Occurrence and Characterization of a *Phytophthora* sp. Pathogenic to Asparagus (*Asparagus officinalis*) in Michigan

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ABSTRACT

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A homothallic *Phytophthora* sp. was recovered from asparagus (*Asparagus officinalis*) spears, storage roots, crowns, and stems in north-west and central Michigan in 2004 and 2005. Isolates ($n = 131$) produced ovoid, nonpapillate, noncaducous sporangia $45\ \mu\text{m}$ long $\times$ $26\ \mu\text{m}$ wide and amphigynous oospores of 25 to $30\ \mu\text{m}$ diameter. Mycelial growth was optimum at 25°C with no growth at 5 and 30°C . All isolates were sensitive to 100 ppm mefenoxam. Pathogenicity studies confirmed the ability of the isolates to infect asparagus as well as cucurbits. Amplified

fragment length polymorphism analysis of 99 isolates revealed identical fingerprints, with 12 clearly resolved fragments present and no clearly resolved polymorphic fragments, suggesting a single clonal lineage. The internal transcribed spacer regions of representative isolates were homologous with a *Phytophthora* sp. isolated from diseased asparagus in France and a *Phytophthora* sp. from agave in Australia. Phylogenetic analysis supports the conclusion that the *Phytophthora* sp. isolated from asparagus in Michigan is a distinct species, and has been named *Phytophthora asparagi*.

Additional keywords: Fusarium crown and root rot, *P. megasperma*, spear rot.

Michigan ranks third nationally for fresh and processing asparagus (*Asparagus officinalis*), following California and Washington (3). In 2007, 10.7 million kg of asparagus spears were harvested from fields located in Mason and Oceana Counties in the northwest and Van Buren County in the southwest. Asparagus is a deep-rooted perennial crop that can have a production span of more than 15 years. In Michigan, asparagus production fields are established from 1-year-old crowns that are produced from seeds in nursery fields. Crowns consist of rhizomes and fleshy, fibrous roots. The thick and somewhat woody rhizomes are sites of nutrient and starch accumulation and contain buds that give rise to spears. The spears are edible, tender, succulent aerial stems with a prominent modified leaf. If not harvested, spears expand into extensively branched ferns with cladophylls, the asparagus true leaves (13).

Several diseases limit asparagus production (4,14,19). *Phytophthora* spear and root rot is a significant problem in most asparagus growing areas in Europe (39), North America (4,50), Australia (10), and New Zealand (6). In the United States, the disease is most prevalent in warm climates, but has also been reported in cooler growing regions (14). Spear and root rot was first reported and described in California in 1938 and a *Phytophthora* sp. was identified as the causal agent (4). The disease was not extensively studied until the 1980s, when 34 to 50% yield losses occurred in California due to spear rot in the field and postharvest disease development, caused predominantly by *P. megasperma* var. *sojae* and other *Phytophthora* spp. (19,20).

Phytophthora spear and root rot varies among growing seasons and sites; the pathogen is highly favored by excessive rainfall and

poor soil drainage (21,22,24). Disease symptoms include soft, water-soaked lesions on shoots slightly above or below the soil line. Under favorable conditions, lesions rapidly elongate and turn light brown in color resulting in shriveling and curved growth of the spear. Infected storage roots are initially firm but become water-soaked, and shrivel as lesions expand. Root mass and vigor can be reduced. Internal tissues of the infected crowns may be yellow to brown in color; however, these symptoms can also be attributed to Fusarium crown and root rot, caused by *Fusarium oxysporum* f. sp. *asparagi* (14).

P. megasperma Dreschsler has been associated with *Phytophthora* spear and root rot, but other *Phytophthora* spp., such as *P. richardiae*, *P. cryptogea* (19,23), *P. megasperma* var. *sojae* (2,5,6,16,17,19,28), and *P. cactorum* (39) have also been identified from several asparagus production areas worldwide. Because morphological characteristics of different *P. megasperma* taxa can be indiscernible, molecular methods such as sequence analysis of various regions of the ribosomal DNA (32,52), nuclear and mitochondrial DNA (27), and restriction fragment length polymorphisms (26), have been used to separate and further clarify the complex *P. megasperma* taxa. Based on molecular parameters, *P. megasperma* isolates have been sorted into nine groups, five of which are host-specific and four non-host-specific. Some isolates that were previously considered to be *P. megasperma* have been renamed as *P. medicaginis* from alfalfa (*Medicago sativa*), *P. sojae* from soybean (*Glycine max*), and *P. trifolii* from clover (*Trifolium pratense*) (30). In addition, host-specific Fraser fir (*Abies fraseri*) and asparagus groups of *Phytophthora* spp. have been determined, and are currently being studied and reclassified (7,26). The non-host-specific *Phytophthora* spp. isolates are included in an undefined broad host range group (27).

