



Pathogenicity and Mefenoxam Sensitivity of *Pythium*, *Globisporangium*, and *Fusarium* Isolates From Coconut Coir and Rockwool in Marijuana (*Cannabis sativa* L.) Production

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Oomycetes and fungi were recovered from coconut coir and rockwool substrates where marijuana (*Cannabis sativa* L. cv. Silver and Citron) plants with root rot and wilt symptoms were grown in a commercial growing facility in Connecticut. The objectives of this study were to identify the isolates collected from these substrates, determine the pathogenicity of the isolates on hemp seedlings *in vitro* and *in vivo*, and evaluate the pathogens' sensitivity to mefenoxam. *Pythium* and *Globisporangium* isolates were identified by sequencing the mitochondrially-encoded cytochrome oxidase genes (COI and COII) and *Fusarium* sp. with the translation elongation factor (EF-1 α) region and internal transcribed spacer region (ITS4 and ITS5) genes. Three isolates were identified as *Globisporangium irregulare* (formerly *Pythium irregulare*), 21 isolates were *Pythium myriotylum*, and one was *Fusarium oxysporum*. All the isolates tested were pathogenic to hemp plants *in vitro* and *in vivo*, with disease incidence between 6.7 and 100%. Inoculated plants were smaller by 32% or more compared with the non-inoculated control. On average, hemp plants infected with *Pythium myriotylum* produced the lowest biomass and relative greenness values. None of the *Pythium* and *Globisporangium* isolates were resistant to mefenoxam—all were sensitive to $\geq 5 \mu\text{g}\cdot\text{mL}^{-1}$ mefenoxam. This is the first report of *G. irregulare* causing root rot on marijuana and hemp plants. The results of this study provide information about the characteristics of pathogens that can be found potentially in soilless substrates in controlled environment agriculture.

Keywords: cannabis, controlled environment agriculture, plant pathogen, substrates, roots

INTRODUCTION

The production of cannabis and hemp (*Cannabis sativa* L.) in controlled environment agriculture (CEA) has increased rapidly in North America and little is known about the diseases that prevail in commercial operations. The production of hemp in the United States increased in production acreage from zero in 2013 to 32,464 in 2018 and to 146,065 in 2019 (Mark et al., 2020). Hemp is grown in controlled environment agriculture for cannabidiol (CBD) (Mead, 2017). Many U.S.

states and providences in Canada have legalized marijuana production for recreational or medical purposes in controlled environment agriculture (i.e., greenhouses or indoor production facilities). The rapid upsurge in production requires a better understanding of potential plant diseases that might affect the industry.

Cannabis sativa L. is the botanical classification of cultivated strains and varieties of marijuana and hemp (Sawler et al., 2015; Barcaccia et al., 2020). There are genetic and biochemical differences between marijuana and hemp (Sawler et al., 2015). Government agencies use plant tissue concentrations of delta-9 tetrahydrocannabinol (Δ^9 -THC), a cannabinoid with psychoactive effects on humans, to regulate these two crops. The U.S. Federal and Drug Administration (US-FDA, 2020) classifies marijuana as any strains of *C. sativa* with >0.3% THC by dry weight (US-FDA, 2020), a Schedule I Controlled Substance. The 2018 Farm Bill defines industrial hemp as plants with <0.3% THC by dry weight (US 115th Congress, 2018). In this project we collected soilless substrate samples from a marijuana production facility and conducted controlled experiments with hemp in our research facility—per local regulation, we could not collect plant tissue from the commercial facility or conduct experiments with marijuana plants.

Pythium and *Fusarium* spp. are causal agents of damping-off, root rot, and wilt diseases on a wide-range of crops (Agrios, 2004). There are only a few reports about root diseases on marijuana and hemp plants. Punja and Rodriguez (2018) confirmed symptoms of root rot, reduced growth, chlorosis, and wilt caused by *P. aphanidermatum*, *P. dissotocum*, *P. myriotylum*, *F. oxysporum*, and *F. solani* in hydroponically-grown marijuana in Canada. We recovered *P. myriotylum* from necrotic roots of a wilted hemp plant grown in a research greenhouse in Connecticut, USA (McGehee et al., 2019). Root rot and wilt on field-grown *C. sativa* L. caused by *Pythium* spp. and *Fusarium* spp. have been reported in Kentucky, Indiana, Israel, California, Canada, Italy, and North Carolina (Beckerman et al., 2017, 2018; Punja et al., 2018; Sorrentino et al., 2019; Jerushalmi et al., 2020; Thiessen et al., 2020; Pitman et al., 2021). *Pythium* spp. and *Fusarium* spp. in greenhouse-grown hemp and marijuana have been reported in Arizona, Canada, and North Carolina (Punja et al., 2019, 2020; Punja, 2020; Thiessen et al., 2020; Hu, 2021). The number of reports on diseases associated with hemp and marijuana production are limited, especially in soilless substrates in indoor production. Therefore, there is a need to identify pathogens associated with these crops in order to develop effective management strategies.

The objectives of this study were to (1) identify fungal and oomycete isolates from soilless substrates obtained from a commercial marijuana growing operation, (2) determine the pathogenicity of the isolates on hemp seedlings, and (3) evaluate the sensitivity of isolates to mefenoxam.

MATERIALS AND METHODS

Isolate Collection

Soilless substrates (coconut coir and rockwool) with no plant tissue were collected from the root zone of symptomatic

plants in a marijuana production facility. Federal and local laws prevented us from collecting plant tissue. The symptoms observed were chlorosis, reduced foliar or root growth compared with surrounding plants of the same stage, and root rot. Coconut coir samples were collected from containers with one *C. sativa* L. 'Silver' plant at a mature stage and from two *C. sativa* L. 'Citron' plants in the early flowering stage. Rockwool was collected from *C. sativa* L. 'Citron' cuttings. The samples were collected from a commercial growing facility on March 19, 2019 in Connecticut, USA.

Dilution plating was conducted using Singleton et al.'s (1992) modified-protocol. Briefly, two grams of each soilless substrate were suspended in 10 mL of sterile-deionized water, followed by three 10-fold serial dilutions. A 100 μ L aliquot of each dilution was transferred onto Petri plates with corn meal agar (CMA; Difco Laboratories, Detroit, MI, USA), potato dextrose agar (PDA; Difco Laboratories, Detroit, MI, USA), PARP (CMA amended with pimarin, ampicillin, rifampicin, and pentachloronitrobenzene; Jeffers and Martin, 1986), or PARP-H (pimarin, ampicillin, rifampicin, pentachloronitrobenzene, and hymexazol), and then spread evenly with a sterile glass rod. Petri dishes were incubated at room temperature in the dark for 48 h. The hyphal tips of individual colonies were transferred to PARP, PARP-H, PDA, and CMA. Twenty-five isolates were collected during one sampling period.

Morphological Identification

Morphological identification was conducted to identify the organisms at the genus level and guide the selection of genes used for molecular identification. Five mycelial plugs of each isolate were placed on four 2.5 cm fragments of autoclaved tall-fescue leaves in sterilized-deionized water and incubated under constant fluorescent light at room temperature for 4 days (Trigiano et al., 2008). The isolates were identified via microscopy following van der Plaats-Niterink identification key for oomycetes (van der Plaats-Niterink, 1981) and the presence of macro and microconidia, false-heads, and chlamydospores for *Fusarium* (Leslie and Summerell, 2006).

Molecular Identification

DNA from each isolate was obtained from mycelial mats. Mycelial mats were produced as follows: five 4 mm diameter plugs of 3-day-old cultures grown on CMA were transferred to an empty Petri dish and then filled with 20 mL of V8 broth. The plates were incubated in the dark for 5 days, and then the mycelial mats were rinsed three times with sterile deionized water, and then placed on sterile filter paper to remove excess water. The mycelial mats (80–100 mg) were placed in a 2-mL centrifuge tube with a metal bead, liquid nitrogen was added and lyophilized in a TissueLyzer II machine (Qiagen, Germantown, MD, USA) for 2 min with a frequency at 30 $1 \cdot s^{-1}$ or until a fine powder was formed in the tube. DNA was extracted using the DNeasy Plant Mini Kit (Qiagen, Germantown, MD, USA) and quantified with a NanoDrop Spectrophotometer ND-1000 (Thermo Fisher Scientific, Wilmington, DE, USA).

