New Frontiers in Plant Disease Losses and Disease Management

The Spatiotemporal Genetic Structure of *Phytophthora capsici* in Michigan and Implications for Disease Management

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Root, crown, and fruit rot caused by *Phytophthora capsici* Leonian is a limiting factor for the production of peppers, tomatoes, and cucurbit crops in Michigan and the United States. Like many species in the genus *Phytophthora*, *P. capsici* has the potential for rapid polycyclic disease development from a limited amount of initial inoculum (6). *P. capsici* produces caducous sporangia that can be spread by wind-blown rain or release 20 to 40 motile zoospores in the presence of free water. The polycyclic phase of disease development is thought to be driven primarily by asexual spore dispersal at a local scale (within and down rows). Sexual reproduction requires both the A1 and A2 compatibility types (CTs) and results in the production of thick-walled oospores. Oospores are thought to serve as the primary survival structure outside of host tissue.

Recommended disease management strategies stress the importance of avoiding excess water in the plant rhizosphere by using well-drained fields, conservative irrigation, and planting on raised beds. Additional recommendations include rotation to nonsusceptible hosts for at least 2 years and the use of fungicides. The phenylamide fungicide (PAF) mefenoxam is a systemic compound with high activity against *P. capsici* and has been used by growers throughout the United States to control *P. capsici*. Insensitivity to PAF has been reported for a number of other oomycetous organisms (*Bremia lactucae*, *P. infestans*, and *P. sojae*, etc.) and appears to be conferred by a single incompletely dominant gene of major effect (1). Growers in Michigan practicing 2+-year rotation in well-drained fields using an array of fungicidal management tools have experienced significant losses to *P. capsici*. Michigan is the number one producer of cucumbers for pickling in the United States and it was at the request of grower groups associated with this industry that research into the epidemiology and reproductive biology of *P. capsici* on cucurbit hosts was initiated.

Although many researchers cite oospores as the most likely propagule for survival outside of host tissue, there have been very few investigations specifically aimed at determining the impact of sexual reproduction in natural populations of *P. capsici*. Our hypothesis was that the sexual stage may play an important role not only in survival but also in the adaptation of *P. capsici* populations to environmental stresses (e.g., fungicides). Our goal was to perform a comprehensive investigation of the phenotypic and genetic diversity present in *P. capsici* populations from the major vegetable production regions of Michigan, with the implicit intention of addressing questions concerning epidemiology, repro-

ductive biology, and the durability of currently recommended management strategies.

METHODOLOGY

Isolate collection and maintenance. Sampling of diseased fields began at the end of the 1997 growing season and continued through September 2000. In all cases, fields were sampled on a grid with quadrants varying from 40 m² to 12 km². A limited number of isolates were collected in 1997. In 1998, the strategy was to collect as many samples from as many fields as possible. This strategy was modified in 1999 and 2000 to focus on specific fields. Isolations from diseased plants were made onto selective media and single zoospore cultures were generated according to standard single sporing techniques (3). Isolates were placed into long-term storage (15°C) using a hemp seed/sterile water technique.

Phenotypic characterization. Single zoospore isolates were screened for CT using known A1 and A2 isolates. In vitro screening techniques published for other *Phytophthora* species for assessing sensitivity to mefenoxam were compared and a novel, simple, high dose screen using 100 ppm of mefenoxam-amended V8 agar was found to separate field isolates into three modal distributions that appeared consistent with the expectations of a single incompletely dominant gene governing mefenoxam insensitivity (e.g., sensitive, intermediately sensitive, and fully insensitive). These putative mefenoxam sensitivity (MS) groupings were tested by performing a series of crosses and testing whether the observed progeny sets met the expectations for Mendelian inheritance of a single incompletely dominant gene controlling insensitivity to mefenoxam. Sexual crosses were conducted on unclarified V8 agar plates and incubated for 3 months in the dark. Individual germinated oospores were recovered after 3 months using previously published techniques (2).

The efficacy of this in vitro mefenoxam screening technique was further tested in pumpkin seedlings using progeny from a cross between parents intermediately sensitive to mefenoxam. Nine isolates from each of the three MS categories were screened for pathogenicity on untreated seedlings. Single sensitive, intermediately sensitive, and fully insensitive isolates were then placed onto the unwounded surface of plants treated with either a field rate of mefenoxam, three times the field rate, or distilled water. Lesion diameters on seedling stems were measured after 4 days.