In spring 2004, a spear and root rot was observed in several asparagus fields in Oceana, Ingham, and Van Buren Counties, Michigan (45). A *Phytophthora* sp. with morphological charac-

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teristics similar to *P. megasperma* was consistently isolated from spears, roots, crowns, and stems of symptomatic plants. The complexity of the taxonomy of this *Phytophthora* group raises the following questions. Which *Phytophthora* spp. infect asparagus in Michigan? Are the isolates specific to asparagus or can they infect other crops commonly grown in the asparagus production regions? Answers to these questions will assist in defining strategies necessary to manage *Phytophthora* spear and root rot of asparagus in this significant asparagus-producing region of the United States. The objective of this study was to describe the phenotypic and genotypic diversity of the *Phytophthora* species causing disease on asparagus in Michigan.

MATERIALS AND METHODS

Field sampling and symptoms. Asparagus spears and crowns growing in fields located in northwest (29 fields), central (2 fields), and southwest (10 fields) Michigan were sampled for *Phytophthora* spp. in the spring of 2004 and 2005. In the northwest, fields were located near the Asparagus Research Station in Hart, an area with mostly Benona sandy soils. In the central region, samples were collected from fields at the Michigan State University Plant Pathology Research Farm in East Lansing. These fields were characterized as having Spinks loamy sandy soils. Fields in the southwest portion of the state were primarily Oshtemo sandy soils and were located near the Michigan State University Southwest Michigan Research and Extension Center in Benton Harbor. Average monthly minimum and maximum temperatures and total rainfall during each growing season (May to October) for 2004 and 2005 were obtained from the Michigan Automated Weather Network in Hart (northwest Michigan), East Lansing (central Michigan), and Benton Harbor (southwest Michigan).

In 2004 and 2005, a minimum sample size of 1,500 asparagus crowns/roots and 1,000 spears were collected from fields and a crown storage facility (2004 only) based on symptoms characteristic of *Phytophthora* spear and root rot.

Pathogen isolation and maintenance. *Phytophthora* isolates were recovered from the samples by excising small sections of tissue (5 × 5 mm) from the lesion margins and placing them onto BARP- (25 ppm of benomyl, 100 ppm of ampicillin, 30 ppm of rifampicin, and 100 ppm of pentachloronitrobenzene) amended V8 juice agar (840 ml of distilled water, 163 ml of unclarified V8 juice, 3 g of CaCO₃, and 16 g of agar) at 25°C for 5 to 7 days under laboratory lighting. Single sporangia were transferred to AR (100 ppm of ampicillin and 30 ppm of rifampicin) amended unclarified V8 juice agar (AR-V8) to obtain axenic cultures. Axenic cultures were maintained on AR-V8 plates at 23 to 25°C and transferred every 2 months. For long-term storage, isolates were placed into sterile microcentrifuge tubes containing two 7-mm mycelial plugs cut from the edge of actively growing axenic cultures, two sterile hemp seeds and 1 ml of sterile distilled water. Long-term isolates were incubated for 2 to 3 weeks at 23 to 25°C, and then stored in an unlit incubator at 15°C.

Colony and spore morphology. *Phytophthora* isolates were identified by comparing colony growth patterns and morphological features of sporangia, oospores, and hyphae (15,51). For evaluation of colony morphology, isolates were grown for 7 days on AR-V8 juice agar plates (100 × 15 mm) and incubated at 23 to 25°C. Sporangia and oospores were produced on dilute V8 juice agar (40 ml of V8 juice, 1.5 g of CaCO₃, and 960 ml of distilled water). The morphology of sporangia and oospores was assessed by measuring the length and breadth of 50 sporangia and the diameter of 50 oospores from 48 representative isolates (35 recovered in 2004 and 13 recovered in 2005) using light microscopy (×200).

Effect of temperature on mycelial growth. To determine the isolates' optimum temperature for growth, AR-V8 juice agar

plates were inoculated with 7-mm diameter mycelial plugs from the edge of actively growing cultures. Plates were incubated in the dark using PTC-1 Peltier-effect temperature cabinets (Sable Systems International, Las Vegas, NV) at 5, 10, 15, 20, 25, and 30°C. Four plates per isolate per temperature were used. Growth rates were assessed 3 and 7 days after inoculation by measuring colony diameters with mycelial plugs subtracted. The test was conducted twice and data from both experiments averaged. Data were analyzed using Proc Mix of SAS (SAS Institute, Cary, NC) with Tukey means separation ($P = 0.05$) used to compare colony diameters of isolates.

Mefenoxam sensitivity. Isolates were screened for sensitivity to the fungicide mefenoxam (Ridomil Gold 4EC, Syngenta, Greensboro, NC) by comparing the growth rate of mycelial plugs placed at the center of mefenoxam-amended V8 juice agar plates with the growth on unamended plates. Mefenoxam was suspended in sterile distilled water and added to medium cooled to 50°C. For each isolate, four 100 ppm and two 0 ppm mefenoxam-amended plates were inoculated with a 7-mm mycelial plug from the edge of actively growing 5-day-old cultures and incubated at 25°C under laboratory lighting. Colony diameters were measured 5 days after inoculation. Percent growth of an isolate on amended medium was calculated by subtracting the inoculum plug diameter (7 mm) from the diameter of each colony and dividing by the diameter of the nonamended control. Isolates were assigned mefenoxam sensitivity ratings based on the percent growth of the control. Isolates were scored as sensitive (S) when percent growth was <30%, intermediate sensitive (IS) when percent growth was 30 to 90%, and resistant (R) when percent growth was >90% (36).