PCR Amplification

The following genes were selected: mitochondrially-encoded cytochrome oxidase I and II (COI and COII), elongation factor 1- α regions (EF1 and EF2), and the internal transcribed spacer regions 4 and 5 (ITS5 and ITS4). Amplification of the COI gene was performed with the OomyCoxI-Levup (5' TCAW CWMGATGGCTTTTTC AAC 3') as the forward primer and OomyCoxI-Levlo (5' CYTCHGGRTGWCCRAAAAACCAAA 3') as the reverse primer (Robideau et al., 2011). Amplification of the COII was performed with FM58 (5' CCACAAATTTCA CTACATTGA 3') as the forward primer and FM66 (5' TAGG ATTTCAAGATCCTGC 3') as the reverse primer (Martin, 2000). Each 20 μ L reaction contained 10 μ L GoTaq™ 2x MasterMix (Promega Corp., Madison, WI, USA), 1 μ L of 0.4 μ M of each primer, and 1 μ L of 50 ng of genomic DNA. All amplifications were performed on a T100™ Thermocycler (Bio-Rad, Hercules, CA, USA). COI amplification was conducted with the following program: 2 min at 95°C, 35 cycles of 1 min at 95°C, 1 min at 55°C, and 10 min at 72°C. COII gene amplification was conducted with the following program: 3 min at 96°C, 35 cycles of 1 min at 96°C, 1 min at 55°C, 2 min at 72°C, and 10 min at 72°C. Elongation factors, EF1 (5' ATGGGTAAGGARGACAAGAC 3'), and EF2 (5' GGARGTACCAGTSATCATG 3') (O'Donnell et al., 1998), and ITS5 (5' GGAAGTAAAAGTCGTAACAAGG 3') and ITS4 (5' TCCTCCGCTTATTGATATGC 3') (White et al., 1990) were used for the *Fusarium* isolate. Amplification of the EF-1 α and ITS genes were performed with the following program: 3 min at 95°C, 35 cycles of 30 s at 95°C, 30 s at 55°C, 1 min at 72°C, 7 min at 72°C (Figueroa et al., 2015). Amplicons were separated on a 1.7% agarose gel in 1x Tris-Acetate-EDTA (TAE) buffer at 90 volts for 80 min. Gels were visualized using Quantity One Software (Biorad, Hercules, CA, USA).

Sequence Analysis

All amplicons were purified using ExcelsaPure PCR purification kit (Edge BioSystems, San Jose, CA, USA) according to the manufacturer's instructions. Purified products were prepared for sequencing with BigDye™ Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Waltham, MA) and Performa® DTR Ultra 96-Well Plates (Edge Biosystems, San Jose, CA, USA) removed dye terminator using Capillary electrophoresis on a 3730xl DNA Analyzer (Applied Biosystems, Waltham, MA, USA). Sanger sequencing was conducted by DNA Analysis Facility at Yale University and Eurofins Genomics LLC laboratories.

Base pairs were trimmed on each end according to the quality graph. The nucleotide sequences were compared with BLAST nucleotide analysis against publicly available sequences in the GenBank online database (www.ncbi.nlm.nih.gov/BLAST). An isolate was considered homologous to published organisms when there was $\geq 99\%$ identity match and $\geq 98\%$ query coverage.

Pathogenicity and Virulence Assays

Pathogenicity was evaluated *in vitro* and *in vivo* on hemp cv. Wife. Twenty-five isolates were tested for pathogenicity using an *in vitro* Petri dish assay based on Broders et al. (2007). The seeds were surface-sterilized with concentrated sulfuric acid (98% H₂SO₄) for 20 s, rinsed under cold tap water for 20 min,

placed on cheese cloth, and tied into a bag. The bag was steeped into 75% ethanol for 2 min, wrung out, steeped in 3% bleach for 20 min, rinsed with sterile deionized water, and imbibed in purified water (Milli-Q®, MilliporeSigma, Burlington, MA, USA) for 48 h. After 48 h, the seed coat was removed using sterile forceps and a scalpel to ensure uniform germination. Five seeds were placed in a 9 cm diameter Petri dish containing water agar and spaced 2 cm from the edge and equidistantly 2 cm from each other. A 2 mm plug from each actively growing isolate cultured on CMA for 4 days was placed in the middle of each Petri dish. The control consisted of hemp seeds placed in a Petri dish with a 2 mm plug of CMA with no isolate present. Petri dishes were maintained at $\sim 21^\circ\text{C}$ in the dark for 7 days. Pathogenicity was evaluated using the following virulence scale: 0 = 100% seed germination with healthy appearance, 1 = 99–70% seed germination, small brown lesions on the radicle or hypocotyl, 2 = 69–30% seed germination, large brown lesions on the hypocotyl and radicle, and 3 = 29–0% germination, coalesced lesions covering the hypocotyl and radicle (Broders et al., 2007; Del Castillo Múnera and Hausbeck, 2016). Seed germination per plate (%) and individual seedling length (cm) were recorded. The experiment was arranged as a complete randomized design with three replicate plates per isolate. The experiment was conducted twice.

An *in vivo* pathogenicity assay was conducted in the greenhouse. Hemp cv. Wife seeds were sown in 42 mm peat pellets (Jiffy, Lorain, OH, USA), with one seed per peat-pellet. After 2 weeks, one seedling was transferred to a square plastic pot (Dillen® Deep Press Fit, A.M.A. Horticulture, Kingsville, ON, CA) with the following dimensions 10.5 \times 10.5 \times 12.7 cm filled with a peat-based potting mix (Promix BK25, SunGro, Agawam, MA, USA). Five pots were transferred to a seedling production tray (27.9 \times 54.3 \times 3.3 cm). A tray with five pots was the experimental unit and received the same treatment. Mycelial mats were used as inoculum (Heungens and Parke, 2000) and were produced as described above. Three mycelial mats of each isolate were placed in a 50 mL centrifuge tube and vortexed at high speed with six sterile silicon beads. The mycelial mats were diluted with 800 mL of sterile deionized water and 50 mL of the suspension was used to inoculate directly into the soilless-substrate in each pot. The negative control consisted of plants treated with 50 mL of sterile deionized water. The trays were maintained in UConn's Plant Science Research greenhouse on benches under high-pressure sodium lights with a photoperiod of 16 h between June and July 2019. Plants were irrigated manually with 15-30-15 at 100 mg·L⁻¹ N. Plants were assessed daily for visual wilting and mortality for 14 consecutive days. The area under disease progress curve (AUDPC) was calculated using the trapezoidal integration method (Campbell and Madden, 1990) from the disease incidence assessments. After 14 days, plant shoots were cut right above the cotyledons, and roots were washed with tap water. Shoots and roots were dried at $21 \pm 1^\circ\text{C}$ for 2 weeks and weighed. SPAD 502 Plus chlorophyll meter (Spectrum Technologies, Inc., IL) was used to compare relative greenness as a way to measure the visual chlorosis symptoms. To confirm that the symptoms observed were caused by inoculant, re-isolation of the pathogen from symptomatic

plant tissue was achieved by placing 10 pieces of 1 cm triple-washed roots on PARP for the oomycete isolates and PDA for the *Fusarium* isolate. Colonies isolated from the root tissue were examined morphologically and compared to the originally isolated organism following the methods previously described. The experimental unit consisted of five hemp seedlings in a tray and there were three replicates per treatment per experimental run. The experiment was conducted twice.

Mefenoxam Sensitivity

The sensitivity to mefenoxam of the 25 isolates was evaluated *in vitro*. Technical grade mefenoxam (99.5% active ingredient; Chem Service Inc., West Chester, PA, USA) was dissolved in dimethyl sulfoxide (DMSO) to a stock concentration of 100 mg·mL⁻¹. Control plates received the same volume of DMSO as fungicide-amended agar. A 2 mm actively growing mycelial plug was transferred to the center of a 9 cm diameter Petri plate containing CMA amended with mefenoxam at 0, 0.5, 5, 10, and 100 µg·mL⁻¹. The plates were incubated for 4 days in the dark at room temperature. The relative growth rate of an isolate at each fungicide concentration was calculated by measuring the hyphal growth in two perpendicular directions and averaging the result of the culture diameter, then dividing it by the radial growth on plates with 0% mefenoxam and multiplying the value by 100 (Olson and Benson, 2011). Isolates were characterized as “sensitive” if the culture diameter had <30% growth compared with the 0% mefenoxam plate, “intermediate resistance” if the culture diameter had between 30 and 50% of growth compared with the 0% mefenoxam plate, or “resistant” if the culture diameter was ≥50% of growth compared with the 0% mefenoxam plate (Olson and Benson, 2011). The experiment was a complete randomized design, with three replicates per isolate at each concentration—where each replicate consisted of one plate. The experiment was conducted twice.

Statistical Analysis

Disease incidence and mefenoxam sensitivity data were analyzed using SAS Version 9.4 (SAS Institute Inc., Cary, NC, USA) to establish effect of isolates on the response variables at $\alpha = 0.05$. Homogeneity of variance and normality were checked for all measured variables using Kolmogorov-Smirnov test, Cramer-von Mises test, and Kuiper test. Data were analyzed by analysis of variance (ANOVA), and means were separated using Tukey's studentized range HSD (Honestly Significant Difference) separation test using PROC MIXED. Virulence data were analyzed using non-parametric Kruskal-Wallis test to determine differences between medians and Tukey's studentized range HSD on ranked data for separation of groups (Minitab® 19, Minitab, LLC, State College, PA, USA).