Genetic characterization. Single zoospore isolates were grown in antibiotic-amended V8 broth for 3 days at room temperature. Mycelial mats were washed, frozen, lyophilized, and ground with a sterile mortar and pestle. DNA was extracted with either a Qiagen Dneasy extraction kit (Qiagen, Valencia, CA) or via a cetyltrimethylammonium bromide (CTAB) procedure. A variety

of methods for generating molecular markers were tested for efficacy including isozyme, random amplified polymorphic DNA, and amplified fragment length polymorphism (AFLP). The AFLP technique resulted in a large number of reproducible markers and was chosen to characterize samples of *P. capsici* from Michigan. The AFLP technique involves cutting genomic DNA with moderately rare cutting (*EcoRI*) and frequent cutting (*MseI*) restriction enzymes, while concomitantly ligating synthetic adaptor fragments of DNA to the sticky ends created by the restriction enzymes (7). The result is a large number of DNA fragments that have ends with known DNA sequences. Amplification of fragment subsets (termed fingerprints) can be accomplished using polymerase chain reaction (PCR) primers complementary to the adaptor sequences with additional "selective" nucleotides. Changing the amount and type of selective nucleotides results in different subsets or fingerprints. Stringent PCR cycling parameters (touchdown technique) are used to ensure the fidelity of the reaction. For the analysis summarized here, adaptor sequences and fluorescent labeled selective primers were purchased as a kit through Perkin-Elmer ABI (Applied Biosystems, Foster City, CA). Using this system, AFLP fragments were resolved on a polyacrylamide gel by an ABI 377 gene sequencer. Fluorescent labels were excited by a laser and band emissions were analyzed in the form of an electropherogram where peaks represent individual bands. The sizing of fragments was particularly robust because a DNA ladder was loaded with every sample into the gel. To test for the reproducibility of fingerprints, DNA was extracted from a single isolate on three separate occasions approximately 3 months apart and subjected to the aforementioned protocol.

Data analysis. Isolates with identical multilocus AFLP fingerprints were considered to be members of the same clonal lineage and only a single representative was used for analysis. Because AFLP markers can only be scored confidently for presence (1) or absence (0), allele frequencies were estimated based on the assumption that populations under investigation meet the criterion for Hardy-Weinberg equilibrium, and that loci have only one "present" allele. The term population refers to all samples taken from a single field during a single year.

Genetic diversity within single populations was assessed by calculating the average number of polymorphic bands and estimating the average heterozygosity. Fixation indices were calculated according to methods of Weir and Cockerham (8) for populations from the same site over multiple years and among populations in Michigan using the program tools for population genetic analysis (TFPGA) (M. P. Miller, Northern Arizona University, Flagstaff). Confidence intervals for *F* statistics at the 95% confidence level were generated by bootstrapping at 1,000 iterations. The program NTSYS-pc version 2.02k (Exeter Software, Setauket, NY) was used to construct a similarity matrix from the presence/absence (1/0) data. Cluster analysis using the unweighted pair group with arithmetic averages (UPGMA) method was performed on the matrix and a tree was generated to give a visual representation of isolate similarity. Excoffier's ARLEQUIN program (L. Excoffier, University of Geneva) was used to assess population differentiation using a phenetic approach termed analysis of molecular variance (AMOVA), which allows for total genetic variation to be partitioned within and among populations using a classical analysis of variance (ANOVA).

RESULTS

Phenotypic results. Five isolates were recovered in 1997 from five different farms (four A1 and one A2 CT). One isolate was fully insensitive to mefenoxam, whereas the other four were fully sensitive. These findings prompted the extensive sampling conducted in 1998 in which 523 isolates (473 from cucurbits and 30 from bell pepper) were collected from 14 farms. A frequency histogram plotting percent growth of control on 100 ppm of

mefenoxam-amended media versus number of isolates revealed a trimodal distribution (3). Putative MS categories were assigned based on these groupings with sensitive (S) <30% growth of control, intermediately sensitive (IS) between 30 and 90% growth of control, and insensitive (I) >90% growth of control. In vitro crosses between isolates representative of the different putative sensitivity categories (S × S, I × S, IS × S, and IS × IS) resulted in progeny sets not significantly different than expected for insensitivity inherited as a single incompletely dominant gene unlinked to CT (*P* = 0.05) (3). In 1998, 55% of the isolates were sensitive to mefenoxam, 32% were intermediately sensitive, and 13% were fully insensitive to mefenoxam. A1 and A2 CTs were recovered in a ratio of approximately 1:1 in 8 of the 14 farms. Oospores were detected in naturally diseased cucurbit fruit from four farms, and 223 oospore progeny were recovered and germinated from a single diseased cucumber. All six possible MS × CT combinations were detected in this naturally occurring oospore progeny set (3).

