosity
SW1-A98	57	72	37 (39)	0.16
SW1-A99	141	72	37 (39)	0.16
SW1-B99	35	69	38 (40)	0.16
SW1-B00	24	69	38 (40)	0.16
SC1-A98	50	68	42 (45)	0.17
SC2-B99	45	71	43 (46)	0.17
C1-A00	48	77	41 (44)	0.17
NW1-A99	37	80	44 (47)	0.19
NW2-B98	24	73	46 (49)	0.18

[a] First two capital letters indicate location in Michigan with S = south, W = west, C = central, and N = north, the number following the location designator indicates the farm, the capital letter following the hyphen is a field designator, and the numbers following the field designator indicate year (eg; 00 = 2000).
[b] Total number of isolates with unique multilocus AFLP profiles.

Seventeen (18%) AFLP loci were fixed for the present state in all populations, 12 (13%) were polymorphic in all populations, and 65 (69%) were fixed for presence or absence in some populations and polymorphic in others. Of the 646 isolates fingerprinted 70% (454) had unique multilocus AFLP fingerprints (Table 3.3). The number of clones detected from single locations in Michigan varied from 3 to 15 and the number of isolates within any single clonal lineage ranged from 2 to 40 (Table 3.3). In all cases isolates with identical multilocus AFLP profiles had identical compatibility types and fell into the same mefenoxam sensitivity category.

Table 3.3: Clonal component of genotypic diversity within sample sets of *Phytophthora capsici* from Michigan.

Population[a]	Total no. of isolates	Unique AFLP genotypes (%)	No. of clonal lineages	Minimum:maximum no.of isolates per clone
SW1-A98	63	57 (0.94)	5	2:2
SW1-A99	200	141 (0.71)	15	2:40
SW1-B99	71	34 (0.48)	12	2:9
SW1-B00	36	24 (0.67)	5	2:8
SC1-A98	57	50 (0.88)	5	2:3
SC2-B99	56	45 (0.80)	5	2:5
C1-A00	51	48 (0.94)	3	2:2
NW1-A99	88	37 (0.42)	12	2:12
NW2-B98	24	18 (0.75)	3	2:3
Totals	646	454 (0.70)	65	-

[a] First two capital letters indicate location in Michigan with S = south, W = west, C = central, and N = north, the number following the location designator indicates the farm, the capital letter following the hyphen is a field designator, and the numbers following the field designator indicate year (eg; 00 = 2000).

Temporal dynamics: F-statistics (Φ_{ST}) for populations of *P. capsici* sampled from field SW1-A over 1998 and 1999, and field SW1-B sampled over 1999 and 2000 were 0.04 and 0.03 respectively (Table 3.4).

Table 3.4: F-statisics (Φ_{ST}) (below diagonal) and geographical distances (in km, above diagonal) between *Phytophthora capsici* sample sets collected from single locations over time and different locations in Michigan.

Populations[a]	SW1-A98	SW1-A99	SW1-B99	SW1-B00	SC1-A98	SC2-B99	C1-A00	NW1-A99	NW2-B98
SW1-A98	-	0	1	1	165	169	150	180	185
SW1-A99	0.04	-	0	1	165	169	150	180	185
SW1-B99	0.18	0.25	-	0	166	170	150	180	185
SW1-B00	0.25	0.24	0.03	-	166	170	150	180	185
SC1-A98	0.36	0.37	0.29	0.29	-	8	135	260	265
SC2-B99	0.33	0.35	0.32	0.33	0.28	-	130	255	260
C1-A00	0.36	0.37	0.33	0.32	0.38	0.40	-	140	145
NW1-A99	0.32	0.34	0.30	0.30	0.32	0.32	0.38	-	5
NW2-B98	0.36	0.37	0.31	0.32	0.33	0.33	0.33	0.27	-

[a] First two capital letters indicate location in Michigan with S = south, W = west, C = central, and N = north, the number following the location designator indicates the farm, the capital letter following the hyphen is a field designator, and the numbers following the field designator indicate year (eg; 00 = 2000).

