Research Report

Genetic Diversity and Fungicide Sensitivity of *Phymatotrichopsis omnivora* Isolates from Cotton in Arizona

az1655 December 2014

P. Chitrampalam and M. W. Olsen

Postdoctoral Research Scientist, North Dakota State University, and
Extension Specialist in Plant Pathology University of Arizona, College of Agriculture and Life Sciences

Abstract

Cotton root rot, caused by Phymatotrichopsis omnivora, is the most destructive disease of dicotyledonous plants in Arizona. There are no known reliable control methods for this disease, and the difficulty in its management is most often directed at its survival deep in soils and its wide host range. Genetic diversity in P. omnivora and its potential role in disease are unknown. Isolating the fungus and reproducing the disease in the greenhouse or laboratory are problematic, making it difficult to assess the efficacy of potential treatments. To overcome at least part of these difficulties in research on the disease, we developed a culture independent method in which mycelial strand pieces from individual roots were used for DNA extraction, and the DNA was used in PCR amplification with P. omnivora specific primers. Sequences of ITS and LSU regions revealed variability in P. omnivora isolates from four cotton fields in two locations (Marana and Safford) in Arizona. Two major groups of the fungus were found with one containing isolates mostly from Marana while the other contained isolates from both locations. Fungicide sensitivity of selected isolates conducted in laboratory culture assays with Topsin-M (thiophanate-methly) and Topguard (flutriafol) fungicides showed Topguard was more effective than Topsin-M with significant inhibition at 0.01 mg/L a.i. Results indicate that genetic diversity in the P. omnivora isolates tested are not a factor in fungicide sensitivity. The culture independent method reported in this study will make further investigations into the diversity among isolates more efficient for testing characteristics such as sensitivity to fungicides.

Introduction

Cotton root rot (CRR) is caused by *Phymatotrichopsis omnivora*, an ascomycete fungus that has a wide host range and is capable of infecting more than two thousand species of dicots (Streets and Bloss, 1973). Cotton, alfalfa, vegetable crops, grapes, fruit and nut trees and many ornamental woody plants are susceptible to the disease (Streets and Bloss, 1973). CRR, also known as Texas root rot, is one of the most destructive diseases of cotton in the United States, causing an annual economic loss up to $100 million to the US cotton crop (Marek et al., 2009). To date, no single control strategy is effective against cotton root rot (Gaxiola, 2007; Uppalapati et al., 2010). Chemical control, biological control and breeding for resistant varieties have failed to control the disease, and avoidance of planting susceptible crops in areas with a history of cotton root rot is considered the best management strategy (Gaxiola, 2007). Recent field trials indicate that Topguard (flutriafol) fungicide is effective in controlling cotton root rot (Isakeit et al., 2012; Norton et al., 2014), and trials continue in Texas and Arizona to improve efficacy with variations in rates and application methods.

P. omnivora is a soil borne pathogen which survives as both mycelial strands and sclerotia in the soil. Mycelial strands are multilayered hyphae, which usually form on the surface of the host roots by interweaving the runner hyphae around the central large hyphae (Uppalapati et al., 2010). Mycelial strands can survive on both newly infected cotton roots and decayed roots from year to year (Wheeler and Hine, 1972) to several years (Rogers, 1942). The significance of mycelial strands to both the life cycle of *P. omnivora* and disease cycle of cotton root rot is still not clearly understood (Uppalapati et al., 2010), and the results relating to the function of mycelial strands in causing disease were inconsistent among studies (Dana, 1929; Neal and Maclean, 1931; Alderman and Hine, 1982). Dana (1929) reported that mycelial strands were capable of inciting disease in cotton while Neal and Mclean (1931) and Alderman and Hine (1982) demonstrated that pieces of mycelial strands, collected from the field to inoculate cotton roots, failed to cause disease. *P. omnivora* forms sclerotia on maturing mycelial strands on the host root surface in many locations. However, sclerotia are rarely found in Arizona soils or on infected cotton plants (Wheeler and Hine, 1972; Alderman and Hine, 1982; *authors' observations*), and there is little evidence that they play an important part of the life cycle of the fungus on any hosts in Arizona (Wheeler and Hine, 1972). Reports on long term viability of sclerotia are inconsistent. Some studies reported that sclerotia can survive in soil for several years in the absence of a host (Streets and Bloss, 1973), while others reported that sclerotia die within 45 minutes of sitting on lab bench (Wheeler and Hine, 1972; Alderman and Hine, 1982). *P. omnivora* may also form spore mats on the soil surface with large numbers of conidia, but they are rarely observed in cotton fields in Arizona. Spore mats appear in alfalfa fields, orchards and landscapes, but the conidia do not germinate in culture, and their role in the life cycle of the fungus is unknown (Streets and Bloss, 1973; Uppalapati et al., 2010).

The absence of both a known sexual stage and a functional asexual spore stage limits the potential for genetic variability in *P. omnivora*. However, *P. omnivora* is a multinucleate fungus, and an individual hyphal cell contains several nuclei. In addition, *P. omnivora* could also maintain a heterokaryon state (Macmil, 2009, dissertation), which could further facilitate the parasexual cycle, leading to significant variability in *P. omnivora* (Hosford and Gries, 1966). Considering there is no previous report on the genetic structure of *P. omnivora*, determining the extent of genetic variability in *P. omnivora* is important for visualizing the evolutionary potential of *P. omnivora* and developing effective management strategies, especially with new fungicides.

