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RESEARCH PAPERS

Characterisation and mefenoxam sensitivity of *Phytophthora* spp. from ornamental plants in Italian nurseries

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Summary. Genetic variability and mefenoxam sensitivity are described for *Phytophthora* spp. isolates recovered from nine ornamental plant species at four locations in Sicily, Southern Italy. Fifty-four isolates of *Phytophthora* spp. were obtained from symptomatic plant tissues. Mating type analyses revealed that all isolates are heterothallic, consisting of mating type A1. Fungicide sensitivity assays determined that all isolates are sensitive to mefenoxam. Characterisation of 48 isolates was performed using molecular and phylogenetic analyses of the internal transcribed spacer of ribosomal DNA (rDNA-ITS), beta-tubulin (β-tub) and elongation factor 1 alpha (EF-1α) regions. The isolates belonged to *Phytophthora nicotianae* (34 isolates), *P. arecae* (syn. *palmivora*) (five) and *P. niederhauserii* (nine). The pathogenicity of three representative isolates was tested on seedlings or cuttings of each host, grown in a growth chamber. All isolates were pathogenic and reproduced symptoms identical to those observed in the nursery. This is the first report of disease caused by *P. nicotianae*, *P. arecae* or *P. niederhauserii* on several ornamental plants in Italy, and of the sensitivity of these *Phytophthora* species to mefenoxam.

Key words: pathogenicity, molecular analysis, root and foliar symptoms, mating type.

Introduction

During the last decade, Italy has significantly increased production of ornamental plants in nurseries, and millions of plants are grown in greenhouses and in open fields. In Sicily (Southern Italy), ornamental production is predominantly concentrated in the eastern area, where it took the place of lemon orchards and represents an important citrus alternative.

Growers of ornamental plants often face severe pressure from pathogens which, due to favourable climatic conditions, can represent threats to nursery production. Every year, many thousands of plants are traded through Sicily, which can lead to the spread of pathogens to new areas, and to the introduction of new pathogens. Oomycetes such as *Phytophthora* and *Pythium* spp. are frequently isolated from symptomatic ornamental crops in Italy (Cacciola *et al*., 2008; Aiello *et al*., 2011; Guarnaccia *et al*., 2015).

Phytophthora species cause root and foliar diseases on a wide range of ornamental plants (Erwin and Ribeiro, 1996; Ferguson and Jeffers, 1999; Werres *et al*., 2001; Schwingle *et al*., 2007; Prigigallo *et al*., 2015; Jung *et al*., 2016). The diseases are widespread in nursery environments during plant propagation stages, because warm temperatures, high humidity and densely grown plants provide favourable conditions for growth and sporulation of *Phytophthora* spp. (Donahoo and Lamour, 2008). *Phytophthora* species can also be dispersed through sporangia, zoospores and chlamydospores in free water, and irrigation sources can act as reservoirs for some species (Yamak *et al*., 2002; Hong and Moorman, 2005). Overhead sprinkler irri-

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gation and the use of recycled irrigation water should be avoided. The most common *Phytophthora* species reported to cause diseases on ornamental plants in European nurseries are *P. nicotianae* (Breda de Haan), *P. ramorum* (Werres, de Cock, Man int Veld), *P. niederhauserii* (Z.G. Abad & J.A. Abad), *P. arecae* (L.C. Coleman) Pethybr. (syn. *Phytophthora palmivora*), *P. syringae* (Kleb.), and *P. cambivora* (Petri) Buisman (Werres *et al*., 2001; Moralejo *et al*., 2009; Aiello *et al*., 2011; Abad *et al*., 2014; Schlenzig *et al*., 2014). In Italy, more than 20 *Phytophthora* species have been reported in nurseries of ornamental plants, and some of these pathogens were identified on new hosts for the first time (Cacciola *et al*., 2008).

Prevention is the first strategy to control *Phytophthora* diseases in nurseries, and particular control methods are the use of healthy plants, sanitation of irrigation water, and early removal of infected plants (Donahoo and Lamour, 2008). Chemical control is also widely used to manage *Phytophthora* diseases. However, the use of phenylamide fungicides such as metalaxyl and mefenoxam (the R-enantiomer of metalaxyl; metalaxyl-M, Ridomil Gold™, Syngenta) should be limited because these pesticides induce a selective pressure for resistant isolates (Parra and Ristaino, 2001; Dunn *et al*., 2010). Therefore, accurate diagnosis of the *Phytophthora* species present in particular areas or in ornamental plant nurseries, and determination of their sensitivity to mefenoxam, are important for development of effective disease management strategies.

Considering the importance of Phytophthora diseases and large economic losses caused by Oomycete pathogens, several surveys were conducted over a 3-year period, in commercial nurseries located in Catania province, eastern Sicily, Italy. Various plant host species showing *Phytophthora*-like symptoms were randomly collected for analysis.