Pathogenicity on asparagus spears. Koch's postulates were fulfilled to establish the pathogenicity of *Phytophthora* sp. The pathogenicity of *Phytophthora* sp. isolated from asparagus was assessed on 15-cm long, 2- to 4-day-old 'Jersey Knight' asparagus spears. Spears were washed with dish soap in tap water, surface disinfested with sodium hypochlorite (5%) for 3 min, rinsed with sterile distilled water, and air-dried at room temperature. The middle of the spears was surface wounded with a needle sterilized with 95% ethanol. The wound was covered with a 7-mm mycelial plug of a *Phytophthora* isolate from the edge of actively growing 5-day-old cultures on AR-V8 juice agar. Plugs were placed mycelial side down on spears and covered with sterile 1.5-cm microcentrifuge tube caps sealed to the plant surface with petroleum jelly. Control spears were inoculated with sterile 7-mm plugs of AR-V8 agar. Five spears were inoculated per isolate and a total of 115 isolates were evaluated. Inoculated spears were placed in aluminum trays with wet paper towels (to maintain high humidity) and covered with plastic wrap. Trays were incubated at 23 to 25°C on laboratory bench tops under ambient lighting, and lesion length was measured 7 days after inoculation. This evaluation was conducted twice. A Student-Newman-Keuls means separation test ($P = 0.05$) was used to compare lesion length on spears inoculated with *Phytophthora* isolates with the noninoculated control.

Pathogenicity on asparagus, soybean, alfalfa, and red clover seedlings. Pathogenicity of *Phytophthora* isolate C009 and *P. sojae* isolate P23 (provided by P. Hart, Michigan State University) was tested on asparagus seedlings of cultivars Jersey Giant and Mary Washington, on soybean (*Glycine max*) seedlings of cultivar Williams, and on alfalfa (*Medicago sativa*) and red clover (*Trifolium* sp.) seedlings in the greenhouse. Plant inoculations were carried out using a method adapted from Falloon (18). Asparagus seeds were disinfested according to Damicone et al. (11). Soybean, alfalfa, and red clover seeds were not disinfested. All seeds were planted in flats filled with soilless media (Baccto Professional Planting Mix, Michigan Peat Company, Houston, TX) and allowed to grow for 2 weeks on greenhouse benches. Inoculum was prepared by culturing both *Phytophthora* sp. and *P. sojae* isolates on AR-V8 agar for 8 to 10 days at 23 to 25°C under ambient lighting. Two 1-cm mycelial plugs were placed into flasks

containing autoclaved oat seeds, and allowed to grow and colonize the seeds for 3 weeks. Inoculum (150 g) was mixed into 4 kg of an autoclaved mix (50:50) of sand and soilless media. Pots (8.9 × 6.7 cm) were filled with the infested sand/soilless mix and one seedling of each of the four plant species were transplanted per pot. Controls contained a mixture of autoclaved oat seeds and sand/soilless mix and were placed on trays separated from infested pots. Eight plants of each cultivar were used. Plants growing in pots infested with the same *Phytophthora* isolate were randomly placed on trays without drainage holes and flooded with approximately 4 cm of water for 3 days. On the third day, pots were transferred to trays with drainage holes and watered as needed. The numbers of wilted and dead plants were recorded weekly for 4 weeks and the test was conducted twice. Data were analyzed using PROC GLM of SAS and Student-Newman-Keuls means separation test ($P = 0.05$) to compare severity of disease on asparagus, soybean, alfalfa, and red clover seedlings.

Pathogenicity on cucurbit fruits. Twenty-five *Phytophthora* isolates were tested for pathogenicity on slicing and pickling cucumber (*Cucumis sativus*), yellow squash (*Cucurbita moschata*), zucchini (*Cucurbita pepo*), and acorn squash (*Cucurbita* sp.) fruits obtained from a local supplier. Fruits were washed with dish soap in tap water, surface disinfested with sodium hypochlorite (5%) for 5 min, rinsed with sterile water, and air-dried at room temperature. Fruits were wounded with a needle sterilized with 95% ethanol. A 7-mm mycelial plug of each *Phytophthora* isolate was placed mycelial side down over the wounds, covered with a sterile 1.5-cm microcentrifuge tube and sealed to the fruit surface with petroleum jelly. Wounded fruits inoculated with sterile AR-V8 agar plugs were used as controls. Three fruits were inoculated per isolate. Inoculated fruits were incubated in aluminum trays containing wet paper towels, covered with plastic wrap, and maintained at 23 to 25°C on laboratory bench tops with ambient lighting. Lesion lengths were assessed 7 days after inoculation and the test was conducted twice. Data were analyzed using PROC GLM of SAS and Student-Newman-Keuls means separation test ($P = 0.05$) to compare lesion length on fruits inoculated with *Phytophthora* isolates with the noninoculated control.