Phylogenetic Analysis

COI gene regions for oomycete isolates and concatenated ITS and EF-1 α regions for *Fusarium* isolates were aligned with MUSCLE (Edgar, 2004). Phylogenetic and molecular analyses were then conducted on the aligned sequences using Geneious Prime version 2020.2.4 (Biomatters, Ltd., Auckland, NZ) (Kearse et al., 2012). An unweighted pair group method with arithmetic

mean (UPGMA) (Michener and Sokal, 1957) was constructed using the Tamura-Nei model (Tamura and Nei, 1993) with bootstrap analysis performed by resampling the data 1,000 times (Felsenstein, 1985). In addition to the sequences obtained in this study, sequences were included from the National Center for Biotechnology Information (NCBI) database for comparison (Tables 1, 2). *Phytophthora capsici* and *Phytophthora nicotianae* were used as out-groups for *P. myriotylum* and *G. irregulare* phylogenies. *F. proliferatum* and *F. subglutinans* were used as out-groups for *F. oxysporum* phylogeny.

RESULTS

Collection and Identification

Twenty-five isolates were obtained from four soilless-substrate samples from symptomatic marijuana plants collected from an indoor-growing facility. The isolates were identified based on morphological characteristics and then using gene amplification and sequencing.

Morphological Characteristics

The isolates from this study were identified as *Globisporangium irregulare* (Buisman), *Pythium myriotylum* (Drechsler), and *Fusarium oxysporum* (Schltdl). *G. irregulare* isolates on tall-fescue leaves presented the following characteristics: limoniform hyphal swellings, mostly aplerotic oospores with an average diameter of $18.6 \pm 2.8 \mu\text{m}$ and average wall diameter of 1.3 ± 0.4 , intercalary or terminal spherical oogonium (21.6 ± 3.4) with a smooth wall, monoclinal antheridia stalked 1–3 per oogonium with some branches, and spherical sporangia. *P. myriotylum* had the following characteristics: filamentous-inflated sporangia, globose-shape smooth-surface oogonia with an average diameter of $27.3 \pm 2.2 \mu\text{m}$, two to six antheridia per oogonium with club-shape and predominately declinous, aplerotic oospores an average diameter of $26 \pm 1.9 \mu\text{m}$ and average wall diameter up to $2.7 \pm 1.1 \mu\text{m}$ thick, and finger-shaped appressoria formed in clusters. *F. oxysporum* had sickle-shaped microconidia with 3–4 septae. Microconidia were produced in false heads on short monophialides. Chlamydospores were single and terminally positioned.

Identification Based on Nucleotide Sequences

Nucleotide sequences of PCR products from genomic DNA were compared (BLASTn) against publicly available sequences in GenBank®. The isolates were identified as *F. oxysporum* ($n = 1$), *G. irregulare*—also known as *P. irregulare* ($n = 3$), and *P. myriotylum* ($n = 21$) (Tables 1, 2). Six isolates of *P. myriotylum* (PM28, PM30, PM40, PM50, PM54, and PM75) and three of *G. irregulare* (GI19, GI22, and GI56) were isolated from coconut coir samples when the cultivar ‘Citron’ was at the early flowering stage. Six isolates of *P. myriotylum* (PM16, PM18, PM26, PM45, PM62, and PM65) were isolated from another coconut coir substrate with ‘Citron’ at the early flowering stage, and nine *P. myriotylum* isolates (PM1, PM7, PM10, PM11, PM13, PM15, PM32, PM71, PM77) were isolated from ‘Silver’ in an 11.4-liter

TABLE 1 | Oomycete isolates obtained from coconut coir substrates of symptomatic marijuana plants at a commercial facility in Connecticut and other oomycetes used in the phylogenetic analysis.

Organisms	Isolate	GenBank accession numbers*		References
		COI	COII	
<i>Globisporangium irregulare</i>	GI19	MT823175	MT192208	This study
<i>Globisporangium irregulare</i>	GI22	MT823165	MT192209	This study
<i>Globisporangium irregulare</i>	GI56	MT823171	MT192212	This study
<i>Pythium myriotylum</i>	PM1	MT823157	MT192191	This study
<i>Pythium myriotylum</i>	PM10	MT823159	MT192206	This study
<i>Pythium myriotylum</i>	PM11B	MT823160	MT192193	This study
<i>Pythium myriotylum</i>	PM13	MT823161	MT192194	This study
<i>Pythium myriotylum</i>	PM15	MT823162	MT192195	This study
<i>Pythium myriotylum</i>	PM16	MT823163	MT192207	This study
<i>Pythium myriotylum</i>	PM18	MT823164	MT192196	This study
<i>Pythium myriotylum</i>	PM26	MT823155	MT192197	This study
<i>Pythium myriotylum</i>	PM28	MT823166	MT192198	This study
<i>Pythium myriotylum</i>	PM30	MT823167	MT192199	This study
<i>Pythium myriotylum</i>	PM32	MT823168	MT192200	This study
<i>Pythium myriotylum</i>	PM40	MT823169	MT192210	This study
<i>Pythium myriotylum</i>	PM45	MT823176	MT192201	This study
<i>Pythium myriotylum</i>	PM50	MT823170	MT192202	This study
<i>Pythium myriotylum</i>	PM54	MT823156	MT192211	This study
<i>Pythium myriotylum</i>	PM62	MT823172	MT192213	This study
<i>Pythium myriotylum</i>	PM65	MT823173	MT192203	This study
<i>Pythium myriotylum</i>	PM7	MT823158	MT192192	This study
<i>Pythium myriotylum</i>	PM71	MT823174	MT192214	This study
<i>Pythium myriotylum</i>	PM75	MT823177	MT192204	This study
<i>Pythium myriotylum</i>	PM77	MT823178	MT192205	This study
<i>Pythium myriotylum</i>	PM11	MN037880	MN037881	McGehee et al., 2019
<i>Pythium myriotylum</i>	CBS 69579	HQ708748	-	Robideau et al., 2011
<i>Pythium myriotylum</i>	CBS 25470	HQ708745	-	Robideau et al., 2011
<i>Globisporangium irregulare</i>	CBS 74996	HQ708646	-	Robideau et al., 2011
<i>Globisporangium irregulare</i>	CBS 25028	HQ708640	-	Robideau et al., 2011
<i>Pythium aphanidermatum</i>	CBS11880	HQ708485	-	Robideau et al., 2011
<i>Pythium aphanidermatum</i>	CBS28779	HQ708486	-	Robideau et al., 2011
<i>Pythium ultimum</i>	BR1037	HQ708917	-	Robideau et al., 2011
<i>Pythium ultimum</i>	CBS72994	HQ708910	-	Robideau et al., 2011
<i>Pythium dissotocum</i>	CBS 16668	HQ708574	-	Robideau et al., 2011
<i>Pythium dissotocum</i>	BR127	HQ708576	-	Robideau et al., 2011
<i>Phytophthora capsici</i>	CBS 25493	HQ708252	-	Robideau et al., 2011
<i>Phytophthora capsici</i>	302	AY129166	-	Martin and Tooley, 2003
<i>Phytophthora nicotianae</i>	CBS 101655	HQ708354	-	Robideau et al., 2011
<i>Phytophthora nicotianae</i>	332	AY129169	-	Martin and Tooley, 2003

**Pythium* spp. and *Globisporangium* spp. isolates were sequenced with the mitochondrially-encoded cytochrome oxidase genes (COI and COII).

container with coconut coir. One isolate of *F. oxysporum* (FO76) was collected from rockwool.

Phylogenetic Analysis

Phylogenetic analysis of oomycete isolates based on the rDNA COI gene consisted of 39 taxa including 21 *P. myriotylum* and three *G. irregulare* isolates from this study, voucher sequences, and *Pythium* spp. from *Cannabis sativa* in published studies

obtained from the NCBI database and *P. capsici* and *P. nicotianae* as out-group taxa. Distance analysis using the UPGMA algorithm with bootstrap values >75% are displayed in **Figure 1**. Within the *G. irregulare* clade two monophyletic groups were formed—one with the isolates from this study (GI19, GI22, and GI56) and another with published sequences (CBS 74996 and CBS 25028) at a 100% bootstrap value. The *P. myriotylum* clade separated in two groups—PM40 parted from all other isolates. Twenty *P.*

TABLE 2 | *Fusarium oxysporum* isolate FO76 obtained from the rockwool substrate of a symptomatic marijuana plant at a commercial facility in Connecticut and other *Fusarium* spp. used in the phylogenetic analysis.