In planta studies using sensitive, intermediately sensitive, and fully insensitive *P. capsici* isolates supported the in vitro screening categories, with sensitive isolates causing no disease on mefenoxam-treated plants, intermediately sensitive isolates being slowed by mefenoxam, and fully insensitive isolates showing no difference in the ability to colonize host tissue between treated and untreated plants at three times the field rate. All the progeny isolates were pathogenic on untreated pumpkin plants (K. H. Lamour and M. K. Hausbeck, unpublished data).

Sixty-three mefenoxam insensitive (18% intermediate and 82% fully insensitive) isolates were recovered from a single southwest Michigan field in 1998. Field experiments were conducted in this field during 1999 and 2000, testing alternative cultural control strategies, and no mefenoxam was applied. Two hundred isolates were recovered from this site over the course of the 1999 season and 34 isolates at the beginning of the 2000 season. Of the 200 isolates recovered in 1999 from this field, 141 had unique AFLP genotypes. Seventy percent of these were fully insensitive to mefenoxam, 28% were intermediately sensitive, and 2% were sensitive. In 2000, 15% of the isolates were intermediately sensitive and 85% were fully insensitive. A single fully insensitive clonal lineage rose in frequency over the course of the 1999 season and comprised 20% of the total number of samples recovered (4).

During 1999 and 2000, approximately 2,500 isolates were recovered from farms in Michigan. Both the A1 and A2 CTs were present in every field sampled, and mefenoxam insensitivity was detected in the majority of farms that had a history of mefenoxam use.

Genetic results. Nine populations from the four major vegetable production areas of Michigan were analyzed with the AFLP procedure (*N* = 641). AFLP analysis resolved a total of 94 clearly discernable markers when considering all the isolates together. No single isolate or group of isolates from a single location contained all 94 markers. The total number of AFLP loci in a single population ranged from 68 to 80. Seventeen (18%) fragments were fixed for the present state across all populations, 12 (13%) fragments were polymorphic in all populations, and 65 (69%) were fixed for presence or absence in some populations and polymorphic in others. The number of polymorphic bands within a single population ranged from 37 to 46 with estimated heterozygosities ranging from 0.18 to 0.22. Clonal reproduction was significant within single fields over the course of the growing season. For example, genotypic diversity in a single field ranged from 100% at the beginning of the growing season (seedling stage) to <30% at the time cucurbit fruit were ready for harvest (4). When considering all nine populations, genotypic diversity ranged from 42 to 96% with an average of 74% of the isolates in any sample set having unique genotypes. Although clonal reproduction was significant within single fields within years, no clones were recovered from single fields between years or among fields separated by at least 1 km. Fixation indices (Φ_{ST}) between the

populations sampled on consecutive years were very close to zero, indicating that gene diversity was not measurably impacted by genetic drift (5). The overall estimated ϕ_{ST} for populations from different locations was 0.35, indicating that approximately 35% of the total genetic diversity present in Michigan *P. capsici* populations is found among populations and 65% is found within any one population. AMOVA partitioned genetic diversity among (40%) and within (60%) populations. The similarity tree based on UPGMA cluster analysis clearly showed that isolates from the

same site sampled over years branched from the same node, with no clustering of isolates based on the year of sampling. Cluster analysis also clearly showed that populations separated geographically branched from population-specific nodes (5).