The lack of selective media and the extensive growth of secondary saprobes during isolation from rotted roots are major difficulties associated with isolation of *P. omnivora*. Therefore, we attempted a culture independent approach to examine the genetic variability in *P. omnivora* by extracting DNA from mycelial strands collected directly from cotton root samples and performing PCR amplification with *P. omnivora* specific primers. The nuclear ribosomal RNA genes (rDNA) and internal transcribed spacer (ITS) regions of the rDNA have been widely used to study the relationship between closely related fungal genera, species or isolates of a single species (O'Donnell, 2000; O'Donnell et al., 2008). Protein-encoding genes such as elongation factor 1-alpha (*EF1-α*), β-tubulin and glyceraldehyde phosphate dehydrogenase have also been used in phylogenic studies in fungi (O'Donnell et al., 2008). In this study, we used ITS and 28S large subunit of ribosomal DNA and *EF1-α* to examine the genetic variability in *P. omnivora*. The main objective of this study was to determine the genetic diversity in *P. omnivora* from cotton in two geographically separated locations in Arizona where CRR may cause yield losses of 10% or more (Mulrean, et al., 1984). Specific objectives were to (1) develop a culture independent method to examine the genetic diversity in *P. omnivora*; and (2) determine the comparative sensitivity of *P. omnivora* isolates to Topguard (flutriafol) fungicide.

Materials and Methods

Sample collection, isolation and pure culturing

Samples were collected from four cotton fields, two in Marana, Arizona, labeled A and B and two in Safford, Arizona, labeled C and D in 2012 (Table 1). In each field, several *P. omnivora* infected cotton plants were collected from each of twenty discreet infection sites across the field in early August when disease was first observed. Each site had from 3 to 10 symptomatic plants in a group, but none was contiguous with another. Plants were dug out using a drain spade to take out as much intact root as possible. The roots were cut from plants, placed in a plastic bag and processed on the same or next day. Roots from each infection site were washed separately in running tap water for at least 5 minutes. Fifteen to 20 pieces of mycelial strands were manually picked off with forceps from one diseased root sample from each site under a dissecting microscope and collected in sterilized water in 1.5 ml centrifuge tubes. The collected mycelial strands were used immediately or stored at -20˚C for DNA extraction.

To validate our culture independent technique, isolations were made from a plant at each infection site. At this stage of disease, when plants are wilting but not dead, the fungus is relatively easy to isolate from symptomatic cotton roots. Roots were washed in running water, cut into small pieces (1cm), surface sterilized with 10% bleach for 3 min, rinsed in sterilized water, placed in 2% water agar Petri dishes (four pieces/dish) and incubated at 28˚C. The plates were checked for *P. omnivora* growth for 3 to 7 days. Newly emerging *P. omnivora* hyphae were isolated on ¼ strength potato dextrose agar (PDA) and incubated at 28˚C for one week. We attempted to generate at least 20 pure culture isolates by hyphal tipping, but only eight hyphal-tipped cultures were successfully grown and transferred. They were maintained as A10, B6, B12, C2, C10, D1, D8 and D10.

DNA extraction, primer design, and PCR amplification

For field samples, DNA was extracted from mycelial strands collected directly from the root. Pure cultured isolates were grown in 50 ml of potato dextrose broth (PDB) in 250 ml flask at 28˚C for 2-3 weeks. Mycelia were harvested and used for DNA extraction. DNA was extracted using the FastDNA extraction kit (MP Biomedicals, Solon, Ohio), quantified using a NanoDrop 1000 spectrophotometer (NanoDrop Products, Wilmington, Delaware), and the final DNA concentration was adjusted to 10 ng/µl using sterilized water for polymerase chain reaction (PCR). The extracted DNA was stored at -20˚C until further use.

Three loci, internal transcribed spacer (ITS), and 28S larger subunit (LSU) of the nuclear ribosomal DNA and translation elongation factor 1-α (*EF1-α*) were initially PCR amplified for 12 representative samples using *P. omnivora* primers. For ITS amplification, *P. omnivora* specific primers previously described (Marek, et al., 2009) were used. *P. omnivora* specific primers for the other two loci, LSU and EF1-α, were designed in this study (Table 2). LSU sequences of several *P. omnivora* isolates and its closely related species were retrieved from GenBank (Table 3) and aligned invoking clustalW in program Bioedit v.5 (Ibis Biosciences, Carlsbad, California). *P. omnivora* specific primers were designed at regions conserved (> 95%) within species but that varied between species (27 to 59 % depending on the species and the primers) and the specificity was verified (Figure 1). As there was no sequence of *EF1-α* available for *P. omnivora* at the GenBank as of on May 2012, *EF1-α* sequence of *Rhizina undulata*, a sister species of *P. omnivora*, retrieved from GenBank (DQ471080) was queried against the *P. omnivora* genome sequence (http://www.genome.ou.edu/fungi.html) to obtain the matching sequence in *P. omnivora*. The resulting sequences were aligned with *EF1- α* sequence from *Rhizina undulata* (DQ471080), and primers were designed at variable regions to amplify a region of *EF1-α* specifically from *P. omnivora*.