Organisms	Isolate	GenBank accession numbers*		References
		EF-1 α	ITS4 and ITS5	
<i>Fusarium oxysporum</i>	FO76	MT197114	MT210898	This study
<i>Fusarium oxysporum</i>	CA-1	MH844832	MH789985	Punja et al., 2018
<i>Fusarium oxysporum</i>	CA-2	MH844833	MH789986	Punja et al., 2018
<i>Fusarium oxysporum</i>	BC-1/F1	MH844828	MH782043	Punja and Rodriguez, 2018
<i>Fusarium oxysporum</i>	BC-2/9B	MH844829	MH782044	Punja and Rodriguez, 2018
<i>Fusarium oxysporum</i>	62A	MT186263	MT180726	Jerushalmi et al., 2020
<i>Fusarium oxysporum</i>	64B	MT186262	MT180727	Jerushalmi et al., 2020
<i>Fusarium oxysporum</i>	CBS 140424	KT794174	KT794176	Jiang et al., 2016
<i>Fusarium oxysporum</i>	CBS 133023	KF255492	KF255448	van Diepeningen et al., 2013
<i>Fusarium oxysporum</i>	CBS 132475	KR071771	KR071660	Al-Hatmi et al., 2016
<i>Fusarium oxysporum</i>	CBS 463.91	KR071772	KR071661	Al-Hatmi et al., 2016
<i>Fusarium oxysporum</i> f. sp. <i>cannabis</i>	NRRL 26410	FJ985290	FJ985497	O'Donnell et al., 2009
<i>Fusarium solani</i>	Can16	MK814525	MK393907	Sorrentino et al., 2019
<i>Fusarium solani</i>	Can18	MK814527	MK393909	Sorrentino et al., 2019
<i>Fusarium solani</i>	Can23	MK814528	MK393910	Sorrentino et al., 2019
<i>Fusarium solani</i>	109A-3	MT254546	MT229135	Jerushalmi et al., 2020
<i>Fusarium solani</i>	CBS 102429	KM231936	KM231808	Lombard et al., 2015
<i>Fusarium solani</i>	CBS 135789	KU711773	KR071699	Al-Hatmi et al., 2016
<i>Fusarium brachygibbosum</i>	G (FB-1)	MH844834	MH789987	Punja et al., 2018
<i>Fusarium brachygibbosum</i>	CBS 131252	JX118981	JX162372	Davari et al., 2013
<i>Fusarium graminearum</i>	CBS 130605	JX118870	JX162262	Al-Hatmi et al., 2016
<i>Fusarium equiseti</i>	CBS 307.94	KR071777	KR071664	Al-Hatmi et al., 2016
<i>Fusarium fujikuri</i>	CBS 262.54	KR071777	KR071664	Al-Hatmi et al., 2016
<i>Fusarium subglutinans</i>	CBS 747.97	KR071768	KR071623	Al-Hatmi et al., 2016
<i>Fusarium proliferatum</i>	CBS 480.77	KR071736	KR071678	Al-Hatmi et al., 2016

**Fusarium* spp. isolates were sequenced with the translation elongation factor (EF-1 α) region and internal transcribed spacer region (ITS4 and ITS5) genes. *F. subglutinans* and *F. proliferatum* were used as out-group taxa in phylogenetic analysis.

myriotylum isolates from this study separated in 10 subclades with bootstrap values $\geq 94.3\%$.

The EF-1 α and ITS concatenated tree analyses for *Fusarium* species consisted of 25 taxa including the *F. oxysporum* isolate obtained in this study, and 24 taxa obtained from the NCBI database (Figure 2). *F. oxysporum* FO76 (from this study) clustered with all other *F. oxysporum* isolates, except *F. oxysporum* f. sp. *cannabis* (O'Donnell et al., 2009). *F. oxysporum* FO76 caused root necrosis, chlorosis, and occasional wilt symptoms in hemp—consistent with the symptoms described by Punja and Rodriguez (2018)—and was >99% identical to the Canadian isolates BC-1 and BC-2 based on a BLASTn analysis. *F. oxysporum* isolate FO76 was 98 and 95% identical to *F. oxysporum* isolates CA-1 and CA-2 collected from marijuana roots in California, respectively (Punja et al., 2018).

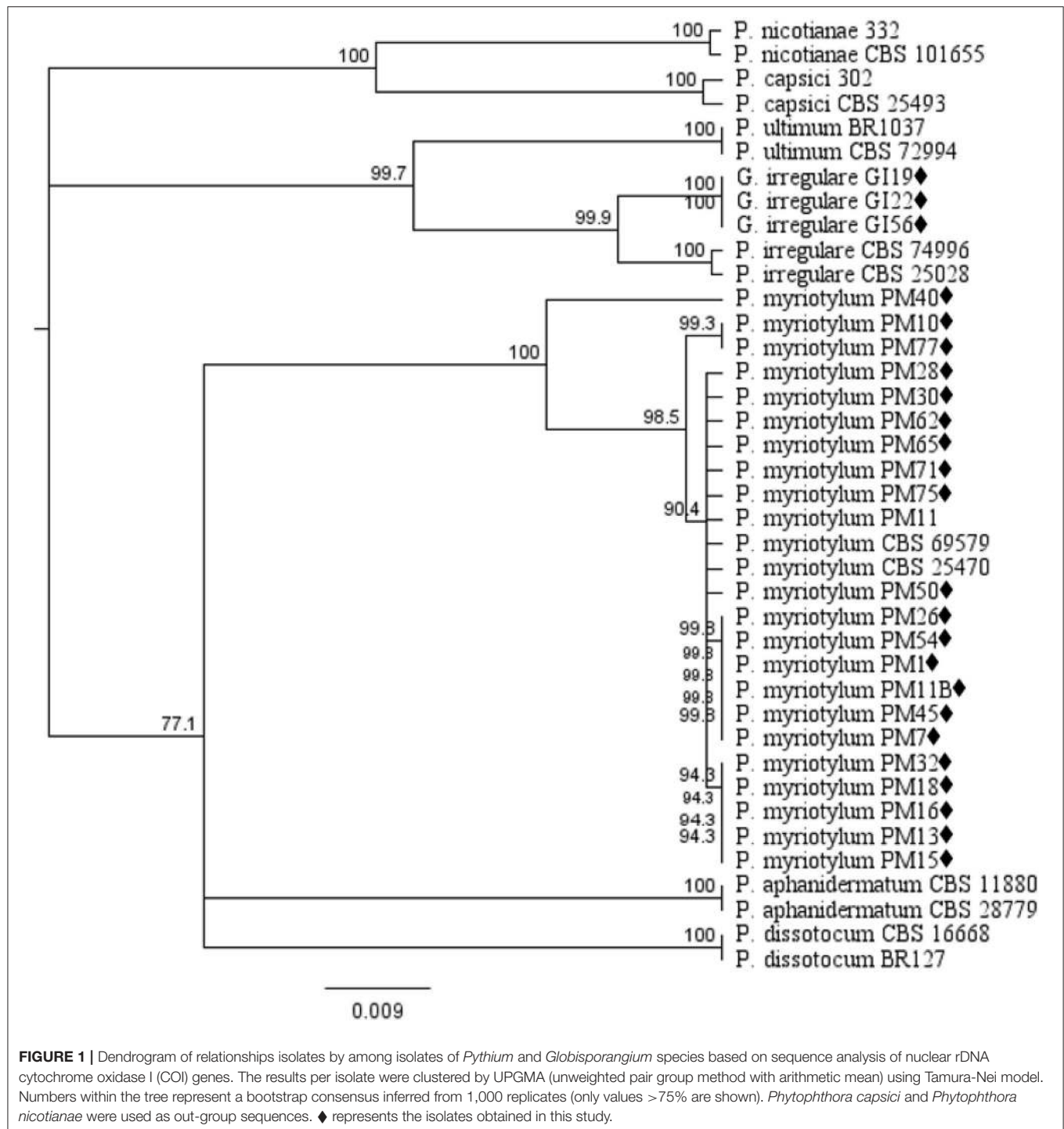
In vitro Pathogenicity Assays on Hemp Seed

All the isolates, except *F. oxysporum* FO76, resulted in the highest virulence score (3.0) and were pathogenic to hemp seeds *in vitro* (Figure 3A). Seed germination was under 30%

when inoculated with the *Pythium* and *Globisporangium* isolates, whereas the control and *F. oxysporum* FO76 resulted in 100 and 80% germination, respectively. *P. myriotylum* PM71 was the only isolate that did not differ from *F. oxysporum* FO76 in germination percentage (Figure 3B). Seedling length was the longest for hemp seeds in untreated control Petri plates. Seedlings infected with *F. oxysporum* were $\geq 60\%$ longer than the ones infected with oomycete isolates, yet all hemp seedlings in contact with isolates were significantly shorter than the negative (non-infected) control ($P < 0.0001$). Seedling length data is not shown because it presented a similar pattern as germination percentage confirmed with a Pearson correlation ($P < 0.001$, $r = 0.935$).