DISCUSSION

During the past 10 years, Michigan has experienced a steady increase in the incidence of root, fruit, and crown rot on cucurbits

Fig. 1. Spore types and signs of infection caused by *Phytophthora capsici* on cucurbit fruit: **A**, infected cucumber, **B**, pumpkin, and **C**, acorn squash fruit. **D**, Scanning electron microscope (SEM) photo of an infected cucumber showing tufts of sporangia produced on the surface of the fruit (Bar = 300 μ m). **E**, Close-up of a single tuft of sporangia (Bar = 30 μ m). **F**, Typical papillate sporangium with a long pedicel (Bar = 20 μ m). **G**, Zoospores exiting sporangia after immersion in water (Bar = 50 μ m). **H**, SEM photo of a single encysted zoospore that germinated and directly penetrated the epidermis of a cucumber fruit (Bar = 4 μ m). **I**, Typical amphigynous oospore (Bar = 10 μ m). **J**, A germinating oospore with multiple germ tubes and a terminal sporangium (Bar = 100 μ m).

caused by *P. capsici*. Rotation to nonsusceptible hosts, in conjunction with cultural and chemical control strategies, have not provided economic control. Correspondence with other vegetable pathologists suggests that this phenomenon is not confined to Michigan, and a similar increase in control failures due to blight by *P. capsici* is being reported throughout the United States.

Investigation of the inheritance of MS demonstrated that MS is inherited as a single incompletely dominant gene unlinked to CT. In 1998, all six possible MS $\times$ CT combinations were present in single fields and insensitivity to mefenoxam was common in Michigan. Typical amphigynous oospores were observed in *P. capsici*-infected cucurbit fruit from multiple locations, and oospore progeny from a single naturally infected fruit showed segregation for MS and CT. These findings strongly support the hypothesis that sexual reproduction is occurring in the field, and also suggest that sexual recombination may directly generate progeny fully insensitive to mefenoxam. Tracking a single mefenoxam insensitive population over 2 years in the absence of mefenoxam selection pressure suggests that costs associated with mefenoxam insensitivity are minimal.

Estimates of average heterozygosity and polymorphism indicate surprisingly high levels of gene and genotypic diversity in all the populations of *P. capsici* analyzed. Tracking a single population through an entire growing season showed that asexual reproduction plays a significant role in disease development within a single season. Sampling single fields over consecutive years suggested that clones do not survive Michigan winters and that oospores are the primary survival propagule. Estimation of fixation indices for samples from the same site over consecutive years suggested that there was not a significant reduction in genetic diversity between growing seasons. This implies that populations are large enough to withstand dramatic effects of genetic drift. Cluster analysis revealed unambiguous groups corresponding to geographical locations with regional populations showing more similarity overall than populations from different regions. Population pairwise fixation indices corroborated this finding. The estimated overall fixation index and AMOVA are in agreement with both, suggesting that most (approx 60%) of the total genetic variability in Michigan is found within any one population, but that a relatively large component (40%) of genetic variability is found among populations.

Recommendations based on our findings are as follows: (i) the fungicide mefenoxam may be of limited usefulness because insensitivity appears to be selected for rapidly and is unlikely to decrease when mefenoxam selection pressure is removed; (ii) fields with epidemics are likely to harbor oospores for an extended amount of time (at least 5 years), and this factor must be considered before replanting to susceptible hosts; and (iii) factors that may contribute to the introduction of *P. capsici* into uninfested fields (e.g., drainage ditches between farms, irrigation ponds, and the dumping of culls) need to be considered and if possible avoided, because once an epidemic is established we have found no evidence that the population will become extinct in an agriculturally meaningful time period.

From an evolutionary perspective, it is clear that *P. capsici* has successfully colonized a number of geographical locations in

Michigan and that each of the populations sampled thus far have similarly high levels of genetic variability. The genetic stability of single populations over multiple years, the high fixation indices between even geographically close populations (1 km), and the clear structuring based on UPGMA cluster analysis all suggest that long-distance dispersal of inoculum is not common and that geographically isolated populations are also genetically isolated. It appears that the sexual stage of the *P. capsici* life cycle plays a significant role in survival as well as maintaining both genic and genotypic diversity, and has likely played a key role in the evolution of mefenoxam insensitivity. The combination of high levels of genetic variability, thick-walled oospores, and polycyclic asexual disease development make *P. capsici* a formidable pathogen (Fig. 1). This work underscores the need for management strategies aimed at preventing the spread of *P. capsici* to uninfested field sites and suggests that management strategies aimed at limiting spread within a single season may be the only option for growers with *P. capsici*-infested fields.

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