At both locations the number and identity of AFLP bands resolved remained identical over time with 72 total bands recovered from populations at SW1-A and 69 bands recovered from populations at SW1-B (Table 3.2). The number and identity of bands polymorphic at the 95% level (37 for SW1-A and 38 for SW1-B) and the estimated average heterozygosity (0.16 for both locations) also remained constant over time (Table 3.2). AMOVA analysis of SW1-A and SW1-B over time partitioned 5% of the total variability between years for SW1-A, and < 1% of the total variability between years at

SW1-B (Table 3.5).

Table 3.5: Results of nested analysis of molecular variance (AMOVA) for *Phytophthora capsici* isolates based on 94 AFLP markers. Variance is partitioned (A) between 1998 and 1999 at SW1-A, (B) between 1999 and 2000 at SW1-B, (C) between combined sample sets from SW1-A and SW1-B, and (D) within and between samples sets from seven locations in Michigan.

Source of variation[a, b]	Degrees of freedom	Sum of squares	Variance component	Percentage of variation	P [c]
(A) SW1-A (98-99)					
Among populations	1	39.658	0.396	5.05	<0.0001
Within populations	197	1461.559	7.457	94.95	
(B) SW1-B (99-00)					
Among populations	1	6.678	0.016	0.27	.0029
Within populations	57	312.399	6.248	99.73	
(C) SW1-A vs SW1-B					
Among populations	1	234.790	2.762	27.34	<0.0001
Within populations	255	1820.294	7.340	72.66	
(C) All locations					
Among populations	6	1169.295	4.814	39.67	<0.0001
Within populations	273	1984.345	7.322	60.33	

[a] First two capital letters indicate location in Michigan with S = south, W = west, C = central, and N = north, the number following the location designator indicates the farm, the capital letter following the hyphen is a field designator, and the numbers following the field designator indicate year (eg; 00 = 2000).
[b] AMOVA analysis for all locations includes sample sets from a single year for locations SW1-A and SW1-B.
[c] P = the probability of obtaining a more extreme component estimate by chance alone based on 1000 sampling realizations.

Significant clonal reproduction was detected at both field sites within a given year with genotypic diversity reduced by 6% for SW1-A98, 29% for SW1-A99, 52% SW1-B99, and 33% for SW1-B00 (Table 3.3). No clonemates were detected between years for either location. Cluster analysis of all the isolates combined showed that samples from SW1-A and SW1-B branched from location specific nodes and that there was no clustering within either of the location specific clusters based on year (Figure 3.1). The ratio of A1:A2 compatibility types at each location was 35:22 for SW1-A98, 75:66 for SW1-A99, 18:16 for SW1-B99, and 12:12 for SW1-B00 (Table 3.6). The percentage of isolates falling into the six mefenoxam sensitivity/compatibility type categories remained relatively similar between years at each location with a breakdown of 0 and 1% A1/S, 7 and 12% A1/IS, 54 and 40% A1/I, 0 and 1% A2/S, 11 and 16% A2/IS, and 28 and 30% A2/I for location SW1-A in 1998 and 1999 respectively (Table 3.6). The percentage of isolates in each of the six categories for SW1-B was 41 and 29% A1/S, 12 and 21% A1/IS, 0 and 0% A1/I, 32 and 21% A2/S, 12 and 21% A2/IS, and 3 and 8% A2/I between 1999 and 2000 respectively (Table 3.6).

Spatial structure: Pairwise F-statistics (Φ_{ST}) between populations separated by geographical distances ranging between 1 and 265 km had values ranging from 0.18 to 0.40 (Table 3.4). A Mantel test comparing the geographical distance matrix to the Φ_{ST} matrix showed essentially no correlation between geographical distance and genetic differentiation with 8 of the 999 permutated data sets having Z-scores ≥ the original Z-score ($r = 0.45$, $P = 0.009$). Although there was not a direct correlation between geographical distance and genetic differentiation, UPGMA cluster analysis based on Jaccard's similarity coefficient (Figures 3.1 and 3.2) indicates that populations located within the same vegetable production region were more similar to each other than to populations located within different regions.

Table 3.6: Location, year, hosts, compatibility type, and mefenoxam sensitivity of genetically unique *Phytophthora capsici* isolates collected in Michigan between 1998 and 2000.