PCR amplification was performed in Bio-Rad DNA Engine thermocycler (Bio-Rad Laboratories, Hercules, California). Reaction mixtures included: 2 µl of genomic DNA (2 ng/µl), 2 µl of 10X buffer, 2.5 µl of Q solution, 0.5 µl of dNTPs, 0.25 µl of Taq polymerase,1 µl each of forward and reverse primer and 15.25 µl of sterilized water. PCR cyclic parameters for all three loci were: initial denaturing at 95˚C for 5 min, followed by 35 cycles of denaturing at 95˚C for 60 s, annealing for 30 s, and extension at 72˚C for 60 s. A final extension was carried out at 72˚C for 5 min. Annealing temperatures were 55, 40 and 59˚C for ITS, *EF1-α* and LSU, respectively. Aliquots of PCR products (6µl) were separated on 1% agarose gel by electrophoresis. Gels were stained with ethidium bromide and visualized under a UV trans-illuminator (Ultra-Violet Products Ltd, Cambridge, United Kingdom). Once the amplifications were confirmed on the gel, PCR products were cleaned with exonuclease I and rAPid (alkaline phosphatase) by following the manufacturer's protocol (USB products, Cleveland, Ohio) and sequenced. The specificity of primers designed in this study for *P. omnivora* was validated by testing these primers in PCR amplification against several other fungi from several ascomycetes (*Alternaria* sp., *Fusarium* sp., *Sclerotinia sclerotiorum*, and *Verticillium* sp.) and a basidiomycete (*Rhizoctonia* sp.).

Genetic diversity of *P. omnivora* in cotton.

Nucleotide variability was performed using representative isolates from all four fields to assess the usefulness of each locus for capturing variability in *P. omnivora.* Only the LSU and ITS regions revealed variability among representative isolates, so these two loci were chosen for further genetic analysis. DNA sequencing was performed at the University of Arizona Genetics Core for bidirectional sequencing using ABI BigDye Terminator v3.1 Cycle Sequencing chemistry on an ABI PRISM 3100 automated sequencer (Applied Biosystems, Foster City, California). The sequences were trimmed, aligned using ClustalW version 2.0.12, and manually adjusted using Mesquite software version 2.72 (Maddison and Maddison 2009; Thompson et al., 1994).

The sequences of both ITS and LSU regions were analyzed separately and together along with six *P. omnivora* isolate sequences retrieved from GenBank (Table 4). Maximum parsimony tree was constructed using MEGA5, and the tree was rooted with *Rhizina undulata* (Marek *et al*., 2009). Maximum parsimony tree was obtained using Tree-Bisection-Regrafting algorithm with search level 1 in which the initial trees were obtained by the random addition of 1000 replicates. The other parameters were set as default. A bootstrap test was performed with 1000 random sequences additions and 1000 bootstrap to examine the confidence in tree topology. Sequences were deposited in Genbank under the accession numbers KJ410052- KJ410130 for ITS, and KJ397625- KJ397704 for LSU.

Sensitivity of *P. omnivora* to fungicides in-vitro

Six representative isolates, three from each of the two major clades described by phylogenetic analysis, were used to determine fungicide sensitivity. *P. omnivora* isolates pure-cultured by hyphal tipping (A10, B6, B12, C2, D1 and D8) were tested for sensitivity to Topguard (a.i: 11.8% flutriafol; Cheminova, Inc., Research Triangle Park, North Carolina 27709), and Topsin-M (a.i: 70% thiophonate-methyl; United Phosphorous, Inc., King of Prussia, Pennsylvania 19406) in-vitro. Topguard was chosen because of its recent success in field trials (Isakeit et al., 2012) and Topsin-M because of its broad range of fungicidal activity and history of use in previous field trials (Isakeit, 2006). Concentrations of 0.1, 1, 10 and 100 mg/L a.i. Topsin-M and of 0.001, 0.01, 0.1, 1, 10 and 100 mg/L a.i. Topguard were tested based on results from a previous fungicide sensitivity trial with *P. omnivora* (Hine, et. al, 1985). Stock solutions of the fungicides were prepared in sterilized water and the required quantity of stock solution was added to PDA (autoclaved and cooled to 55 C) to obtain the desired concentrations of active ingredient. Mycelial plugs (3-mm-diameter) from 10 day old PDA cultures were placed in the center of PDA plates amended with fungicides and incubated at 28 C for 21 days. Fungal growth was measured by colony diameters at two axes after 14 and 21 days incubation and averaged for each time point. Mycelial plugs on PDA plates without fungicide served as a negative control. There were five plates for each treatment. Measurements taken after 21 days incubation were used in statistical analyses. Analysis of variance was performed on the fungicide data using Sigmastat software package (Systat Software Inc., San Jose, California, USA).