The aims of this study were: to identify the species of *Phytophthora* recovered from ornamental plants, using morphological characteristics and molecular analysis; to evaluate the sensitivity of different *Phytophthora* isolates to mefenoxam; and to confirm the pathogenicity of representative *Phytophthora* isolates on the host species from which they were isolated.

Materials and methods

Field survey and fungal isolation

During 2011–2013, surveys were conducted in four ornamental nurseries located in Catania province (eastern Sicily). Approximately 20 plants per species showing root rot, foliar blight or leaf lesions were randomly collected for analysis. Small sections from the edges of symptomatic tissues were surface disinfected with 1.2% (v/v) sodium hypochlorite for 1 min, rinsed in sterile distilled water (SDW), dried on sterile absorbent paper. The tissue sections were then

Table 1. Ornamental host species, disease symptoms and geographical location of *Phytophthora* spp. isolates collected from ornamental nurseries.

Isolate Di3A[a] number	Host	Symptoms	Geographical location
Di3A-AA1-6	*Citrus aurantium*	Crown and root rot	Carruba, Sicily, nursery 1
Di3A-CC1-5	*Cistus creticus*	Crown and root rot	Grotte, Sicily, nursery 2
Di3A-CS1-5	*Cistus salvifolius*	Crown and root rot	Grotte, Sicily, nursery 2
Di3A-DV1-6	*Dodonaea viscosa*	Foliar blight	Praiola, Sicily, nursery 3
Di3A-PMH1-7	*Polygala myrtifolia* × *oppositifolia* 'Bibi Pink'®	Foliar blight	Grotte, Sicily, nursery 2
Di3A-CVE1-5	*Grevillea juniperina* 'CVE'	Crown and root rot	Mascali, Sicily, nursery 4
Di3A-WIN1-4	*Grevillea thelemanniana* × *oppositifolia* 'Wimpara Gem'	Crown and root rot	Mascali, Sicily, nursery 4
Di3A-GRR1-7	*Grevillea gracilis* 'Rosea'	Crown and root rot	Mascali, Sicily, nursery 4
Di3A-BIGN1-9	*Bignonia venusta*	Leaf lesion	Carruba, Sicily, nursery 1

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plated on corn meal agar amended with pimaricin, ampicillin, rifamycin, PCNB and hymexazol. Plates were incubated at 25 ± 1°C in darkness. Pure cultures were obtained by transferring mycelial tips from colony margins onto fresh V8 juice agar (V8A) (Erwin and Ribeiro, 1996). Single spores were selected and transferred into V8A plates to establish monospore isolates. For long-term storage, isolates were placed on V8A slants, allowed to grow for 3 to 4 d, covered with sterile mineral oil, and then stored at room temperature. A total of 54 isolates were obtained (Table 1), and these were used for morphological characterisation and mefenoxam sensitivity testing. Among these, 48 isolates were characterized by molecular and phylogenetic analyses (Table 2).

Morphological characteristics and mating type

To characterize colonies and sporangia, the isolates were observed on corn meal agar after incubating for 10 d at 25°C under continuous fluorescent light. If sporangia had not been produced after 10 d, cultures were flooded with clarified V8 broth (50 mL of clarified V8 juice and 950 mL of SDW (Ferguson and Jeffers, 1995), and then incubated under continuous fluorescent light for a further 7 d. Clarified V8 juice was prepared by adding 2 g of calcium carbonate to 100 mL of V8 juice, centrifuging at 7,000 rpm for 10 min and collecting the supernatant (Ferguson and Jeffers, 1995). To determine the mating type of each isolate, a 6 mm diam. plug, taken from a 10-d-old culture of each isolate, was placed on unclarified V8A (UCV8), approx. 2 cm away from one plug of known A1 or A2 mating types of *P. nicotianae* (CBS 410.87 and CBS 411.87) or *P. arecae* (CBS 179.26 and CBS 236.30) isolates. Plates were incubated in the dark for at least 1 week before being inspected under a light microscope for the formation of sexual structures (oogonia, antheridia and oospores). Isolates which produced oospores with the A1 mating type standard, but not with the A2 mating type standard, were determined to be mating type A2, and *vice versa* (Dunn *et al*., 2010). Plates containing plugs from both the A1 and A2 standard strains of both *P. nicotianae* and *P. arecae* were included as positive controls.

Mefenoxam sensitivity test

Fifty-four isolates of *Phytophthora* spp. were assayed for sensitivity to mefenoxam. According to the protocol reported by Dunn *et al*. (2010), agar disks (6 mm diam.) were removed from the edges of actively growing cultures using a sterile cork borer. Plugs were transferred to the center of clarified unamended V8A (control), or with 5 or 100 μg mL^{-1} mefenoxam as Ridomil Gold™ EC (45.3% mefenoxam; Syngenta Crop Protection). Three replicate plates per treatment were inoculated for each isolate. Plates were incubated in the dark at 25°C for 7 d under constant light. Radial colony growth of resulting colonies was measured twice at perpendicular angles for each plate, averaged, and compared to colony diameters from nonamended (control) medium.