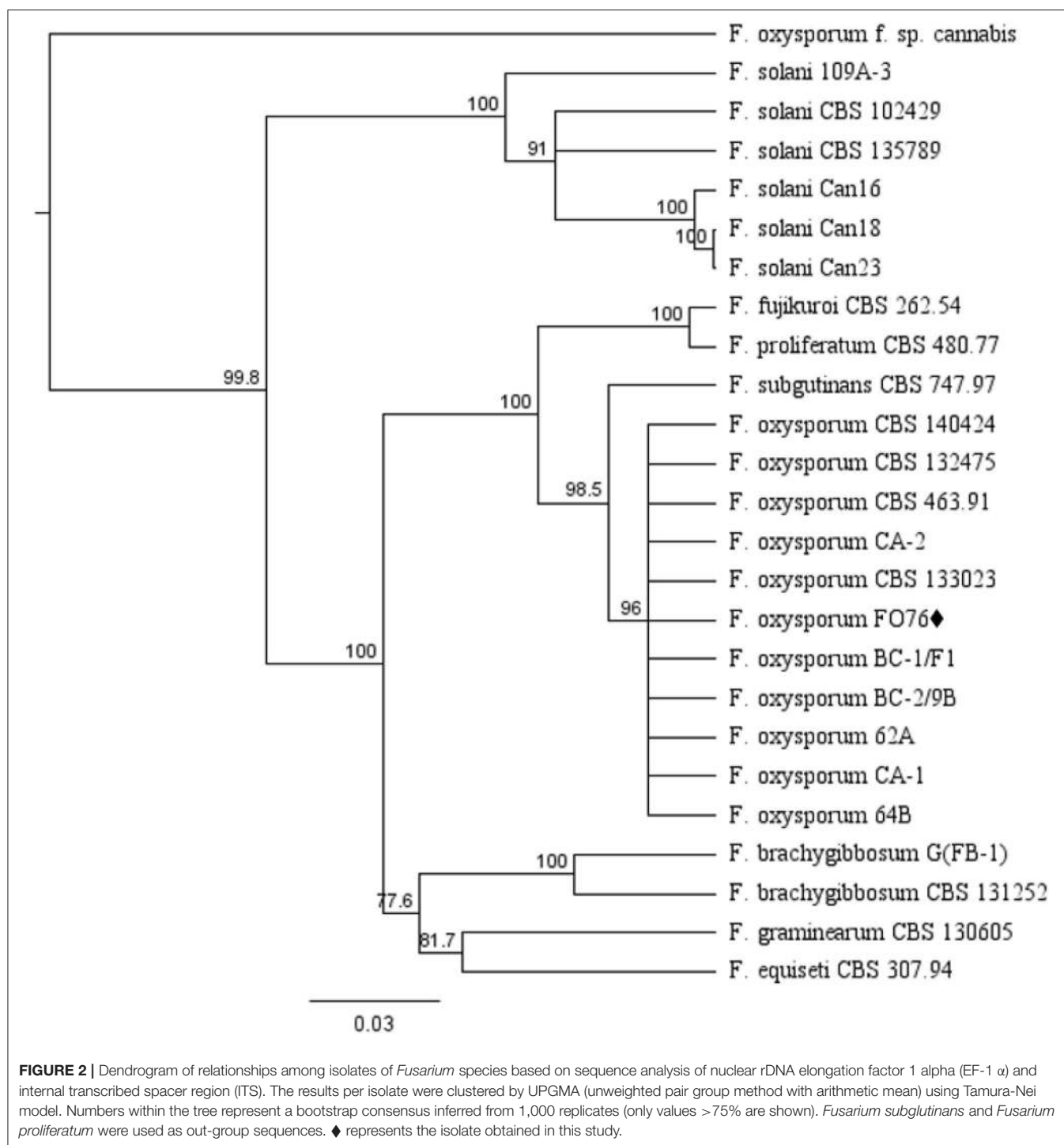
Pathogenicity Assay in the Greenhouse

Data were analyzed together for both experimental runs due to homogeneity among experimental runs ($P > 0.05$), except relative greenness and disease incidence represented as the area under the disease progress curve (AUDPC). The control plants did not display any symptoms. In general, plants inoculated with *P. myriotylum* had the lowest biomass, followed by *G. irregulare* and *F. oxysporum* (Figure 4). The control plants had the highest shoot ($\geq 32\%$) and root ($\geq 48\%$) biomass compared



with all other treatments (**Figure 4**). Early disease symptoms, wilt and chlorosis, were observed 6 days after plants were inoculated with *P. myriotylum* isolates. For *G. irregulare*, disease symptoms occurred at day 8 for isolate GI56 and day 13 for isolate GI22. Disease symptoms were observed for the *F. oxysporum* isolate FO76 9 days after inoculation. Analysis of variance for the AUDPC values based on disease incidence showed a significant

difference among isolates for both experimental runs (**Figure 5**). *P. myriotylum* isolates resulted in the highest AUDPC values in both experimental runs (**Figure 5**). In general, the AUDPC of *G. irregulare* and *F. oxysporum* did not differ from the control and *P. myriotylum* had the highest disease incidence (**Figure 5A**). The control and *G. irregulare* isolates resulted in the highest relative greenness (SPAD), indicating no symptoms

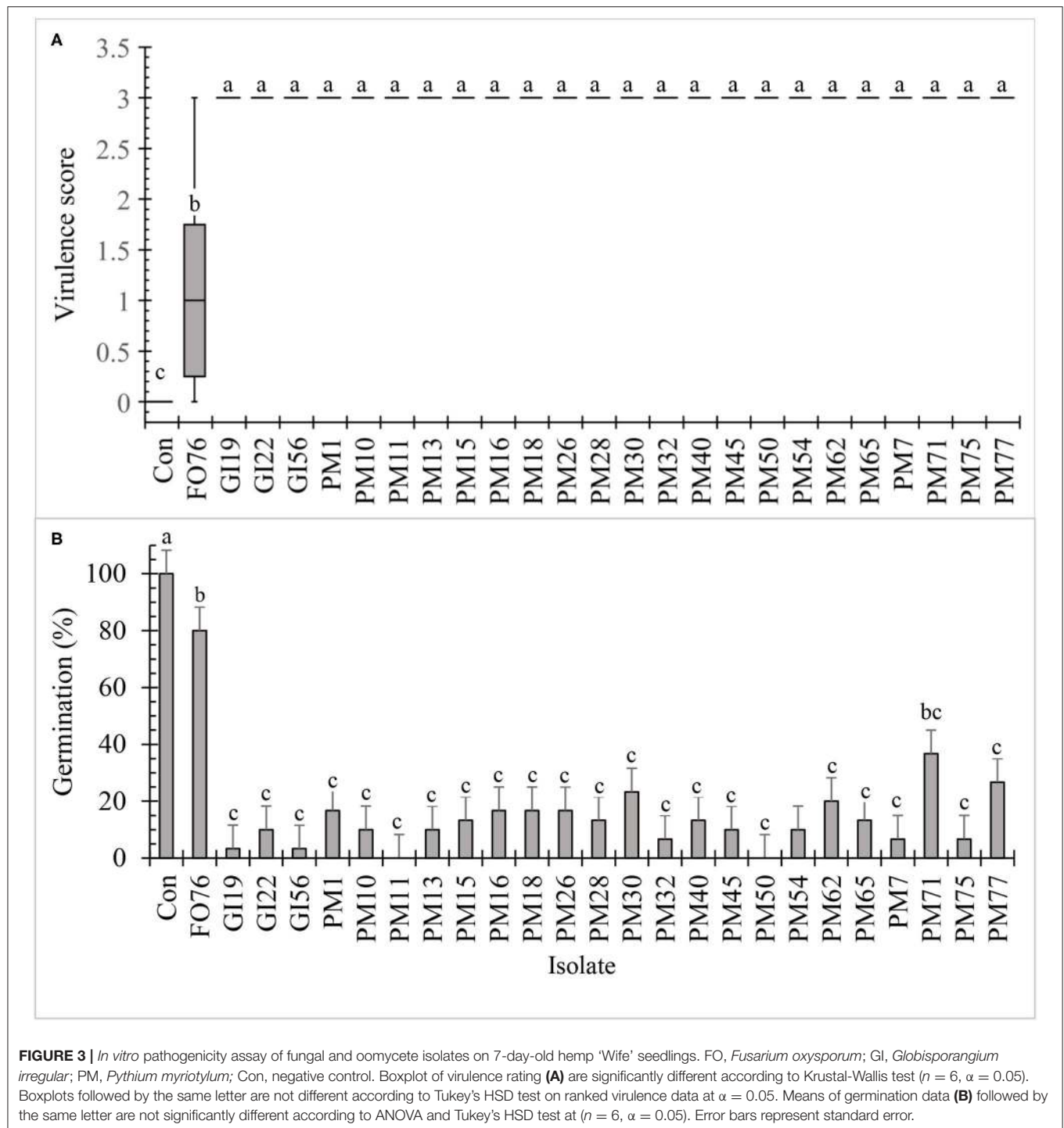


of chlorosis (**Figure 6**). Plants inoculated with *P. myriotylum* isolates had the lowest SPAD readings. *F. oxysporum* FO76 resulted in lower SPAD values compared to the control, but higher than *P. myriotylum* (**Figure 6**). Symptoms for the hemp plants infected with pathogenic isolates included reduced growth, chlorosis, and wilting (**Figure 7**). Morphological identification of the organisms grown from root fragments cultured on PARP and

PDA confirmed that the plants were infected with the organisms originally inoculated (data not shown).