Results and Discussion

Confirmation of specificity of primers

Polymerase chain reaction amplification was successful with *P. omnivora* specific primers designed in this study. The primer pairs Po28SF1& Po28SR1 and PoEF2 & PoEFR2 specifically amplified the region of 28S large subunit (LSU) of ribosomal DNA and *EF1- α*, respectively from DNA extracted directly from mycelial strands from diseased roots. Expected amplicon size of 1200 bp and 500 bp were obtained for LSU, and *EF1- α*, respectively in PCR (Fig. 1). Both primer pairs failed to yield a product in PCR with any of the non-*P. omnivora* DNA tested, such as *Alternaria* sp., *Fusarium* sp., *Rhizoctonia* sp., *Sclerotinia sclerotiorum*, and V*erticillium* sp., confirming the specificity of these primers to *P. omnivora* (Figure 1). Both non-*P. omnivora* DNA and *P. omnivora* mycelial strand DNA yielded a product with universal ITS primers (ITS1 and ITS4) (data not shown), suggesting the previous negative amplification for non-*P. omnivora* DNA was not associated with DNA but exclusively due to the specificity of primers to *P. omnivora.*

Successive sequencing of the PCR products yielded an unambiguous sequence for every sequenced sample validating both the culture independent DNA extraction approach and the *P. omnivora* specific primers designed either in this study (*EF 1-α* and LSU specific primers) or a previous study (ITS specific primer) (Marek et al., 2009). No differences between the sequences obtained from DNA of the culture independent DNA extraction method and DNA of pure culture were observed, emphasizing the suitability and advantage of the culture independent approach used in this study.

One potential problem that may arise with this approach is the presence of more than one strain of the fungus on a single root. If more than one genetically distinct strain attacks the same plant then there is a possibility that the mycelial strand pieces collected from that particular plant could be a mixture of both strains and potentially yield an ambiguous sequence if there is any polymorphism at the sequenced region. This problem could be reduced either by collecting a single mycelial strand or restricting mycelial strand collection to a single area of root under the dissecting microscope as was followed in this study. *P. omnivora* is a multinucleate fungus and heterokaryon formation is possible (Macmil 2009). Sequencing from a heterokaryotic isolate would be expected to yield an

ambiguous sequence at the polymorphic sites. With the culture independent approach, it is difficult to differentiate whether the ambiguous sequences at the polymorphic sites are from a heterokaryon or DNA of genetically distinct strains. To overcome this confusion, an additional pure culturing step would be necessary for a particular isolate.

Genetic diversity of *P. omnivora* in cotton

The aligned partial LSU and ITS regions consisted of 1094 and 515 aligned nucleotide characters, respectively, of which 9 (0.82%) and 36 (7%) characters were parsimony informative for LSU and ITS, respectively. Partition homogeneity test revealed that LSU and ITS data were congruent (P= 0.0769), and the combined ITS and LSU data set consisted of 1609 aligned nucleotide characters and 43 informative sites.

A heuristic search revealed 14 most-parsimonious trees (tree length = 168) with consistency index (C.I) equal to 0.66 and a retention index (R.I) equal to 0.95 (Table 5). One of the most parsimonious trees is shown in Figure 2. *P. omnivora* isolates from cotton fields were grouped into two major clades. In clade-I all isolates were from fields A and B in Marana, AZ except two from field D in Safford, AZ. However, the two isolates from Safford differed from the isolates from Marana in clade-I (BP 79%). Clade-II contained cotton isolates from both Marana and Safford, AZ (Figure 2). Isolates within clade-II were highly variable, and there were five phylogenic lineages within clade-II (Figure 2), of which three groups were supported with bootstrap value > 50%. Isolates from field C and D were grouped together in several groups in clade-II. Isolates from field A and B from Marana were grouped separately as distinct lineage or as distinct node within lineage in clade-II. All other isolates retrieved from Genbank were nested in clade-II (Figure 2) except the cotton isolate from Arizona (ATCC 32445) that nested in clade-I.

The biological significance of two genetic clusters and multiple phylogenetic lineages within cluster-II in *P. omnivora* is unknown. The observance of association of genetic cluster-I mainly to Marana isolates as well as the distinction of isolates from the two areas within cluster-II suggested a restricted genetic flow between the two cotton producing areas. The areas are located about 150 miles apart geographically, and there is no movement of commercial farm equipment between them. *P. omnivora* has no known sexual stage, but occasionally spore mats form on the soil surface that produce large numbers of conidia. These conidia could easily be spread from field to field and location to location by wind, but they do not germinate in culture and have never been observed to do so under natural conditions. Their role in both the life cycle of the fungus and pathogenicity is unknown, and their role in gene flow is further questioned (Uppalapati et al., 2010.). Taken together, physical separation and little possibility of spread of the fungus, the conduits to genetic flow between these two cotton areas are highly limited.

P. omnivora reproduces asexually by producing sclerotia and mycelial strands, and the resulting population is expected to be clonal with limited variability mainly from mutation. On the contrary, in this study high genetic variability was observed in *P. omnivora* especially in clusters II which contained five phylogenetic lineages. The possible reason (s) for the observed high genetic variability in *P. omnivora* is unknown at this point.