Isolates were considered sensitive to mefenoxam if colony growth at 5 μg mL^{-1} was less than 40% of the growth on nonamended medium. Isolates were considered intermediately sensitive if growth on medium amended with 5 μg mL^{-1} was greater than 40% that of the growth on nonamended medium, but growth on medium amended with 100 μg mL^{-1} was less than 40% that on nonamended medium. Isolates were considered resistant to the pesticide if growth on medium amended with 100 μg mL^{-1} was greater than 40% that of the growth on nonamended medium.

Molecular characterisation and phylogenetic analysis

The 48 *Phytophthora* spp. isolates used in the phylogenetic analysis are listed in Table 2. Mycelium samples were lyophilized, and DNA was extracted from lyophilized mycelia using the DNeasy Plant Mini Kit (Qiagen) according to the manufacturer's instructions. Primers ITS4 and ITS5 (White *et al*., 1990)were used to amplify the internal transcribed spacer (ITS) region of ribosomal DNA for each isolate. A subset of 27 isolates also had the translation elongation factor 1-alpha gene (EF-1α) amplified using primers ELONGF1 and ELONGR1, and the β-tubulin gene amplified using primers TUBUF2 and TUBUR1 (Kroon *et al*., 2004). Polymerase chain reaction (PCR) conditions were as follows: initial denaturation at 95°C for 5 min; 35 cycles of 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min; and final extension at 72°C for 10 min. PCR products were checked visually by gel electrophoresis to confirm single products in the appropriate size range of 800–1000 bp. PCR products were cleaned using the DNA Clean and Concentrator -25 kit (Zymo Research) according to the manufacturer's instructions, and products were quantified using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific Inc.).

Table 2. Identity and Genbank accession numbers of *Phytophthora* spp. isolates collected from ornamental plants in Italy.

Isolate code[a]	Host	Species identification	GenBank Accession		
			ITS	EF1α	β-tubulin
Di3A-CC1	*Cistus creticus*	*Phytophthora arecae* (syn. *palmivora)*	MH062246	MH101827	MH101854
Di3A-CS2	*Cistus salvifolius*	*Phytophthora arecae*	MH062247	MH101828	MH101855
Di3A-CS4	*Cistus salvifolius*	*Phytophthora arecae*	MH062248	MH101829	MH101856
Di3A-CS5	*Cistus salvifolius*	*Phytophthora arecae*	MH062249	-	-
Di3A-DV2	*Dodonaea viscosa*	*Phytophthora arecae*	MH062250	MH101830	MH101857
Di3A-AA1	*Citrus aurantium*	*Phytophthora nicotianae*	MH062212	MH101831	MH101858
Di3A-AA2	*Citrus aurantium*	*Phytophthora nicotianae*	MH062213	MH101832	MH101859
Di3A-AA3	*Citrus aurantium*	*Phytophthora nicotianae*	MH062214	MH101833	MH101860
Di3A-AA4	*Citrus aurantium*	*Phytophthora nicotianae*	MH062215	MH101834	MH101861
Di3A-AA5	*Citrus aurantium*	*Phytophthora nicotianae*	MH062216	-	-
Di3A-AA6	*Citrus aurantium*	*Phytophthora nicotianae*	MH062217	-	-
Di3A-BIGN1	*Bignonia venusta*	*Phytophthora nicotianae*	MH062238	MH101847	MH101874
Di3A-BIGN3	*Bignonia venusta*	*Phytophthora nicotianae*	MH062239	MH101848	MH101875
Di3A-BIGN4	*Bignonia venusta*	*Phytophthora nicotianae*	MH062240	-	-
Di3A-BIGN5	*Bignonia venusta*	*Phytophthora nicotianae*	MH062241	-	-
Di3A-BIGN6	*Bignonia venusta*	*Phytophthora nicotianae*	MH062242	-	-
Di3A-BIGN7	*Bignonia venusta*	*Phytophthora nicotianae*	MH062243	-	-
Di3A-BIGN8	*Bignonia venusta*	*Phytophthora nicotianae*	MH062244	-	-
Di3A-BIGN9	*Bignonia venusta*	*Phytophthora nicotianae*	MH062245	-	-
Di3A-CC2	*Cistus creticus*	*Phytophthora nicotianae*	MH062218	MH101835	MH101862
Di3A-CC3	*Cistus creticus*	*Phytophthora nicotianae*	MH062219	MH101836	MH101863
Di3A-CC4	*Cistus creticus*	*Phytophthora nicotianae*	MH062220	-	-
Di3A-CC5	*Cistus creticus*	*Phytophthora nicotianae*	MH062221	-	-
Di3A-CS1	*Cistus salvifolius*	*Phytophthora nicotianae*	MH062222	MH101837	MH101864
Di3A-CS3	*Cistus salvifolius*	*Phytophthora nicotianae*	MH062223	MH101838	MH101865
Di3A-CVE1	*Grevillea juniperina*	*Phytophthora nicotianae*	MH062224	MH101842	MH101869
Di3A-CVE2	*Grevillea juniperina*	*Phytophthora nicotianae*	MH062225	MH101843	MH101870
Di3A-CVE3	*Grevillea juniperina*	*Phytophthora nicotianae*	MH062226	MH101844	MH101871
Di3A-CVE4	*Grevillea juniperina*	*Phytophthora nicotianae*	MH062227	-	-
Di3A-CVE5	*Grevillea juniperina*	*Phytophthora nicotianae*	MH062228	-	-
Di3A-GRR1	*Grevillea gracilis*	*Phytophthora nicotianae*	MH062229	MH101845	MH101872
Di3A-GRR5	*Grevillea gracilis*	*Phytophthora nicotianae*	MH062230	MH101846	MH101873
Di3A-PMH1	*Polygala myrtifolia* × *oppositifolia*	*Phytophthora nicotianae*	MH062231	MH101839	MH101866