Population[a]	Hosts[b]	No. of isolates[c]	Compatibility type and mefenoxam sensitivity[d]					
			A1/S	A1/IS	A1/I	A2/S	A2/IS	A2/I
SW1-A98	S, PK	57	-	4	31	-	6	16
SW1-A99	S	141	1	17	57	1	23	42
SW1-B99	S	34	14	4	-	11	4	1
SW1-B00	S	24	7	5	-	5	5	2
SC1-A98	C	50	10	17	2	10	11	-
SC2-B99	C	45	-	6	22	-	2	15
C1-A00	P	48	20	-	-	28	-	-
NW1-A99	S, C	37	25	-	-	12	-	-
NW2-B98	P	18	10	-	-	7	1	-
	Totals	454	87	53	112	74	52	76

[a] First two capital letters indicate location in Michigan with S = south, W = west, C = central, and N = north, the number following the location designator indicates the farm, the capital letter following the hyphen is a field designator, and the numbers following the field designator indicate year (eg; 00 = 2000).
[b] S = squash, C = cucumber, PK = pumpkin, and P = pepper.
[c] Total number of isolates with unique multilocus AFLP profiles.
[d] Mefenoxam sensitivity determined by in vitro screening on 100 ppm AI amended media with S = < 30% growth of control (GC), IS = between 30 and 90% GC and I = >90% GC.

The SW1-A and SW1-B populations which were located approximately 1 km apart on the same farm had the lowest Φ_{ST} scores ranging from 0.18 to 0.25 when comparing each SW1-A population to each SW1-B population. AMOVA analysis based on comparing combined sample sets from SW1-A to combined sample sets from SW1-B partitioned the total variation into 73% within and 27% between locations (Table 3.5).