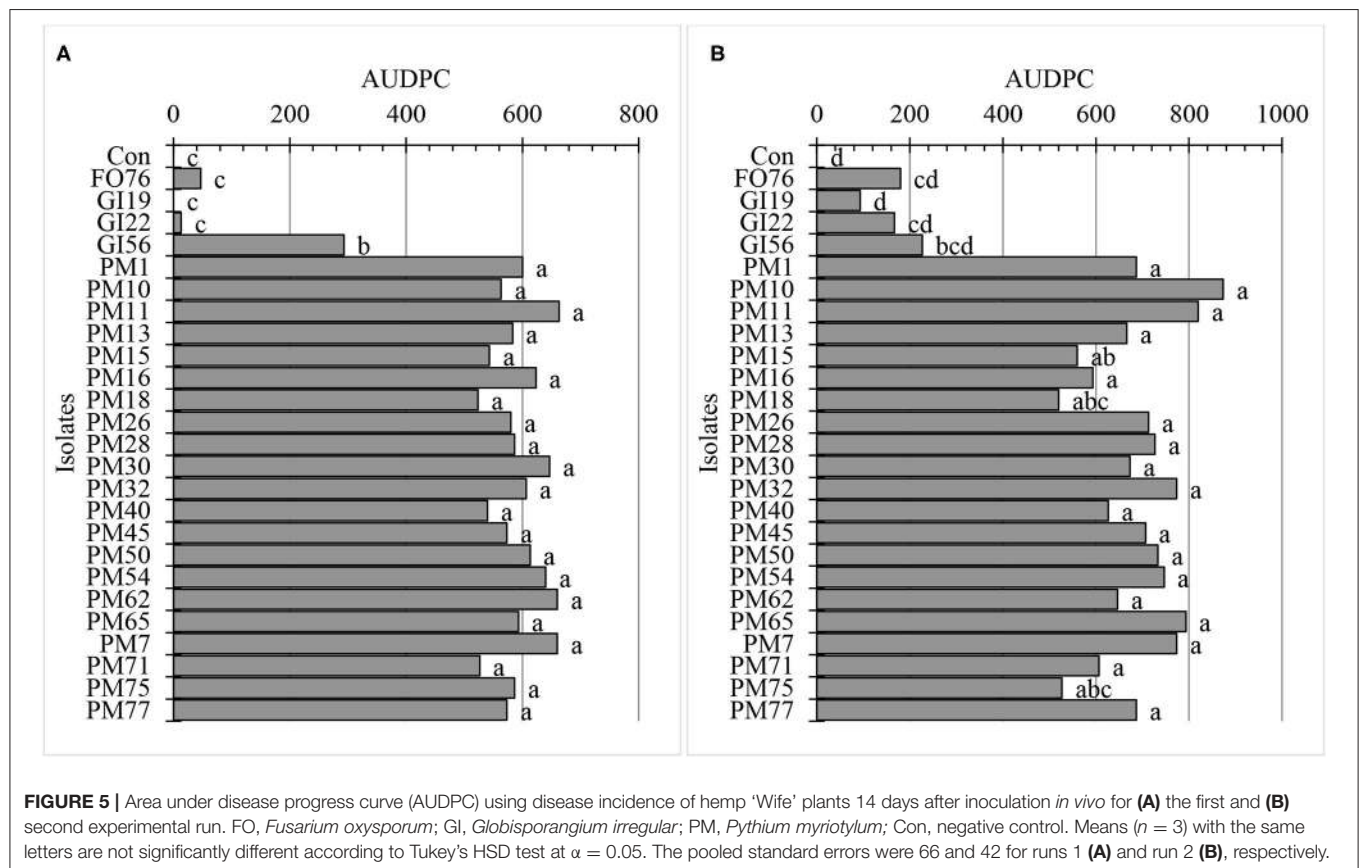
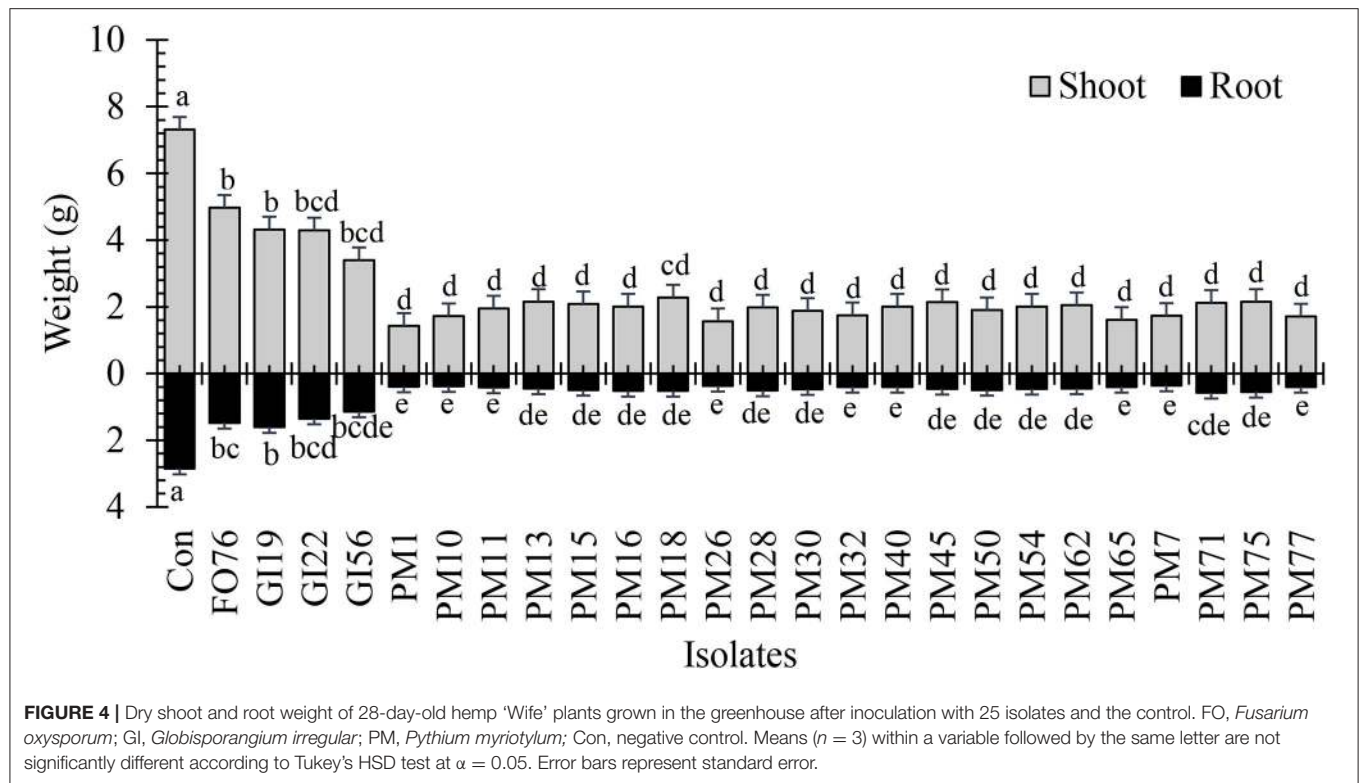
Mefanoxam Sensitivity Assay