Elongation factor 1-α and β tubulin genes are extensively used for determining genetic variability within and between fungal species (O'Donnell et al., 2008; Qin et al., 2006), but the β tubulin gene was not phylogenetically informative in a previous *P. omnivora* study (Marek et al., 2009), so it was not used in this study. Similarly, sequencing of *EF 1-α* did not show any sequence variability among the representative *P. omnivora* isolates even though the *EF 1-α* gene was phylogenetically informative in number of other soil borne pathogens such as *Fusarium solani* and *Verticillium* sp. etc. (O'Donnell, 2000; O'Donnell et al., 2008; Inderbitzin et al., 2011). Neither the sequences of *EF 1-α* of *P. omnivora* in GenBank nor a completely annotated genome sequence of *P. omnivora* were available before this study to amplify large region of *EF 1-α* gene from *P. omnivora*. With the use of *EF1-α* sequence of *Rhizina undulata*, a sister species of *P. omnivora*, from GenBank (DQ471080.1) and with the blastx search facility in *P. omnivora* genome sequence database (http://www.genome.ou.edu/fungi.html), we were able to design a *P. omnivora* specific primer to obtain a 500 bp fragment. The observance of invariability at this locus may be due to either lower fragment size or the low number of representative samples tested for this locus.

Sensitivity of *P. omnivora* to fungicides in-vitro

All isolates tested for fungicide sensitivity in-vitro were more sensitive to Topguard than to Topsin-M (Table 6). Topguard significantly inhibited growth of four of the six *P. omnivora* isolates at concentrations of 0.01 mg/L a.i. and higher when compared to the control, but none was significantly inhibited at 0.001 mg/L a.i. Differences among isolates were significant at 0.01 and 0.1 mg/L, indicating some variability, but a larger sample size will be needed to

make further conclusions. Topsin-M inhibited growth of all isolates at 1.0 mg/L a.i and higher with evidence of variability among isolates at the 1.0 mg/L concentration.

Laboratory trials with soils treated with Topguard in the field showed Topguard significantly inhibited growth of *P. omnivora* for at least three irrigations and at depths below the treated soils (Olsen, et al., 2014). However, the percentage of dead plants and yield data from field plots were variable (Norton, et al., 2014). Since our results showed genetic distinctions in *P. omnivora* in these two cotton producing areas of Arizona, where cluster-II contained isolates mainly from Marana, we questioned if this genetic variability could explain the observed differences in efficacy of flutriafol fungicide. Results of in-vitro fungicide sensitivity assays show that representative isolates of both clusters were equally sensitive to Topguard (Table 6). In an ANOVA for location, isolates from fields A and B in Marana and from C and D in Safford were not significantly different. However, the number of isolates tested was limited, and further work is needed to confirm these findings.

Conclusions

The culture independent approach developed in this study, extracting the DNA directly from mycelial strands from diseased samples followed by PCR amplification with *P. omnivora* specific primers, overcomes the limitations associated with isolation of *P. omnivora* from diseased samples. Isolating *P. omnivora* from symptomatic plants is problematic since infected plants often wilt and die very quickly and roots are usually severely rotted when hosts are symptomatic. Our studies were conducted on cotton, one of the easiest of hosts from which the fungus can be isolated. Isolation from most other hosts, including many woody ornamentals, wine grapes and fruit trees are particularly difficult. Growth and storage of the fungus also pose problems since *P. omnivora* produces no viable spores and growth from hyphal tipping is often unsuccessful. The culture independent method is not only useful in investigating overall genetic variability in the fungus but also in testing efficacy of new fungicides for growth inhibition of genetically distinct isolates. We concluded from results of this study that differences in efficacy of flutriafol in two different field trial locations probably were not due to genetic differences in *P. omnivora* populations but to other factors, such as fungicide application method, timing and placement that need further investigation.

Literature cited

Alderman, S. C. and Hine, R. B. 1982. Vertical distribution in soil and induction of disease by strands of *Phymatotrichum omnivorum*. Phytopathology 72:409–412.

Dana, B. F. 1929. Recent developments in root-rot investigations. Farm and Ranch 48:22-23.

Gaxiola, J. M. 2007. Research perspectives on *Phymatotrichopsis omnivora* and the disease it causes. Agric. Tec. Mex. 33:309-318.

Hastie, A. C.1973. Hybridization of *Verticillium albo-atrum* and *Verticillium dahliae*. Trans Br Mycol Soc 60: 511–523.

Hine, R. B., Whitson, R., Armstrong, A., Howell, D. and Cluff, R. 1985. Fungicide Evaluations for the Control of Phymatotrichum Root Rot. Cotton: A College of Agriculture Report, 370063, Series P-63, The University of Arizona, http://arizona.openrepository.com/arizona/bitstream/10150/204052/1/370063-143-148.pdf.

Hosford, R. M. and Gries, G. A. 1966. The nuclei and parasexuality in *Phymatotrichum omnivorum.* American Journal of Botany*,* 53: 570-79.

Inderbitzin, P., Davis, R. M., Bostock, R. M. and Subbarao, K. V. 2011. The Ascomycete *Verticillium longisporum* is a Hybrid and a Plant Pathogen with an Expanded Host Range. PLoS ONE 6(3): e18260. doi:10.1371/journal.pone.0018260.

Isakeit, T. 2006. Chemical Control of Cotton Root Rot (Phymatotrichum root rot) in drip irrigated cotton. Cotton Incorporated Project 04-467TX. Project summary: http://www.cottoninc.com/fiber/Agricultural-Research/Project-Summaries/detail.asp?SelectedYear=2005&projectID=2186

Isakeit, T., Minzenmayer, R. R., Drake, D. R., Morgan, G. D., Mott, D. A., Fromme, D. D., Multer, W. L., Jungman, M. and Abrameit, A. 2012. Fungicide management of cotton root rot (*Phymatotrichopsis omnivora*). Beltwide Cotton Prod. Res. Conf. 2012: 235-237.