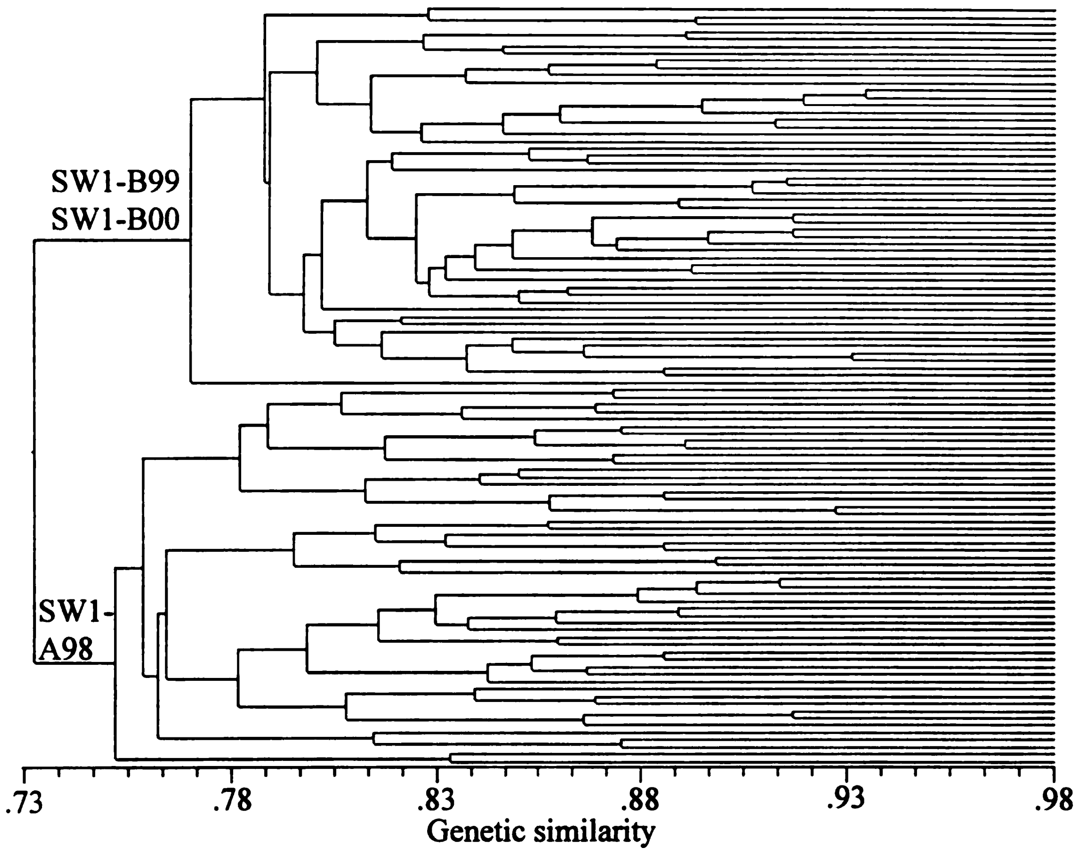

Fig 1: UPGMA cluster analysis of *Phytophthora capsici* isolates from location SW1-B over 1999 and 2000 (N=58) and SW1-A in 1998 (N=57) based on the Jaccard similarity coefficient using 94 amplified fragment length polymorphism (AFLP) markers. Nodes contain isolates exclusively from single locations. Location identifiers precede the inclusive node and are indicated by region (S = south, N = north, W = west, and C = central) and a farm identifier (1,2,...n) prior to the hyphen with a field indicator (A, B,...n) and the year of sampling (eg; 00 = 2000) following.

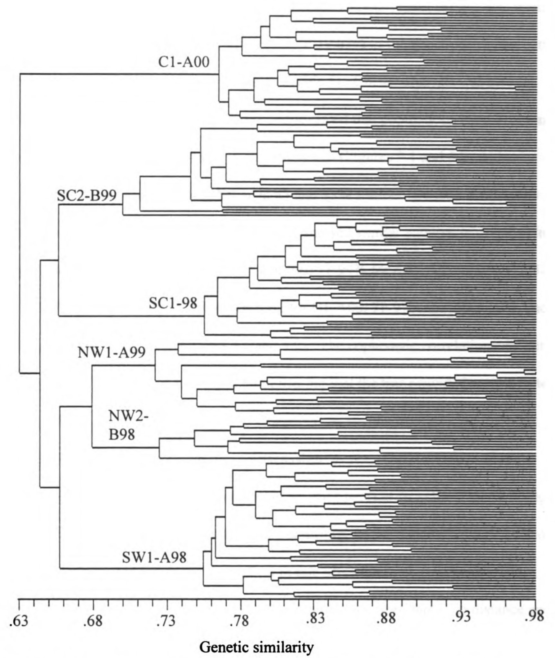

Fig 2: UPGMA cluster analysis of 255 *Phytophthora capsici* isolates from seven locations in Michigan based on the Jaccard similarity coefficient using 94 amplified fragment length polymorphism (AFLP) markers. Nodes contain isolates exclusively from single locations. Location identifiers precede the inclusive node and are indicated by region (S = south, N = north, W = west, and C = central) and a farm identifier (1,2,...n) prior to the hyphen with a field indicator (A, B,...n) and the year of sampling (eg; 00 = 2000) following.

Genetic structure: The overall Φ_{ST} value when analyzing sample sets from all 7 locations combined was 0.34 (s.d.= 0.03). An AMOVA analysis of sample sets from all locations corroborated this finding and attributed 39.67% of the genetic variation between populations and 60.33% within populations (Table 5). Cluster analysis was also in agreement with the overall fixation index and revealed that populations from different geographical locations branched from specific nodes (Figures 3.1 and 3.2) with population specific clusters being between 63 and 75% similar. Genetic similarities between individuals within each of the clusters showed similar patterns with individuals ranging between 75-95% for SW1-A(98 and 99), 77-94% for SW1-B(99 and 00), 75-94% for SC1-A98, 69-92% for SC2-B99, 76-95% for C1-A00, 71-97% for NW1-A99, and 72-93% similar for NW2-B98 (Figures 3.1 and 3.2). The cophenetic correlation coefficient for the overall tree (Figure 3.2) was 0.84 indicating that the tree provided a good fit to the data matrix.