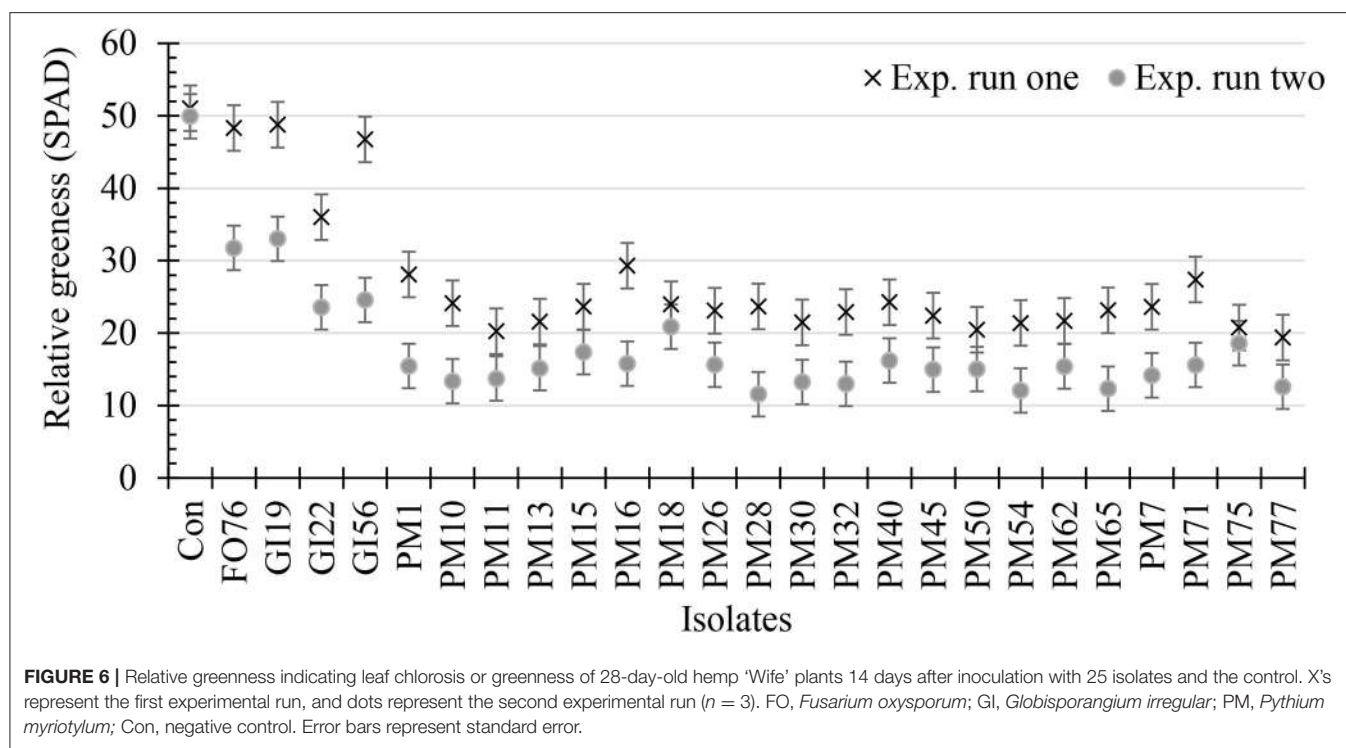
Dose concentrations, pathogenic isolates, and the interaction between the two were significant ($P < 0.0001$). The mycelial radial growth rate at $0 \mu\text{g}\cdot\text{mL}^{-1}$ of mefenoxam resulted in



100% radial growth for all oomycete isolates. *G. irregularis* isolates had intermediate resistance ($>30\%$ mycelial growth) to $0.5 \mu\text{g}\cdot\text{mL}^{-1}$ mefenoxam (Figure 8). At $5 \mu\text{g}\cdot\text{mL}^{-1}$, the radial growth ranged from 3.5 to 12.1% and 0 to 12.7% for *G. irregularis* isolates, and for *P. myriotylum*, respectively. At $10 \mu\text{g}\cdot\text{mL}^{-1}$, the radial growth ranged from 2.8 to 10.2%, and 0 to 10%

for *G. irregularis* isolates and *P. myriotylum*, respectively. *P. myriotylum* isolates were sensitive ($<30\%$ radial growth) at all doses. All oomycete isolates were sensitive to mefenoxam at $5 \mu\text{g}\cdot\text{mL}^{-1}$ or higher. The oomycetes in this study were not resistant to mefenoxam doses. *F. oxysporum* was not included in this study.





DISCUSSION

This study aimed to investigate if soilborne plant pathogens were present in soilless substrates used at a commercial facility growing marijuana. Our study is limited in scope because we were unable to collect plant tissue from the facilities and we could only collect samples one time. We obtained 21 isolates of *P. myriotylum*, three of *G. irregulare*, and one of *F. oxysporum* and confirmed in two separate experiments that these isolates are pathogenic to hemp 'Wife' seeds and seedlings. Plants inoculated with each isolate resulted in varying degrees of growth reduction, chlorosis, and wilting symptoms. *P. myriotylum* consistently caused the highest virulence (*in vitro*), lowest germination (*in vitro*), smallest plants (*in vivo*), highest AUDPC (*in vivo*), and most chlorosis (*in vivo*).

P. myriotylum, the species recovered at the highest frequency in this study, has been previously identified as the causal agent of wilting, stunting, and chlorosis on marijuana and hemp plants grown in soilless systems (Punja and Rodriguez, 2018; McGehee et al., 2019) and *G. irregulare* had not been reported in hemp or marijuana before. Punja and Rodriguez (2018) found *P. dissotocum* at the highest frequency (70%). *P. aphanidermatum* isolates were recovered from diseased cannabis rooted cuttings with symptoms of damping-off, and from flowering plants grown in cocofibre substrate under hydroponic conditions in a commercial greenhouse that were associated with chlorosis of plants and mortality (Punja and Rodriguez, 2018). Thiessen et al. (2020) reported that *Pythium* root and crown rot caused by *P. aphanidermatum*, *P. myriotylum*, and *P. ultimum* was observed in up to 8% of hemp samples from greenhouses received in the plant diagnostic clinic in North Carolina. These three *Pythium*

species have been associated with other soilless production of leafy greens in hydroponic systems (Stanghellini et al., 1998; Utkhede et al., 2000; McGehee et al., 2018). *P. myriotylum* has been recovered from other crops grown in soilless substrates including tobacco (Anderson et al., 1997), pepper (Schuerger and Hammer, 2009), tomato (Jenkins and Averre, 1983), and poinsettia (Miyake et al., 2014). These studies suggest that soilless substrates and indoor-production systems might provide favorable conditions for the proliferation of a few *Pythium* spp. (*P. aphanidermatum*, *P. myriotylum*, and *P. ultimum*). Our study was the first to isolate *G. irregulare* from any *Cannabis sativa*. The abundance and diversity of oomycete species in soilless media should be further studied with non-cultured techniques such as high-throughput sequencing metabarcoding.

We observed that *P. myriotylum* caused more damage to hemp plants than *G. irregulare* and that *G. irregulare* exhibited high disease incidence and severity *in vitro*, but not *in vivo*—suggesting that *G. irregulare* isolates may be pre-emergent pathogens. *G. irregulare* is known to cause severe pre-damping off. For example, 100% seed rot on alfalfa (Berg et al., 2017) and 100% damping-off on carrot seedlings (Howard et al., 1978) have been reported. If *G. irregulare* does cause pre-emergent damping off, that might explain why *G. irregulare* has not been previously identified on *Cannabis* spp.—since most studies studied plants at more advanced crop stages. Alternatively, environmental conditions such as temperature have the potential to influence the interaction of oomycete species with the host, since various *Pythium* species have temperature-mediated virulence (Matthiesen et al., 2016). In our study, the *in vitro* experiments were conducted at room temperature