DNA isolation and genotyping. Isolates were cultured and treated according to Lamour and Finley (35) for high throughput DNA isolation. Amplified fragment length polymorphism (AFLP) marker generation was carried out as described by Habera et al. (29). An overnight restriction-ligation reaction was carried out using 150 ng of DNA in an 11- μ l volume format at room temperature. Restriction-ligation products were diluted to 200 μ l with 10 μ M Tris and a nonfluorescent pre-selective amplification was performed using primers Eco+0 and MseI+0. Adapter sequences were as described by Vos et al. (49). Ten microliters of the preselective amplification was run on a 1% agarose gel to confirm amplification and the remaining 10 μ l was diluted and used for subsequent selective amplification using primers Eco(AC) and Mse(CA). A second selective amplification using a fluorescent-labeled primer (Prologo, Boulder, CO) for Eco(AC) and non-fluorescent primer for Mse(CA) (Integrated DNA Technologies, Skokie, IL) was performed. Fluorescent polymerase chain reaction (PCR) products were analyzed on a CEQ 8000 Genetic Analysis System (Beckman Coulter, Fullerton, CA) following the manufacturer's protocols.

Internal transcribed spacer (ITS) sequencing and phylogenetic analysis. Sequence for the ITS region was generated as described by Cooke et al. (7). PCR products were resolved on 1% agarose gel to insure a single PCR product and were then purified using Qiagen PCR clean up columns (Qiagen, Valencia, CA) following the manufacturer's instructions and diluted with 5 mM Tris. The resulting amplicon was sequenced in both directions using an ABI3700 sequencer (Sequencing Facilities at the University of Tennessee). Sequences were checked for quality using Phred v2.3.1a software and visualized using Sequencher (Gene-

Codes, Ann Arbor, MI). The sequence was submitted to a BLASTn search (1) of the National Center for Biotechnology Information GenBank database. Results of the BLASTnquery from six additional *Phytophthora* spp. that exhibited the closest levels of similarity were also downloaded and included in the phylogenetic analysis. The following six species were included: *P. cryptogea* (L76538.1), *P. drechsleri* (L76547), *P. gonapodyides* (AY787027), *P. megasperma* (AY995359), *P. humicola* (AF266792), and *P. inundata* (AF266791.1). *P. sojae* (AF266769) was included as an outgroup. Sequences were aligned using CLUSTALW (48), exported as an NBRF file, and imported into MacClade v4.08. Minor insertions or deletions were adjusted visually within MacClade.

Maximum likelihood analyses. Phylogenetic trees were estimated under the maximum likelihood (ML) criterion implemented in phylogenetic analysis using parsimony (PAUP*) 4.0b10 (47). Nucleotide substitution models for the molecular data set were selected using Modeltest 3.06 (42). An optimal ML tree was then created via heuristic searches of tree space using tree-bisection-reconnection (TBR) branch swapping in a random stepwise addition of taxa repeated 10 times. Node support was evaluated by nonparametric bootstrap resampling (25).

RESULTS

Field sampling and pathogen isolation. In 2004, dormant crowns held in bulk bins in cold storage prior to planting exhibited water-soaked lesions and/or shriveling, but the tissue was firm at the lesion site (Fig. 1A). Spears observed in the field exhibited a shepherd's crook symptom, with soft water-soaked lesions (Fig. 1B) and/or shriveling occurring slightly above or below the soil line (Fig. 1C and D). In 2005, water-soaked lesions were rarely observed on either spears or storage roots sampled from the field; necrotic lesions on storage roots and spears with a shepherd's crook were the most prevalent symptoms. In 2004, *Phytophthora* sp. was isolated from most of the asparagus plants sampled, primarily from symptomatic spears (Table 1). A total of 115 isolates were recovered; 48% of the isolates were from spears, 25% from storage roots, 14% from crowns, 8% from stems, and 5% from asparagus seedling stems (Table 1). The pathogen was not recovered from samples collected from fields located in the southwest region of Michigan, although the spears and fern exhibited shriveling, stunting, and a shepherd's crook symptom associated with *Phytophthora* infection. In 2005, hundreds of samples were collected from asparagus fields located in the three asparagus growing regions. Only 13 *Phytophthora* isolates were recovered from samples collected from two commercial asparagus fields in northwest Michigan. The pathogen was not recovered from samples collected in central and southwest Michigan in 2005.

In 2004, excessive rainfall occurred in the three asparagus growing regions during spear harvest. In the northwest region, the total rainfall during May 2004 was 246 mm, which fell on 21 days. On four of these days, the rainfall was >2.0 cm, an amount established by Ristaino (43) as a saturation event for light soils. During the same period in central Michigan, rain occurred on 13 days (205 mm total), 1 day received rain >2.0 cm. In the southwest region, rain occurred on 16 days, producing a total of 100 mm of rain. No single rain event registered >2.0 cm in a 24-h period in the southwest region. The amount of rainfall registered during the 2005 growing season was very low. Rainfall totals of only 51, 33, and 34 mm were recorded in northwest, central, and southwest Michigan, respectively. These totals represented a fraction of the total rainfall recorded in the northwest (21%), central (16%), and southwest (34%) growing regions in May 2004. In 2005, no rain events of >2.0 cm in a 24-h period were recorded in any of the three growing regions included in this study.