Discussion

The formulae, or tools, of population genetics provide an indirect methodology for characterizing the evolutionary forces which contribute to the structure of a population. The two evolutionary forces we focused on in this investigation are migration and reproduction. In the case of *P. capsici* it would appear, a priori, that long distance aerial dispersal similar to that described for *P. infestans* is not common (5). If this is true, then the observed increase in disease at new locations in Michigan and recurrence of *P. capsici* at previously diseased sites may be due to spread through waterways, the movement of infected plant material, or in the case of previously infested sites, to survival of *P. capsici* as dormant inoculum in the soil. The advantage of using population genetic theory to unravel the history of a population is that an understanding of the mechanism of dispersal is unnecessary. In theory, the history should be evident in the patterns of genetic variation at the individual and group level. One very important point is that there must be some level of genetic variability from which to make inferences. If there was only asexual reproduction, or if *P. capsici* was exclusively self-fertile, then it would be very difficult to draw conclusions concerning the evolutionary forces acting upon a population.

A 1998 survey of phenotypic diversity suggested that sexual reproduction is active and that recombination via meiosis may play an important role in integrating mefenoxam insensitivity into a population (10), especially because the mating between, or self-fertilization of, intermediately sensitive isolates would directly generate the fully insensitive phenotype. This study was followed by an intensive investigation of a single geographical location (SW1-A) over time (11). During 1999 and 2000, location SW1-A was chosen as an experimental site for testing alternative management strategies for the production of squash, none of which included the use of mefenoxam. Sampling was conducted over the course of the 1999 and 2000 growing seasons to assess whether there is a significant cost incurred by mefenoxam insensitivity. In order to measure the frequency of MS genotypes unambiguously it was important to separate clonal from non-clonal lineages. AFLP markers proved to be useful in this respect due to the large number of loci resolved for a single isolate and also provided an opportunity to estimate the amount of genetic diversity present within and between years at this location. Results of this study indicated that the amount of genic and genotypic diversity was high and did not decrease significantly between years. Clonal reproduction within a single season was found to be significant, but no clones were recovered between years. The overall conclusion was that this population appeared to be large enough to withstand the chance effects of genetic drift and the frequency of mefenoxam insensitivity did not decrease over time (11). Almost 100% of the isolates recovered from this site were either intermediately or fully insensitive to mefenoxam between 1998 and 2000, whereas nearby sites (as close as 1 km) had many sensitive isolates. The phenotypic data alone suggests that extensive migration, or introduction, from geographically separated sites did not contribute significantly to population structure at SW1-A.

The inheritance of MS in sexual crosses between isolates with differing levels of mefenoxam sensitivity was reported previously. It was not possible, based solely on phenotype, to determine how many of the progeny were the products of self-fertilization versus true hybridization between the parents. AFLP analysis of the progeny from the cross between OP97 x SFF3 indicates that all 107 progeny investigated have the co-presence of bands only present in one parent and thus are all products of hybridization. Chi-square analysis of the inheritance of 17 bands used in the overall population analysis indicates that all but one AFLP marker was inherited as a simple Mendelian character. This is consistent with research based on mapping of the *P. infestans* genome (28). It also suggests that hybrid individuals may be more viable than self-fertilized, or inbred, individuals. Genetically determined self-incompatibility is common in plants (7) and may play some role in our observed results.

Location SW1-B, which is approximately 1 km from SW1-A, had a serious *P. capsici* epidemic on squash in 1994 and was planted to corn or soybeans until 1999. In 1999 and 2000, the grower planted squash in this field and *P. capsici* was isolated from infected plants during both years. The overall trends based on phenotype and AFLP analysis of the SW1-B isolates were consistent with the findings from SW1-A. Clonal lineages were common within a single season, but no clones were recovered between years. Genic and genotypic diversity remained high in both years with 40% of the AFLP markers polymorphic in a given year and between 48 and 67% of the isolates having unique multilocus AFLP profiles in 1999 and 2000 respectively. When comparing sample sets between years, both SW1-A and SW1-B had fixation indices approaching zero (0.04 and 0.03) indicating that genic diversity was maintained between growing seasons. AMOVA analysis corroborated this finding and partitioned 5% of the total genetic variability between years for SW1-A and <1% between years for SW1-B. A major difference between the two sites was that 81% of the isolates with unique genotypes recovered at SW1-B were fully sensitive to mefenoxam, whereas 1% of the genetically unique isolates recovered at SW1-A were sensitive to mefenoxam. The structuring revealed by screening isolates for mefenoxam sensitivity was supported by the estimated pairwise fixation indices based on AFLP markers. Results indicate that between 75 and 82% of the total genetic diversity present at these two sites is found in any one population and that 18 to 25% is found at one site and not the other. AMOVA analysis corroborated this finding and partitioned 27% of the total genetic variability between populations. Cluster analysis clearly shows that isolates from SW1-B cluster together from a single node with no blurring of the tree due to isolates from SW1-A. SW1-B is not irrigated from a water source common to SW1-A, nor are there streams or drainage ditches which connect the two sites or run near SW1-B. To our knowledge there were not any fields planted to susceptible hosts, other than SW1-A, within at least 10 km of SW1-B during 1999 or 2000. Without a sample set from the 1994 epidemic at SW1-B it is not possible to say conclusively, but there is a strong probability that the 1999 epidemic at SW1-B originated from dormant inoculum generated five years earlier.