Kohli, Y. and Kohn, L.M. 1998. Random association among alleles in clonal populations of *Sclerotinia sclerotiorum*. Fungal Genet Biol 23:139-149.

Macmil, S. 2009. Sequence and analysis of the *Phymatotrichopsis omnivora* genome and expressed sequences tags (doctoral dissertation). Norman-Oklahoma: Univ. Oklahoma. 218 p.

Maddison, W. P. and Maddison, W. R. 2009. Mesquite: A modular system for evolutionary analysis. Version 2.72 http://mesquiteproject.org.

Marek, S. M., Hansen, K., Romanish, M. and Thorn, R. G. 2009. Molecular systematic of the cotton root rot pathogen, *Phymatotrichopsis omnivora*. Personia 22:63-74.

Mulrean, E. N., Hine, R. B. and Mueller, J. P. 1984. Effect of Phymatotrichum Root Rot on Yield and Seed and Lint Quality in *Gossypium hirsutum* and *G. barbadense*. Plant Disease 68:381-383.

Neal, D. C. and McLean, L. G. 1931. Viability of Strand Hyphae of the Cotton Root-Rot Fungus. Journal of Agricultural Research 43:499-502.

Norton, R., Olsen, M. W. and Mostafa, A. 2014. Efficacy of Flutriafol Fungicide for Control of Cotton Root Rot in Arizona in 2012 and 2013 Field Trials. Proceedings of 2014 Beltwide Cotton Conf., New Orleans, LA Jan 7-9, 2014. http://ncc.confex.com/ncc/2014/webprogram/Paper15266.html

O'Donnell, K. 2000. Molecular phylogeny of the *Nectria haematococca-Fusarium solani* species complex. Mycologia 92:919-938.

O'Donnell, K., Sutton, D. A., Forthergill, A., McCarthy, D., Rinaldi, M. G., Brandt, M. E., Zhang, N. and Geiser, D. M. 2008. Molecular phylogenetic diversity, multilocus haplotype nomenclature, and in vitro antifungal resistance within the *Fusarium solani* species complex. J. Clin. Microbiol. 46:2477-2490.

Olsen, M. W., Norton, R. and Mostafa, A. 2014. Growth of *Phymatotrichopsis omnivora* in Flutriafol Treated Field Soils. Proceedings of 2014 Beltwide Cotton Conf., New Orleans, LA Jan 7-9, 2014. https://ncc.confex.com/ncc/2014/webprogram/Paper15084.html

Qin, Q. M., Vallad, G. E., Wu, B. M. and Subbarao, K. V. 2006. Phylogenetic analyses of phytopathogenic isolates of *Verticillium* spp. Phytopathology 96:582-592.

Rogers, C. H. 1942. Cotton root rot studies with special reference to sclerotia, cover crops, rotation, tillage, seedling rate, soil fungicides, and effects on seed quality. *Tex. Agric. Exp. Stn. Bull.* 614: 1–45.

Streets, R, B. and Bloss, H. E. 1973. Phymatotrichum root rot. Am. Phytopathol. Soc. Monogr. 8:1-38.

Thompson, J. D. Higgins, D. G. and Gibson, T. J. 1994. CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res*. 11:4673-4680.

Uppalapati, S. R, Young, C. A., Marek, S. M. and Mysore, K. S. 2010. Phymatotrichum (cotton) root rot caused by *Phymatotrichopsis omnivora*: retrospects and prospects. Molecular Plant Pathology 11:325-334.

Wheeler, J. E. and Hine, R. B. 1972. Influence of soil temperature and moisture on survival and growth of strands of *Phymatotrichum omnivorum*. Phytopathology 62:828-832.

Table 1. *P. omnivora* isolates collected from cotton in 2012 in Arizona.

Field	No. of samples collected[a]	Isolate code[b]	Location
A	20	A1 to A20	Marana, AZ
B	20	B1 to B20	Marana, AZ
C	20	C1 to C20	Safford, AZ
D	20	D1 to D20	Safford, AZ

[a] Twenty diseased root samples were collected from discrete infection foci in each field.
[b] About 20 pieces of mycelial strands were collected from each diseased root sample under the dissecting scope and used for DNA extraction.

Table 2. *P. omnivora* specific primers used in this study.

Target region[a]	Primer name	Sequence 5'→3'
LSU	Po28SF1	ACCGGGTGTCCGAGTTGTAAT
	Po28SR1	CGCTCCCTACGCTTCGTCACGG
EF1-α	PoEF2	AGTTCCGGTAAGACTCTT
	PoEFR2	AGACCAAATCTGAGACT
ITS	PoITSA	CCTGCGGAAGGATCATTAAA
	PoITSB	GGGGGTTTTCTTTGTTAGGG

[a]Primer sequences for ITS region were obtained from a previous study (Marek et al., 2009), and primer sequences for LSU and *EF1-α* were designed in this study.

Table 3. List of 28S large subunit (LSU) of ribosomal DNA and elongation factor 1-alpha (*EF1-α*) sequences retrieved from GenBank for designing *P. omnivora* specific primers.