(Continued)

Table 2. (Continued).

Isolate code[a]	Host	Species identification	GenBank Accession		
			ITS	EF1α	β-tubulin
Di3A-PMH2	*Polygala myrtifolia* × *oppositifolia*	*Phytophthora nicotianae*	MH062232	MH101840	MH101867
Di3A-PMH3	*Polygala myrtifolia* × *oppositifolia*	*Phytophthora nicotianae*	MH062233	MH101841	MH101868
Di3A-PMH4	*Polygala myrtifolia* × *oppositifolia*	*Phytophthora nicotianae*	MH062234	-	-
Di3A-PMH5	*Polygala myrtifolia* × *oppositifolia*	*Phytophthora nicotianae*	MH062235	-	-
Di3A-PMH6	*Polygala myrtifolia* × *oppositifolia*	*Phytophthora nicotianae*	MH062236	-	-
Di3A-PMH7	*Polygala myrtifolia* × *oppositifolia*	*Phytophthora nicotianae*	MH062237	-	-
Di3A-GRR2	*Grevillea gracilis*	*Phytophthora niederhauserii*	MH062207	MH101852	MH101879
Di3A-GRR3	*Grevillea gracilis*	*Phytophthora niederhauserii*	MH062208	MH101853	MH101880
Di3A-GRR4	*Grevillea gracilis*	*Phytophthora niederhauserii*	MH062209	-	-
Di3A-GRR6	*Grevillea gracilis*	*Phytophthora niederhauserii*	MH062210	-	-
Di3A-GRR7	*Grevillea gracilis*	*Phytophthora niederhauserii*	MH062211	-	-
Di3A-WIN1	*Grevillea thelemanniana* × *oppositifolia*	*Phytophthora niederhauserii*	MH062203	MH101849	MH101876
Di3A-WIN2	*Grevillea thelemanniana* × *oppositifolia*	*Phytophthora niederhauserii*	MH062204	MH101850	MH101877
Di3A-WIN3	*Grevillea thelemanniana* × *oppositifolia*	*Phytophthora niederhauserii*	MH062205	MH101851	MH101878
Di3A-WIN4	*Grevillea thelemanniana* × *oppositifolia*	*Phytophthora niederhauserii*	MH062206	-	-

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PCR products were sequenced bi-directionally, using the above primers, at the Cornell University Biotechnology Resource Center (Ithaca, NY, USA).

Raw sequence data were checked, and missed calls were edited manually using MEGA V.7 (Kumar *et al*., 2016). Sequences were then identified using the National Center for Biological Information (NCBI) Basic Local Alignment Search Tool (BLAST). Following species identification, all 48 sample ITS sequences were aligned, along with reference strain sequences, using ClustalW in MEGA V.7 (Kumar *et al*., 2016). Reference strains used in the phylogenetic analysis are listed in Table 2, and included three isolates per species and two *Pythium* spp. isolates as outgroups. The resulting consensus sequences were used to generate a neighbour-joining tree in MEGA. The Tamura-Nei model was used with 1,000 bootstrapping repetitions, and pairwise deletion was used to handle alignment gaps.

A second neighbour-joining tree was constructed using elongation factor 1 alpha (EF-1α) and β-tubulin sequence data. Sequences were checked and aligned as described for the ITS data and concatenated. Reference strains, listed in Table 3, included three isolates from each of the three species identified here, along with one isolate belonging to each *Phytophthora* clade as defined by Blair *et al*. (2008). The resulting concatenated sequences were used to generate a neighbour-joining tree using the same parameters as described for the ITS data. Sequences derived in this study were deposited in GenBank.

Pathogenicity

Pathogenicity tests were performed with one representative isolate of each species (*P. nicotianae*, *P. arecae* and *P. niederhauserii*) on potted, healthy seed-

Table 3. Reference isolates used to construct phylogenetic trees.