Fig. 1. Symptoms caused by *Phytophthora* sp. on asparagus. **A**, Infected storage roots become water-soaked and shrivel as the lesion expands. Root mass and vigor may be reduced. **B and C**, Infected spears present soft, water-soaked lesions on shoots slightly above or below soil line. Lesions often shrivel, causing the spear to curve and eventually to collapse. **D**, Shriveled and distorted harvested spears.

Colony and spore morphology. *Phytophthora* sp. recovered from asparagus exhibited morphological traits attributed to *P. megasperma* (12,46). Colonies of *Phytophthora* sp. on AR-V8 juice agar had a stellate to rosaceous growth pattern (Fig. 2E). Both sporangia and oospores were produced on AR-V8 juice agar with oospores more profusely produced than sporangia. Abundant, uniform, and long lasting sporangia, as well as uniform oospores, were produced on dilute V8 juice agar, but few sporangia were produced on V8 juice agar. *Phytophthora* isolates produced ovoid, obpyriform, nonpapillate, noncaducous sporangia (Fig. 2A) borne on simply- or sparingly-branched sporangio-phores and/or on internal proliferation (Fig. 2D), after evacuation of zoospores (Fig. 2C). Isolates produced amphigynous oospores (Fig. 2B), and chlamydospores were not produced. Sporangia mean length ($n = 50$) ranged from 42 to 47 μm and mean breadth from 23 to 40 μm . Oospores mean diameter ($n = 50$) ranged from 25 to 30 μm .

Effect of temperature on mycelial growth. Temperature had an effect on mycelial growth of the 46 *Phytophthora* isolates examined. When inoculated onto AR-V8 juice agar, all isolates grew when incubated at temperatures between 10 and 25°C, with optimum growth at 25°C ($P = 0.0001$) (Fig. 3). The average colony diameter after 3 days of mycelial growth at 25°C was 34 mm. At 7 days after inoculation, the diameter of the majority of the isolates measured approximately 75 mm, almost the entire diameter of the AR-V8 juice agar plate (Fig. 3). Sporangia and oospores were produced at temperatures between 10 and 25°C at 7 days after inoculation. Mycelial growth patterns were similar on both evaluation dates, and no growth was recorded on plates incubated at 5 and 30°C (Fig. 3).

Mefenoxam sensitivity. All isolates ($n = 131$) of *Phytophthora* sp. were highly sensitive to mefenoxam. On V8 juice agar with no mefenoxam, the average radial growth of the mycelium after 5 days was 42 mm. On V8 juice agar amended with 100 ppm mefenoxam all isolates failed to grow, indicating sensitivity to the fungicide (data not shown).

Pathogenicity on asparagus spears. A preliminary study (data not shown) showed that wounding of asparagus spear tissue was needed for disease development by *Phytophthora* sp. under laboratory conditions. Inoculation of wounded asparagus spears with the 115 *Phytophthora* isolates listed in Table 1 resulted in water soaking, spear shriveling, and collapse 7 days after inoculation. Regardless of the origin of isolates (geographic location of sample/plant tissue infected), there were no significant differences in overall average lesion length among isolates, although isolate SP112 resulted in slightly longer lesions (62 mm) than other isolates. Lesion length among the remaining 114 isolates was similar and ranged from 56 to 21 mm, with 97% of isolates causing lesions that exceeded 30 mm in length. No lesions developed on uninoculated spears.

Pathogenicity on asparagus, soybean, alfalfa, and red clover seedlings. In the greenhouse, seedlings of both asparagus cultivars Jersey Giant and Mary Washington wilted 2 weeks after inoculation with *Phytophthora* isolate C009, and died between weeks 3 and 4 postinoculation. Asparagus seedlings remained healthy when inoculated with *P. sojae*, but the organism was reisolated from asparagus roots (Fig. 4). *Phytophthora* sp. from asparagus did not infect soybean seedlings, although the pathogen was reisolated from the roots following inoculation. Plant wilt and death were recorded at 1 and 3 weeks, respectively, after inoculation with the *P. sojae* isolate (Fig. 4). Neither *Phytophthora* sp. nor *P. sojae* caused significant disease to alfalfa and red clover seedlings, but each organism was recovered from the roots of inoculated plants. Control plants remained healthy throughout the experiment.

Pathogenicity on cucurbit fruits. Inoculation of fruits from five different cucurbits with 25 isolates of *Phytophthora* sp. from asparagus resulted primarily in water-soaked lesions on zucchini,

water-soaked and necrotic lesions on yellow squash, and water-soaked lesions with some mycelial growth on slicing and pickling cucumbers. No lesions were observed on acorn squash or on any of the control fruits. The average lesion length on the fruits infected with the *Phytophthora* isolates ranged from 2.6 to 4.8 cm. Due to similarities in pathogenicity of the *Phytophthora* isolates, results of just five representative isolates are presented (Table 2).