The considerable genetic variation observed within and between sites SW1-A and SW1-B turned out to be representative for the entire state. Although populations within a given region appear to be slightly more similar to each other than to populations from different regions, there was no correlation between Φ_{ST} and geographical distance. Populations 1 to 8 km distant had only slightly lower fixation indices than populations up to 265 km apart. The populations in total were surprisingly uniform in their levels of genetic diversity with the total number of AFLP markers resolved in any one population, the number of polymorphic fragments, and the estimated average heterozygosities all being surprisingly similar. Cluster analysis provided a useful visual representation of the trends within and between populations. Isolates clustered unambiguously from location specific nodes with populations from the same region showing slightly more similarity. The range of genetic similarities between individual isolates within any one population specific cluster was similar across all populations. The levels of genetic similarity between populations as visualized by UPGMA cluster analysis were supported by both the overall fixation index of 0.34 and the overall AMOVA analysis which partitioned total genetic variability into a 40% between and 60% within population component.

The null hypothesis that migration significantly contributes to the genetic structure of populations of *P. capsici* in Michigan is not supported by this investigation. If significant migration occurred between sites the expectation is that there would be less diversity between populations. Certainly, migration from some source population(s) had to occur at some point in time for each of these locations and there is no way to rule out the possibility that migration via the same, or some alternative route, does not still occur. What can be concluded is that migration does not exert enough of an equalizing force between geographically separated populations to counterbalance what are likely a combination of founder effects, genetic drift, mutation, and selection pressures. An aspect of *P. capsici*'s life history which may also contribute to the apparent genetic stability of populations over time is the survival and non-synchronous germination of oospores. An interesting example of how dormant inoculum (eg; seeds) may act as a stabilizing force against the effects of genetic drift is provided by Epling et al. who followed small populations of *Linanthus parryae* over a 20 year period. It was found that *L. parryae* had an extensive bank of dormant seeds that could remain viable for at least six years and it was concluded that this dense seed store counterbalanced the effects of genetic drift by limiting the effects of chance reductions in population size (2).

The finding that migration does not appear to play a significant role in shaping or maintaining population structure puts the amount, and apparent stability, of genetic variability within individual populations of *P. capsici* in Michigan into perspective. The implications are that the founding individuals in each of these separate geographical locations contained what appears to be a high level of genetic variability and that, according to the results from SW1-A and SW1-B, this variability is maintained over time. A possible explanation for the population structure of *P. capsici* in Michigan is that oospores formed by hybridization between genotypically diverse parents may have a significant survival or viability advantage compared to either oospores produced by self-fertilization or clonal inoculum. This hypothesis is supported by the fact that all the oospores recovered and analyzed from the cross between OP97 and SFF3 were hybrid, and our inability to detect clonal overwintering in natural populations.

An evaluation of currently employed management strategies in light of these findings suggests that rotation to non-susceptible hosts for up to five years may not protect against epidemics due to *P. capsici*. These results also suggest that factors which may contribute to the initial colonization (eg; source of irrigation water, the disposal of infected plant material, infected transplants) need to be carefully considered and, if possible, every possible action taken to avoid the introduction of *P. capsici* into un-infested field sites.

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