Species	Isolate	Reference sequence	GenBank Accession numbers	Reference
Phytophthora arecae (syn. *palmivora*)	CBS 305.62	ITS	HQ643146	Robideau *et al.*, 2011
Phytophthora arecae	CBS 179.26	ITS	HQ643305	Robideau *et al.*, 2011
Phytophthora arecae	UQ1249	ITS	AF266780	Cooke *et al.*, 2000
Phytophthora arecae	P10213	EF1α and β-tubulin	EU080811, EU080810	Blair *et al.*, 2008
Phytophthora arecae	P11011	EF1α and β-tubulin	EU079726, EU079725	Blair *et al.*, 2008
Phytophthora arecae	P11010	EF1α and β-tubulin	EU079720, EU079719	Blair *et al.*, 2008
Phytophthora cactorum	P0714	EF1α and β-tubulin	EU080279, EU080278	Blair *et al.*, 2008
Phytophthora capsici	P10386	EF1α and β-tubulin	EU079545, EU079544	Blair *et al.*, 2008
Phytophthora heveae	P1102	EF1α and β-tubulin	EU079732, EU079731	Blair *et al.*, 2008
Phytophthora infestans	P10646	EF1α and β-tubulin	EU079614, EU079613	Blair *et al.*, 2008
Phytophthora mirabilis	P3005	EF1α and β-tubulin	EU079777, EU079776	Blair *et al.*, 2008
Phytophthora megakarya	P8516	EF1α and β-tubulin	EU079971, EU079970	Blair *et al.*, 2008
Phytophthora megasperma	P3136	EF1α and β-tubulin	EU080060, EU080059	Blair *et al.*, 2008
Phytophthora nicotianae	CBS 101655	ITS	HQ643303	Robideau *et al.*, 2011
Phytophthora nicotianae	CBS 303.39	ITS	HQ643301	Robideau *et al.*, 2011
Phytophthora nicotianae	UQ848	ITS	AF266776	Cooke *et al.*, 2000
Phytophthora nicotianae	P1452	EF1α and β-tubulin	EU080505, EU080504	Blair *et al.*, 2008
Phytophthora nicotianae	P10116	EF1α and β-tubulin	EU079964, EU079963	Blair *et al.*, 2008
Phytophthora nicotianae	P10718	EF1α and β-tubulin	EU079654, EU079653	Blair *et al.*, 2008
Phytophthora niederhauserii	P10617	ITS	HQ261701	Robideau *et al.*, 2011
Phytophthora niederhauserii	P10616	ITS	HQ261702	Robideau *et al.*, 2011
Phytophthora niederhauserii	P16237	ITS	HQ261699	Robideau *et al.*, 2011
Phytophthora pseudosyringae	P10437	EF1α and β-tubulin	EU079564, EU079563	Blair *et al.*, 2008
Phytophthora quercetorum	PD_01105	EF1α and β-tubulin	EU080902, EU080901	Blair *et al.*, 2008
Pythium undulatum	IMI337230	ITS	AF271230	Cooke *et al.*, 2000
Pythium undulatum	P10342	EF1α and β-tubulin	EU080442, EU080440	Blair *et al.*, 2008
Pythium vexans	UQ2074	ITS	AF271224	Cooke *et al.*, 2000
Pythium vexans	P3980	EF1α and β-tubulin	EU080485, EU080484	Blair *et al.*, 2008

lings or cuttings of host species from which they were isolated. Thirty plants of each host species were inoculated with a zoospore suspension (10^6 cfu mL^{-1}) obtained from 10-d-old cultures. *Polygala myrtifolia* L. × *Polygala oppositifolia* 'Bibi Pink'® and *Bignonia venusta* Ker Gawl., (syn. *Pyrostegia venusta*) plants were inoculated by spraying the zoospore suspension on the canopy. *Cistus salvifolius* L., *C. creticus* L., *Grevillea juniperina* R. Br. 'CVE' and *G. gracilis* 'Rosea' were inoculated at the stem bases with a soil drench. Un-inoculated plants served as a controls, for all hosts. After inoculation, the plants were covered with plastic bags and incubated in a growth chamber at 25 ± 1°C and 90–95% relative humidity for 2–4 months. Each

experiment was conducted twice, and similar results were obtained in both tests.

Results

Field survey and pathogen isolations

Symptoms referable to *Phytophthora* spp. were found over a 3 year period in four nurseries and on 11 different ornamental species (Figure 3, Table 1). The diseases were observed during the propagation stages on seedlings and cuttings (1–3 months old) in greenhouses and on established plants (1–2 years old) in open fields. The symptoms observed consisted of crown and root rot, foliar blight and leaf lesions (Table 1). Crown rot was characterized by brown to dark-brown lesions at the soil line or above, which expanded to girdle the plant stems, and internal dark-brown discoloration of cortical tissues. Root rot was observed in association with these symptoms. The affected roots were dark-brown or black and were partially or completely destroyed. As a consequence of crown and root rot, affected plants wilted and died. Symptoms of leaf spot included interveinal and marginal irregular necrotic lesions that progressed to partial or total leaf blight. Foliar blight consisted of light brown to almost black spots that typically expanded and affected the entire leaves, petioles, and stems of the young apical and lateral shoots. Occasionally, stem lesions were detected at the crown level. In advanced stages of the disease, the canopies of plants were partially or completely killed. The roots of these plant did not show any symptoms.