AFLP fingerprinting. AFLP analysis of 99 *Phytophthora* isolates recovered from asparagus in northwest and central Michigan in 2004 (86 isolates) and in northwest Michigan in 2005 (13 isolates) revealed identical fingerprints. Twelve clearly resolved fragments were present for each isolate using the Eco+AC/Mse+CAA primer combination (Fig. 5). There were no clearly resolved polymorphic fragments (Fig. 5). Based on these results, the different isolates are considered to be of one clonal lineage.

ITS sequencing and phylogenetic analysis. Because AFLP results indicated that the isolates were identical, one representative isolate from each year was selected for sequencing of the

ITS region. A representative sequence of the ITS region was deposited in GenBank (accession EF185089). The results of the BLASTn query (1) indicated that the *Phytophthora* sp. isolated from asparagus in Michigan has an exact match to *Phytophthora* sp. asparagus (e value = 0, accession AF266796) described elsewhere (7). A phylogenetic analysis based on ITS sequences (using the six closest matches from the BLASTn search) supports the conclusion that this asparagus *Phytophthora* sp. is a distinct species and is not closely related to isolates considered to be *P. megasperma* (Fig. 6). Therefore, it is proposed that this new *Phytophthora* sp. be named *P. asparagi*. *P. asparagi* isolate SP326 has been deposited with the Cornell Plant Pathology Herbarium (CUP-67873) and registered with the MycoBank (MB511931).

DISCUSSION

The identification of spear and root rot caused by *Phytophthora* sp. in the asparagus growing region of Michigan increases the risk faced by producers who were already concerned with Fusarium crown and root rot, a devastating soilborne disease caused by *F.*

TABLE 1. Characteristics of *Phytophthora* sp. isolates recovered from asparagus in Michigan in 2004 and 2005

Location	Plant part	<i>Phytophthora</i> sp.		Number of isolates tested for pathogenicity on asparagus spears ^x and seedlings ^y	Year collected
		No. of isolates from plant tissue	No. of isolates subjected to AFLP		
NW-1	Crown, root	29	21	R017, R018, R019, R020, R022, R023, R024, R025, R026, R027, R028, R030, R031, R036, R037, C039, R043, R050, R051, R052, R131, R155, R156, R157, C041, C042, (<i>n</i> = 26) ^z	2004
NW-2	Spear	8	7	SP021, SP033, SP047, SP048, SP053, SP070, (<i>n</i> = 6)	2004
NW-3	Spear, crown	15	14	R001, R011, R012, C003, C005, C006, C007, C008, C009, C010 C013, C014, C015, C016, SP029 (<i>n</i> = 15)	2004
NW-4	Spear	4	3	SP114, SP149 (<i>n</i> = 2)	2004
NW-5	Spear	2	2	SP044, SP045 (<i>n</i> = 2)	2004
NW-6	Spear	2	2	SP046, SP049 (<i>n</i> = 2)	2004
NW-7	Spear	9	4	SP054, SP057, SP060, SP063, SP064, SP068, SP095, SP183 (<i>n</i> = 8)	2004
NW-8	Spear	14	6	SP032, SP034, SP035, SP055, SP056, SP058, SP059, SP061, SP065, SP071, SP112 (<i>n</i> = 11)	2004
NW-9	Spear	19	15	SP 086, SP090, SP091,SP092,SP093, SP094, SP096, SP097, SP098, SP099, SP100, SP101, SP102, SP103, SP104, SP107 (<i>n</i> = 16)	2004
NW-10	Stem, spear	11	8	ST076, ST077, ST078, ST079, STO80, ST081, ST082, ST083, SP084 (<i>n</i> = 9)	2004
C-11	Seedling stem	5	4	SD178, SD179, SD180, SD181, SD182 (<i>n</i> = 5)	2004
NW-12	Spear	3	3	SP401, SP402, SP403 (<i>n</i> = 3)	2005
NW-13	Crown, root	10	10	R405, R406, R407, R408, R409, R410, R412, C4013, R415, R417 (<i>n</i> = 10)	2005
Total		131	99	(<i>n</i> = 115)	

^x Letters used to designate the plant parts from which *Phytophthora* sp. was isolated: SP = spear, ST = mature plant stem, SD = seedling stem, C = crown, and R = root.
^y Pathogenicity on asparagus seedlings was evaluated in the greenhouse by inoculating ‘Mary Washington’ and ‘Jersey Giant’ seedlings with isolate C009 and evaluating the number of dead seedlings 4 weeks after inoculation.
^z *n* denotes the number of isolates tested for pathogenicity on 15-cm long ‘Jersey Giant’ spears.