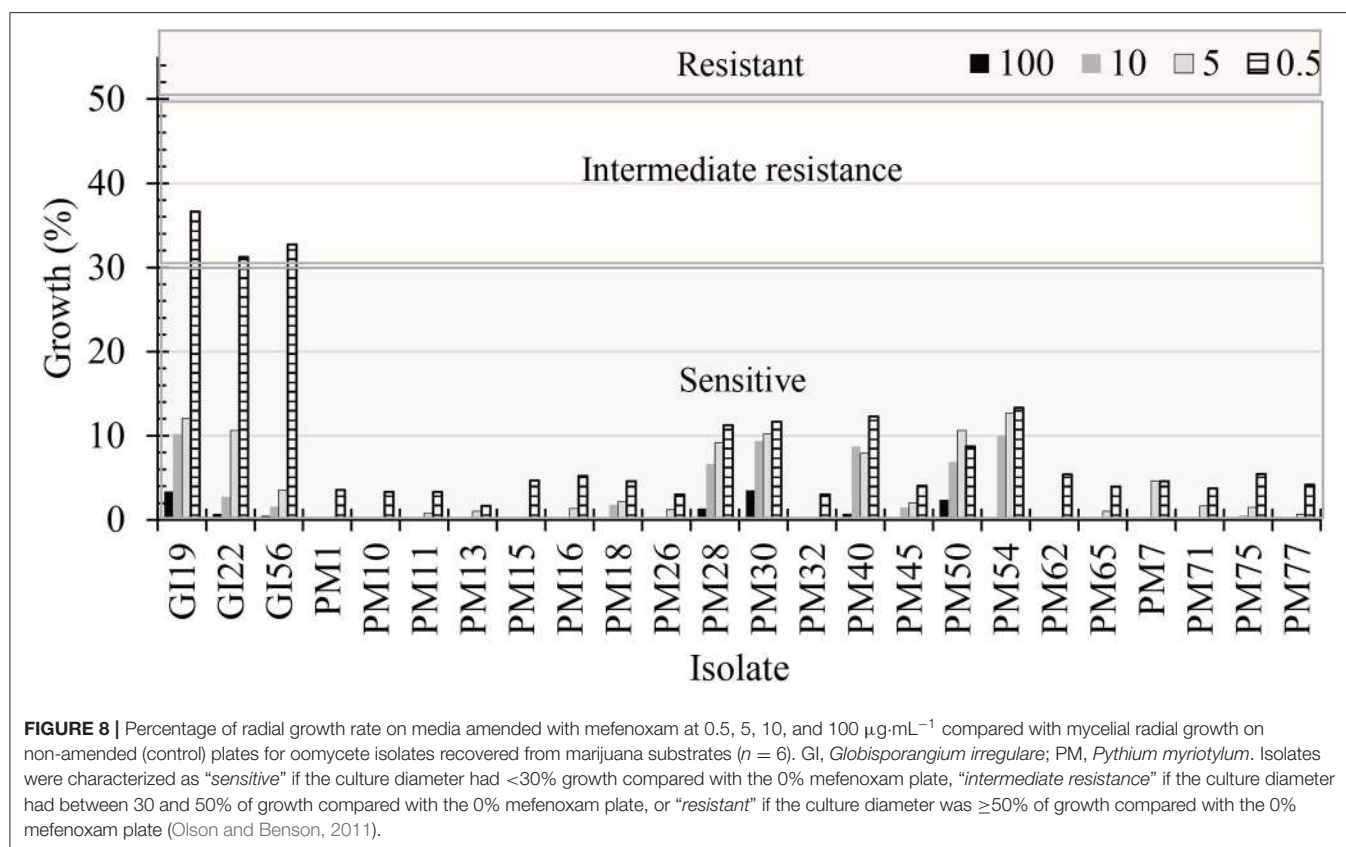
FIGURE 7 | Hemp 'Wife' plants 14 days after inoculation with pathogenic isolates *in vivo*, plus the control. FO, *Fusarium oxysporum*; GI, *Globisporangium irregulare*; PM, *Pythium myriotylum*.

(~21°C), while the *in vivo* assays were conducted in the greenhouse at a higher average temperature of $25 \pm 5.9^\circ\text{C}$. *G. irregulare* differs from *P. myriotylum* in that it prefers cooler environments. For instance, a study showed *P. aphanidermatum* and *P. myriotylum* caused damping-off on cucumber seedlings at temperatures of 20, 24, 28, and 32°C , whereas *G. irregulare* caused disease only at 20 and 24°C (Ben-Yephet and Nelson, 1999). Therefore, the variability in virulence seen with *G. irregulare* isolates may also explain differences in temperature in the two pathogenicity assays.

Phylogenetic analysis verified accurate taxonomic identification for pathogenic isolates collected from this study to reference sequences from curated databases. *F. oxysporum* isolates recovered from *Cannabis sativa* L. samples reported in California, Canada, Italy, and Israel were grouped in the same clade as our isolate FO76, indicating phylogenetic similarities. *G. irregulare* isolates from this study had a high similarity with curated reference sequences (Robideau et al., 2011). However, they formed two separate groups within the *G. irregulare* clade. Phylogenetic analysis of the *P. myriotylum* isolates of this study

resulted in several subgroups, suggesting some diversity within the isolates and low likelihood that the isolates are a single organism isolated multiple times. These results suggest a diverse population of *P. myriotylum* within the production facility that was sampled in this project. The morphological characteristics of *P. myriotylum* isolates recovered from this study were similar to those of PM11 collected from hemp roots in 2019 (McGehee et al., 2019). Both isolates were recovered from the root-zone of *Cannabis sativa* L. plants in Connecticut, yet the environment and genetic make-up of the hosts differ.

Globisporangium irregulare and *P. myriotylum* isolates were not resistant to mefenoxam—suggesting low selection pressure of fungicides in the facility. In a previous study, 32 isolates of *P. myriotylum* recovered from the roots of greenhouse-grown floriculture crops were all sensitive to mefenoxam at $100 \mu\text{g}\cdot\text{mL}^{-1}$ and 89 out of 127 *G. irregulare* isolates were resistant (Lookabaugh et al., 2015). Moorman et al. (2002) observed a similar trend with *P. myriotylum* and *G. irregulare* presenting resistance to mefenoxam at $100 \mu\text{g}\cdot\text{mL}^{-1}$. Moorman and Kim (2004) reported 8 out of 13 *P. irregulare* isolates collected



from commercial greenhouses were resistant to $0.078 \mu\text{g}\cdot\text{mL}^{-1}$ mefenoxam. Another study reported that 43 percent ($n = 55$) of *P. irregulare* isolates were resistant to mefenoxam, with 50% of the resistant isolates obtained from greenhouse grown geranium roots (Del Castillo Múnera and Hausbeck, 2016). The State of Connecticut does not allow registration or application of synthetic fungicides for use on marijuana crops grown for human consumption. While we cannot speculate about the exact source of the pathogens in this operation, we presume that it might not come from other agricultural sites where mefenoxam resistance is common.

While the results of this study are limited to a small set of samples from one commercial facility, they provide insights into potential problems and management strategies in cannabis production. The results of our study are also limited by our inability to complete Koch's postulates with the original crop—marijuana—due to legal restrictions. Hemp and marijuana have similar genetics and biosynthetic pathways, but their differences might result in differences in plant disease development. Despite these limitations, our research indicates that isolates in soilless substrates can cause diseases on hemp significantly reducing its potential to grow. Based on this study and previous ones (Punja and Rodriguez, 2018; McGehee et al., 2019) we can also conclude that *P. myriotylum* is frequently found in the rhizosphere of cannabis plants grown in soilless substrates. Further research should investigate whether some soilless substrates are more or less conducive to harbor specific groups of pathogens, as we observed from our sampling. Except for *P. myriotylum* (McGehee

et al., 2019), the pathogens described here have not been reported to cause root rot and wilt in cannabis plants in Connecticut. Both *P. myriotylum* and *F. oxysporum* have been identified causing root rot in Canada (Punja et al., 2018). Further studies and surveys are needed to determine the prevalence of these species and the economic impact these root diseases have on greenhouse cannabis production in other U.S. states.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repository and accession number(s) can be found below: <https://www.ncbi.nlm.nih.gov/genbank/>, MT823175 <https://www.ncbi.nlm.nih.gov/genbank/>, MT823165 <https://www.ncbi.nlm.nih.gov/genbank/>, MT823171 <https://www.ncbi.nlm.nih.gov/genbank/>, MT823157 <https://www.ncbi.nlm.nih.gov/genbank/>, MT823159 <https://www.ncbi.nlm.nih.gov/genbank/>, MT823160 <https://www.ncbi.nlm.nih.gov/genbank/>, MT823161 <https://www.ncbi.nlm.nih.gov/genbank/>, MT823162 <https://www.ncbi.nlm.nih.gov/genbank/>, MT823163 <https://www.ncbi.nlm.nih.gov/genbank/>, MT823164 <https://www.ncbi.nlm.nih.gov/genbank/>, MT823155 <https://www.ncbi.nlm.nih.gov/genbank/>, MT823166 <https://www.ncbi.nlm.nih.gov/genbank/>, MT823167 <https://www.ncbi.nlm.nih.gov/genbank/>, MT823168 <https://www.ncbi.nlm.nih.gov/genbank/>, MT823169 <https://www.ncbi.nlm.nih.gov/genbank/>, MT823176 <https://www.ncbi.nlm.nih.gov/genbank/>,

MT823170 <https://www.ncbi.nlm.nih.gov/genbank/>, MT823156 <https://www.ncbi.nlm.nih.gov/genbank/>, MT823172 <https://www.ncbi.nlm.nih.gov/genbank/>, MT823173 <https://www.ncbi.nlm.nih.gov/genbank/>, MT823158 <https://www.ncbi.nlm.nih.gov/genbank/>, MT823174 <https://www.ncbi.nlm.nih.gov/genbank/>, MT823177 <https://www.ncbi.nlm.nih.gov/genbank/>, MT823178 <https://www.ncbi.nlm.nih.gov/genbank/>, MT197114 <https://www.ncbi.nlm.nih.gov/genbank/>, MT210898.

AUTHOR CONTRIBUTIONS

CM and RR contributed to the design, implementation, and data analysis of the study. CM conducted the experiments,

analyzed the data, and wrote the first draft of the manuscript. RR supervised the findings of this work, verified data analysis, and contributed to the interpretation of results and discussion. All authors contributed to the final manuscript and approved the final version.

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