Morphological characteristics, mating type and mefenoxam sensitivity test

All isolates showed typical features of *Phytophthora* spp. (colony, sporangial morphology). All isolates of *Phytophthora* spp. produced oogonia and oospores with amphyginous antheridia, and were shown to be of mating type A1.

All the *Phytophthora* spp. isolates assayed were sensitive to mefenoxam, and did not grow on media amended with 5 or 100 μg mL^{-1} of mefenoxam.

Molecular characterisation and phylogenetic analysis

All 48 of *Phytophthora* isolates included in this study shared between 96 and 99% similarity with ITS sequences of reference isolates, based on BLAST search results. Thirty-four of the isolates were identified as *Phytophthora nicotianae*, nine as *Phytophthora niederhauserii*, and five as *Phytophthora arecae* (Table 2).

The neighbour-joining trees based on ITS data (Figure 1) and EF1α and β-tubulin data (Figure 2) showed that isolates within each species grouped together, with 100% bootstrap support.

All three species identified in this study belong to separate clades within the *Phytophthora* genus (Blair *et al*., 2008). Both phylogenies, based on ITS and the EF-1α/β-tubulin combination, are in agreement with previous phylogenetic analyses of the genus *Phytophthora*, and place *P. niederhauserii* (clade 7) basal to *P. arecae* (clade 4) and *P. nicotianae* (clade 1) (Cooke *et al*., 2000; Kroon *et al*., 2004; Blair *et al*., 2008; Yang *et al*., 2017).

Pathogenicity

The three isolates used in the pathogenicity tests were pathogenic to the different hosts inoculated, and produced symptoms identical to those observed on diseased plants in the nurseries (Figure 3).

The tested *P. nicotianae* isolate infected *P. myrtifolia* 'Bibi pink'® cuttings, and symptoms were observed 4 d after inoculation (Figure 3). All inoculated plants were killed within 10 d. This species was also pathogenic on leaves of *B. venusta* and caused necrotic lesions after 4–5 d. Infected leaves were totally blighted within 7 d. *Phytophthora nicotianae* caused symptoms on all *Cistus* spp. plants when inoculated on the bases of stems, and the inoculated plants died within 1 month (Figure 3).

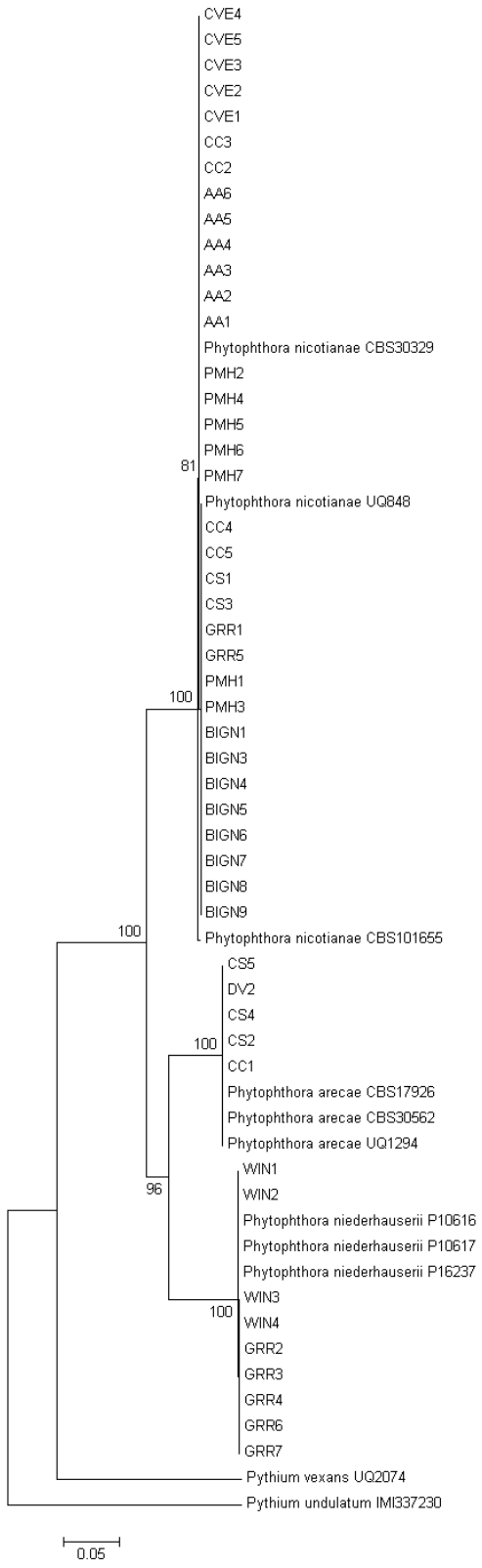
Phytophthora arecae caused high disease incidence (DI) (from 80 to 100%) on both *Cistus* species inoculated, and the first symptoms were observed after 15–20 d. All inoculated plants died within 1 month.