Fig. 2. Morphology of *Phytophthora* sp. isolated from asparagus on dilute V8 juice agar. **A**, Ovoid, nonpapillate, noncaducous sporangium with mean length of 42 to 47 μ m and mean breadth of 23 to 40 μ m. **B**, Oospore with amphigynous antheridia with mean diameter of 25 to 30 μ m. **C**, Sporangium produced by internal proliferation. **D**, Sporangium containing encysted zoospores. **E**, Colony morphology of *Phytophthora* sp. grown on V8 juice agar for 7 days at 25°C.

oxysporum f. sp. *asparagi* and *F. proliferatum* (30,44). *Phytophthora* sp. and *Fusarium* spp. can reduce the lifespan of an asparagus production field (14).

Phytophthora isolates from asparagus were morphologically similar to *P. megasperma*. The finding that the ITS sequence for the *Phytophthora* sp. infecting asparagus in Michigan is identical to isolates of *Phytophthora* sp. recovered from asparagus in Europe and agave in Australia (10) suggests that this species has been widely dispersed and is not specific to asparagus. It would be helpful to obtain *Phytophthora* isolates from the European and New Zealand populations to determine if the fingerprint type is identical or varies. Isolates recovered in Michigan from different locations over 2 years were genetically homogeneous likely as a result of the self-fertilizing sexual stage. Inbreeding maintains homozygosity across the genome. This particular *Phytophthora* sp. produces large numbers of oospores in culture.

In May 2004, excessive rainfall during harvest in the northwest region of Michigan preceded the outbreak of *Phytophthora* spear and root rot. Multiple saturation events occurred in succession and did not allow adequate time for water to drain from the fields. Rain splashing moved infested soil onto emerging spears, resulting in water-soaked tissue and disease. In contrast, the southwest region of the state was relatively dry in 2004 with no saturation events and *Phytophthora* sp. was not readily recovered from asparagus. The drought conditions observed during the 2005 harvest season across the Michigan asparagus growing region inhibited the disease and relatively few symptomatic spears were observed.

Saturated soil conditions from rainfall or excessive irrigation have been implicated in the increase of *Phytophthora* root rot disease outbreaks of many perennial and annual crops (33,38,41). Wet soil conditions stimulate the release of zoospores from sporangia, increasing inoculum levels. *Phytophthora* rot of asparagus in California was associated with a period of heavy and prolonged rainfall and the common practice of flooding certain asparagus production areas to prompt early plant growth (4). Falloon et al. (24) reported that more *P. megasperma* var. *sojae* isolates were recovered from asparagus in a year when rain totaled 300 mm during the harvest season, than in the following year which had only 77 mm of rain. Parra and Ristaino (41) reported rapid progress of root and crown rot of bell pepper (*Capsicum annuum*) caused by *P. capsici* in fields after a single rainfall >2 cm at a very low inoculum of 2 to 5 colony forming units (CFU)/g of soil. Plant-to-plant spread of *P. capsici* occurring with heavy rainfall episodes had a major impact on final disease incidence and on yields (41).

Our temperature study indicated that mycelial growth of *Phytophthora* sp. occurred between 10 and 25°C, with the greatest growth at 25°C and no growth above 25°C. This is comparable to

Fig. 3. Effect of temperature on mycelial growth of 46 isolates of *Phytophthora* sp. on AR-V8 juice agar 3 and 7 days after inoculation.

the findings of Boesewinkel (6) who determined a temperature optimum of 20 to 22°C for mycelial growth of a *Phytophthora* sp. infecting asparagus in New Zealand. Falloon et al. (19) noted that high losses occurred in the early part of the asparagus harvest season after periods of heavy rainfall when soil temperatures were between 9 and 24°C.

Wounding of asparagus plants may exacerbate *Phytophthora* spear rot disease. Blowing sand and soil particles may cause abrasions on the spears during rain storms and provide an avenue for infection. In the laboratory, wounding was necessary for disease development when testing for virulence of *Phytophthora* sp. on detached asparagus spears. Stomata serve as a point of entry for many pathogens and when spears are detached from plants, the stomata close, requiring wounding for some pathogens to cause infection. Lacy (34) reported that wounding of spears was needed in the laboratory for infection of asparagus spears by *Stemphylium vesicarium* to occur, while in the field wounding was not essential because stomata are open and facilitate infection by purple spot.

Spears are succulent, with high levels of soluble sugars, high water (90 to 92%), and low fiber content. During our study, the pathogen was readily isolated from spears with water-soaked symptoms or from dormant crowns in storage. In contrast, the pathogen was rarely isolated from spears that lacked the water-soaked lesion but displayed a shepherd's crook symptom. Crown and storage root tissue is fibrous with less water and soluble sugars than spears (9,37). It was difficult to obtain a *Phytophthora* isolate from this crown tissue associated with symptomatic or even dying fern. Our results are in agreement with those of Falloon and Grogan (23) who suggested that isolation from spears was the most reliable method to detect *Phytophthora* spp.