	GenBank accession numbers	
Species	LSU	*EF1-α*[a]
Balsamia magnata	U42683	NA
Barssia oregonensis	U42684	NA
Helvella cf. compressa	AY544655	NA
Psilopezia deligata	EU722509	DQ471080
Psilopezia juruensis	DQ220390	NA
Psilopezia cf. *nummularialis*	AY945852	NA
Rhizina undulata	DQ220410	NA

[a] NA= Sequence is not available in GenBank.

Table 4. *P. omnivora* sequences retrieved from GenBank for the phylogenetic analysis.

Species	Isolate code	Host[a]	Location[a]	GenBank Accession numbers	
				ITS	LSU
P. omnivora	ATCC 32445	Cotton	Arizona	EF494043	EF494060
P. omnivora	TXCO3-9	NA	NA	AY549455	EF494054
P. omnivora	TAMD-C04	Cotton	Texas	EF494040	EF494058
P. omnivora	OKAIf8	NA	NA	AY549456	EF494053
P. omnivora	PC04	Cotton	Texas	EF494039	EF494057
P. omnivora	ATCC32448	Peach	Mexico	EF494038	EF494056

[a] NA= Information is not available.

Table 5. Tree statistics for the individual ITS and LSU and combined data sets.

Locus	Size (bp)	PI[a]	MPT[b] length	CI[c]	RI[d]	No. of MPT trees
LSU rRNA	1094	9	43	1.0	1.0	88
ITS rRNA	515	36	115	0.79	0.98	20
LSU + ITS rRNA	1609	43	168	066	0.95	14

[a] Parsimony informative characters, [b] most parsimony tree length, [c] consistency index, and [d] retention index.

Table 6. Efficacy of Topsin-M (thiophanate-methyl) and Topguard (flutriafol) fungicides on in-vitro mycelial growth of *P. omnivora* from four cotton fields in Arizona, Marana (A and B isolates) and Safford (C and D isolates).

	Colony diameter (cm)[xy]								
	Topsin-M (thiophanate-methyl) mg/L a.i.				Topguard (flutriafol) mg/L a.i.				
Isolate [w]	0	0.1	1.0	10	0	0.001	0.01	0.1	1.0
A10	8.0 $_{aA}$	8.0 $_{aA}$	0.0 $_{bD}$	0.0 $_{bA}$	8.0 $_{aA}$	7.9 $_{aA}$	7.8 $_{aA}$	3.9 $_{bA}$	0 $_{cA}$
B6	8.0 $_{aA}$	8.0 $_{aA}$	3.5 $_{bA}$	0.0 $_{cA}$	8.0 $_{aA}$	7.9 $_{aA}$	5.7 $_{bA}$	3.4 $_{cB}$	0 $_{dA}$
B12	8.0 $_{aA}$	8.0 $_{aA}$	4.2 $_{bA}$	0.0 $_{cA}$	8.0 $_{aA}$	8.0 $_{aA}$	7.0 $_{bA}$	2.6 $_{cC}$	0 $_{dA}$
C2	8.0 $_{aA}$	8.0 $_{aA}$	2.0 $_{bB}$	0.0 $_{cA}$	8.0 $_{aA}$	8.0 $_{aA}$	6.0 $_{bA}$	3.5 $_{cAB}$	0 $_{dA}$
D1	8.0 $_{aA}$	8.0 $_{aA}$	0.8 $_{bCD}$	0.0 $_{bA}$	8.0 $_{aA}$	8.0 $_{aA}$	7.5 $_{bA}$	2.8 $_{cC}$	0 $_{dA}$
D8	8.0 $_{aA}$	7.9 $_{aA}$	1.5 $_{bC}$	0.0 $_{cA}$	8.0 $_{aA}$	8.0 $_{aA}$	8.0 $_{aA}$	3.7 $_{bAB}$	0 $_{cA}$

[w]Hyphal tipped pure culture from diseased cotton root from four cotton fields in Arizona.
[x]Colony diameter was measured at two axes and averaged after 21 days incubation. Each value is the average of five replicate cultures.
[y]Small letters indicate the analysis of variance (ANOVA) between the active ingredients concentration and should be read left to right. Capital letters indicate the ANOVA between isolates and should be read top to bottom. Columns or rows with different letters are significantly different according to Hom-Sidak method ($P < 0.05$) based on F-test in ANOVA.