Phytophthora niederhauserii was pathogenic on *Grevillea* spp., causing high DI (from 80 to 100%) after 20 d.

Under growth chamber conditions (25°C and 90–95% RH), white mycelia covered portions of the infected plants (Figure 3). The pathogens were re-isolated from the artificially inoculated plants and identified as previously described. No symptoms were observed on control plants.

Discussion

In this study, 54 *Phytophthora* isolates were recovered from nine ornamental plant species in nurseries



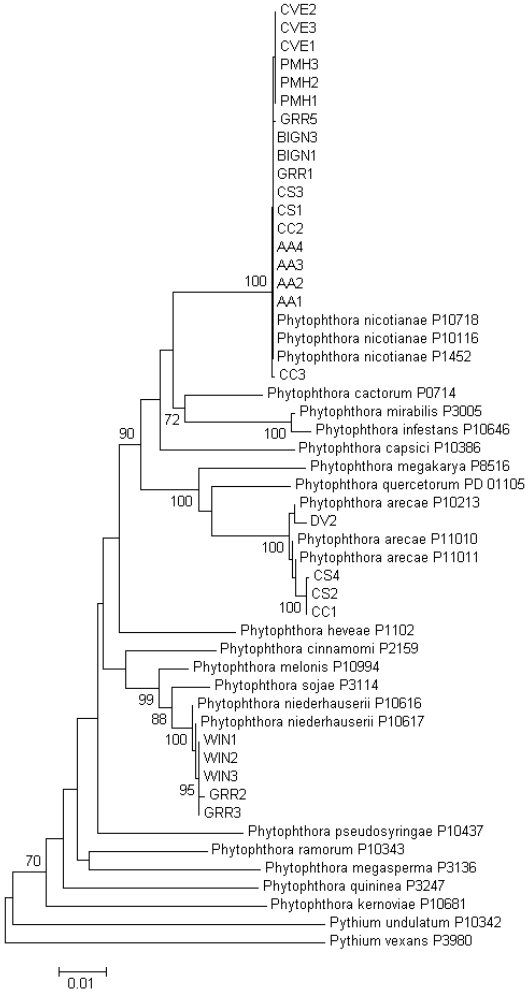


Figure 2. Molecular phylogenetic neighbour-joining tree based on elongation factor 1 alpha and β-tubulin DNA sequence data from 27 *Phytophthora* isolates collected from ornamental host species in Italy. Three reference strains for each species were also included, along with one reference strain from each *Phytophthora* clade (Blair et al. 2008) and two *Pythium* species as outgroups. Bootstrap values are shown at branch nodes, and branch lengths represent the number of substitutions per site.

Figure 1. Molecular phylogenetic neighbour-joining tree based on internal transcribed spacer ribosomal DNA sequence data from 48 *Phytophthora* isolates collected from ornamental host species in Italy. Three reference strains for each species were also included, along with two *Pythium* species as outgroups. Bootstrap values are shown at branch nodes, and branch lengths represent the number of substitutions per site.

Figure 3. a-e. Natural symptoms caused by *Phytophthora* spp. a-b, Crown and root rot leading to death of cuttings of *Cistus salvifolius*. c-d, Leaf lesion symptoms on *Bignonia venusta*. e, Crown and root rot on *Grevillea juniperina* 'CVE'. f-j, Symptoms caused by *Phytophthora* spp. after artificial inoculation. f, Inoculated *C. creticus* plants compared with healthy plants. g-h, Foliar blight of *Polygala myrtifolia* × *oppositifolia*. i-j, Un-inoculated compared with symptomatic cuttings of *Grevillea gracilis* (i) and *G. juniperina* (j).

of eastern Sicily (southern Italy) over a 3-year period. Their mating type and mefenoxam sensitivity were also determined. Among these, 48 isolates were characterized by molecular and phylogenetic analysis, and were identified as *Phytophthora nicotianae* (34 isolates), *P. arecae* (five isolates) or *P. niederhauserii* (nine isolates).

Phytophthora disease management is based on integrated approaches, including the use of healthy plants, sanitation of irrigation water, early removal of infected plants, and the application of fungicides. Mefenoxam is one of the most commonly used fungicides on ornamentals (Erwin and Ribeiro, 1996), although due to its site-specificity of activity, this has a high intrinsic risk of resistance development in target pathogens. The occurrence of mefenoxam resistance in *Phytophthora* species has been well documented (Timmer *et al*., 1998; Hwang and Benson, 2005; Hu *et al*., 2005, 2008), and in several cases this has been associated with repeated use of this pesticide. Based on our study, all *Phytophthora* isolates tested were sensitive to mefenoxam, even though widespread use of fungicides in Sicilian nurseries has already led to a reduction of pathogen sensitivity to other chemical compounds (Guarnaccia *et al*., 2014). A possible explanation could be that growers practice fungicide rotation or use mixtures of pesticides with different modes of action and with efficacy for *Phytophthora* management. In this study, we tested 54 isolates collected in a short period (2011–2013), and more extensive sampling might be needed to gain a true assessment of the prevalence of mefenoxam-resistant isolates in Sicily. Due to the selective pressure of the fungicide imposed on *Phytophthora* populations, frequency of resistant isolates could increase with time, especially if this fungicide is used intensely and exclusively.