The fungicide mefenoxam is registered for use on asparagus and may be applied prior to the first cutting of the season to manage spear and crown rot. Mefenoxam and metalaxyl are phenylamide fungicides used to manage disease caused by oomycetes. All isolates of *Phytophthora* spp. obtained from

Fig. 4. A and B, Pathogenicity of *Phytophthora* sp. isolated from asparagus and *P. sojae* on asparagus 'Mary Washington' and 'Jersey Giant', soybean cultivar William, alfalfa, and red clover (unknown cultivars). Bars with a letter in common are not significantly different (Student-Newman-Keuls; $P = 0.05$).

Michigan asparagus were sensitive to 100 ppm mefenoxam in vitro. An earlier field study in Michigan showed a significant reduction of *Phytophthora* symptoms (shepherd's crook) on asparagus fern when mefenoxam (Ridomil Gold 4EC, Syngenta) was periodically applied directly to crowns via drip irrigation (8). The beneficial effects of metalaxyl-based products were previously reported by Falloon et al. in New Zealand (22) and California (19). In both studies, fewer infected spears and increased yields were observed when metalaxyl was applied compared with an untreated infected plot. In addition, harvesting plots treated with metalaxyl started earlier with better quality of spears at the end of the harvest season compared with the control plot (19). Although promising, the use of mefenoxam-based products for management

of *Phytophthora* spear and root rot of asparagus should be approached with caution, since resistance to mefenoxam by some *Phytophthora* spp. has been reported (31,33,36,40). Further, the fungicide may offer limited protection when applied as a spray because the product may not reach the crown that may be located 38 cm or more below the surface.

The *Phytophthora* sp. affecting asparagus may be a source of inoculum for cucurbits also grown in this region of Michigan. To date, *P. capsici* has been the only *Phytophthora* isolated from cucurbits. In this study, *Phytophthora* isolates from asparagus infected and/or reproduced on cucumber, zucchini, and yellow squash fruits, although they did not cause extensive disease. *Phytophthora* sp. was isolated from the roots of inoculated but

TABLE 2. Lesion length (cm) caused by inoculation of *Phytophthora* sp. isolates on fruits of five cucurbits

Isolate ^x	Slicing cucumber	Pickling cucumber	Zucchini	Yellow squash	Acorn squash
ST087 ^y	4.2 ab ^z	4.8 a	2.9 ab	4.3 a	0.0 a
C039	4.8 a	5.4 a	2.8 ab	3.5 ab	0.0 a
SP062	3.9 ab	3.8 b	3.0 ab	4.7 a	0.0 a
R037	4.9 a	3.7 b	3.5 a	4.1 ab	0.0 a
SD180	4.0 ab	4.3 ab	2.6 ab	4.0 ab	0.0 a

^x Data represent the mean of two replicated trials in which three fruits were tested. Fruits were inoculated with the isolates, incubated at room temperature, and evaluated 7 days after inoculation.

^y Five isolates are a representative sample of the 25 isolates evaluated on fruits of five cucurbit cultivars.

^z Numbers in a column followed by the same letter are not significantly different at $P = 0.05$ (Student-Newman-Keuls).

Fig. 5. Partial amplified fragment length polymorphism profiles for *Phytophthora* sp. isolates recovered from asparagus in A, northwest (2004), B, north central (2004), C, central (2004), and D, central (2005) Michigan. Sizes of the DNA fragments are indicated on the x axis and intensity of fluorescent signal on the y axis.

Fig. 6. Maximum likelihood phylogenetic tree based on internal transcribed spacer sequences. *Phytophthora* sp. isolate from this study showed identical location as *P. asparagi* described elsewhere. Bootstrap scores above nodes were calculated from analysis based on 1,000 replications.

asymptomatic soybean, alfalfa, and red clover plants growing in the greenhouse, suggesting that the organism is sustained in the roots and contributes to inoculum buildup. In Michigan, further studies are warranted to determine the potential host range of this *Phytophthora* sp. Such studies will assist growers in decision-making regarding land that has been cropped to asparagus and is being considered for other crop uses.

Current recommendations suggest that growers focus on methods to limit further spread of the pathogen. A key measure is to establish production fields from disease-free propagules. One-year-old dormant crowns dug from nursery fields are planted in production fields or are held in storage until field conditions are suitable for transplanting. When bulk boxes of asparagus crowns held in storage were sampled, *Phytophthora* was detected on 40% of the dormant crowns/roots. Historically, most Michigan crown nurseries have not fumigated and the nursery sites may have been cropped to asparagus previously, facilitating the movement of infected crowns and infested soils within and between fields, and between geographical regions. Further, until recently, the crowns were not subject to a visual inspection by Michigan Department of Agriculture unless they were to be shipped out of the state.

With the knowledge gained from this study, effective and reliable management strategies for *Phytophthora* spear and root rot can be formulated. Our results indicate that growers should consider practices that limit the negative effects of spear and root rot caused by *Phytophthora* sp., such as utilizing crowns grown in fumigated nurseries and/or direct seeding or transplanting greenhouse-produced seedlings into fumigated production fields. Judicious use of the fungicide, mefenoxam, may also be helpful (24).

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