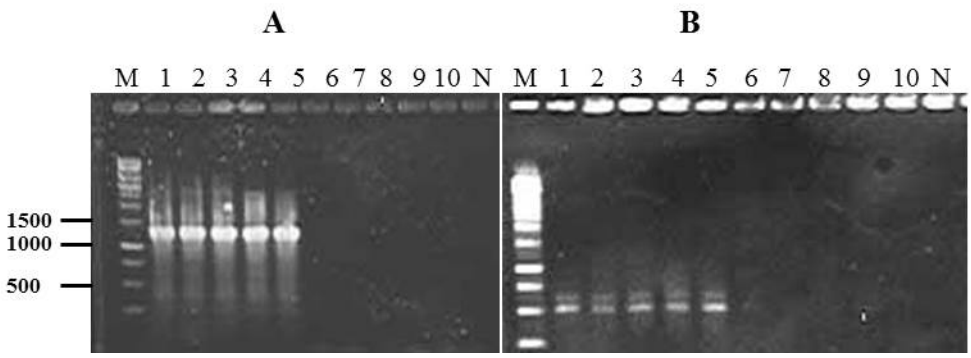

Figure 1. Validation of the specificity of the primer pair to *P. omnivora* in PCR. (i) PCR amplification for 28S ribosomal large subunit, (ii) PCR amplification for elongation factor 1- α. PCR was performed separately for each locus using *P. omnivora* specific primers. The PCR products were separated on 1% agarose gel and stained with ethidium bromide. Lanes 1 to 5: DNA from mycelial strands of *P. omnivora* collected from five different individual cotton root, lanes 6- 10: DNA of pure culture of non-*P. omnivora* fungi (6-*Verticillium* sp, 7- *Fusarium* sp, 8-*Alternaria* sp. 9- *Sclerotinia sclerotiorum*, 10- *Rhizoctonia* sp). N- Negative control and M- 1kb DNA Marker.

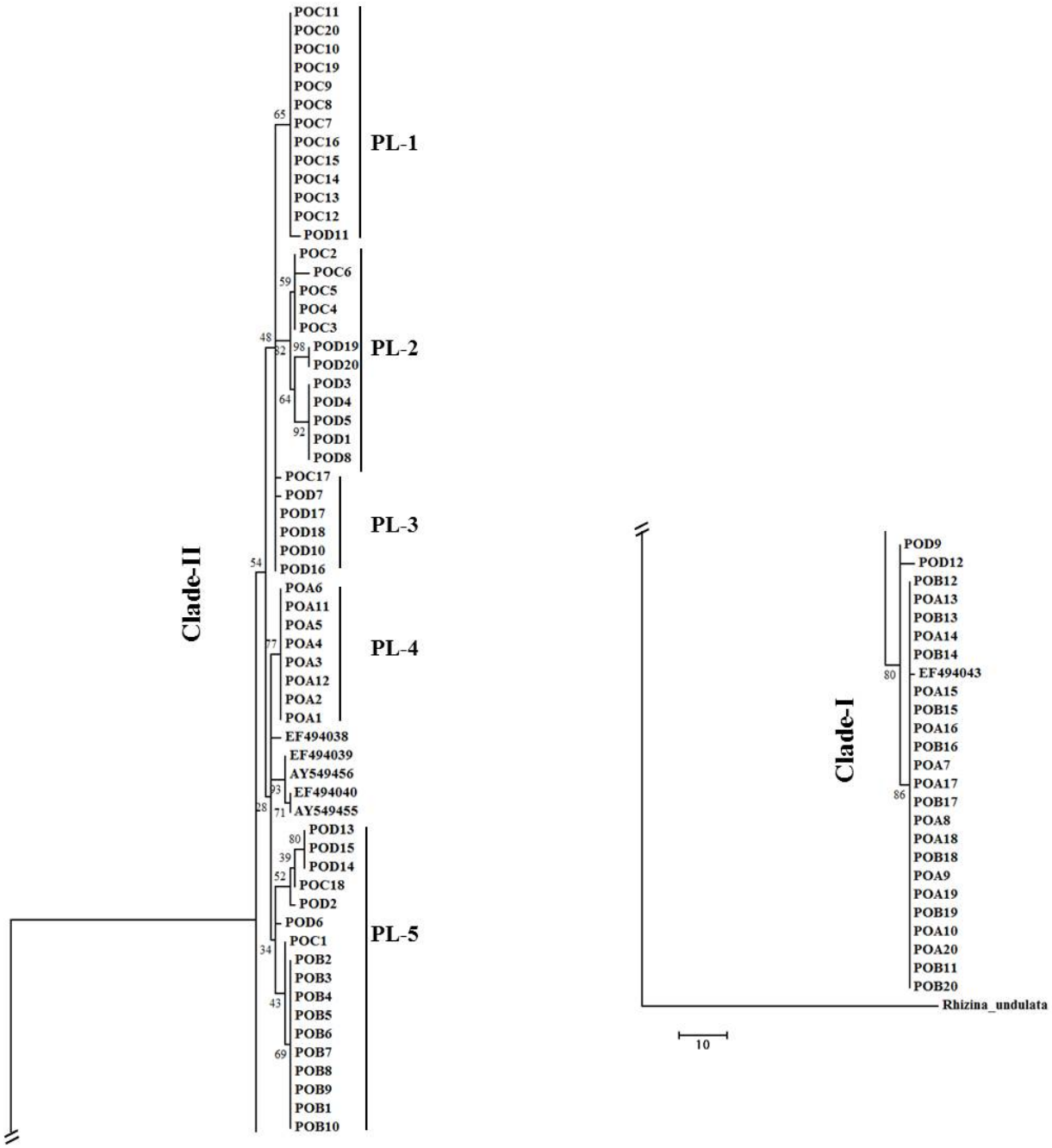

Figure 2. Maximum parsimony analysis of *P. omnivora* from cotton based on the combined data from ITS and LSU regions of rDNA. Bootstrap values are indicated at the node. Additional sequences were retrieved from Genbank, PL-phylogenetic lineage.