Identification of *Phytophthora* species was traditionally based on morphological characters. However, several species were not morphologically distinguishable from one another, and the production of several morphological structures and physiological features require specific environmental conditions. DNA sequence data provide accurate species-level identification. In the present study, ITS and EF-1α and β-tubulin phylogenies were in agreement with previous phylogenetic analyses of the genus *Phytophthora*, and placed *P. niederhauserii* (clade 7) basal to *P. arecae* (clade 4) and *P. nicotianae* (clade 1) (Cooke *et al*., 2000; Kroon *et al*., 2004; Blair *et al*., 2008; Yang *et al*., 2017).

Phytophthora arecae is a polyphagous pathogen that has been reported worldwide on different hosts, mainly causing bud, root and crown rot diseases (Uchida, 1994; Orlikowski and Szkuta, 2006; Torres *et al*., 2016). Recently, this pathogen has become widespread in Italy, where it has been reported on different ornamentals in nurseries (Cacciola *et al*., 2003; Aiello *et al*., 2011). However, no reports of disease caused by *P. arecae* on *C. creticus* and *C. salvifolius* have been published. Previous studies reported *P. nicotianae* on *C. canariensis* (Moralejo *et al*., 2009) and on *Cistus* sp. (Jung *et al*., 2016), and *P. niederhauseri* on several *Cistus* spp. (Cacciola *et al*., 2009; Moralejo *et al*., 2009; Jung *et al*., 2016). Isolates of *P. arecae* from *Dodonaea viscosa* Jacq. found in the present study were mating type A1, while Aiello *et al*., (2011) reported the isolates recovered from the same host species as mating type A2. The importance of *P. arecae* has increased due to the ability of this species to form caducous sporangia and abundant zoospores on diseased tissues, and zoospores are readily disseminated by rain splash and irrigation water. In nurseries, the disease may have been favoured by overhead sprinkler irrigation.

Phytophthora nicotianae, the species most frequently found in the present study, infects many herbaceous and woody plants, causing destructive diseases (Erwin and Ribeiro, 1996). However, no reports of disease caused by *P. nicotianae* on *C. creticus* or *C. salvifolius*, or on *G. juniperina* 'CVE', have been published. A previous study reported *P. arecae* on *C. salvifolius* in Italy (Cacciola *et al*., 2003). The survey reported here is also the first to identify *P. nicotianae* on the *Polygala myrtifolia* × *oppositifolia* "Bibi Pink"® hybrid, and the first report of disease caused by *Phytophthora* species on *Bignonia venusta*. Several *Phytophthora* spp., including *P. cryptogea* Pethybr. & Laff., *P. drechsleri* Tucker, *P. multivora* P.M. Scott & T. Jung and *P. nicotianae*, have been reported on *Polygala myrtifolia* in Australia, Italy and Spain (Cook and Dubé, 1989; Moralejo *et al*., 2009; Jung *et al*., 2016). Previous research reported *P. nicotianae* in Australia on *Grevillea thelemanniana* Hügel **ex** Endl. (Hardy and Sivasithamparam, 1988; Erwin and Ribeiro, 1996) and *P. niederhauserii* on *G. olivacea* A.S. George in the United Kingdom (Abad *et al*., 2014), while *P. niederhauserii* was not reported on *G. thelemanniana* × *olivacea* 'Wimpara Gem'.

Some ornamental hosts recovered in our survey (*C. salvifolius*, *D. viscosa* and *Bignonia* spp.) are also susceptible to other soil-borne pathogens such as binucleate *Rhizoctonia* sp. and *R. solani* Kühn (Aiello *et*

al., 2017). Pathogenic *Phytophthora* spp. could represent serious threats to nursery production. The environmental conditions during cultivation in greenhouses could provide suitable conditions for the development of diseases caused by soil-borne pathogens. Furthermore, the use of non-disinfected soil or plant growth substrate, or the re-use of these, could be possible sources of inoculum and increase the infection risks. Thus, prevention is the first strategy to control these diseases, together with the identification of pathogens present in a particular area or nursery.

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Literature cited

Abad Z.G., J.A. Abad, S.O. Cacciola, A. Pane, R. Faedda, E. Moralejo, A. Pérez-Sierra, P. Abad-Campos, *et al*., 2014. *Phytophthora niederhauserii* sp. nov., a polyphagous species associated with ornamentals, fruit trees and native plants in 13 countries. *Mycologia* 106, 431–447.

Aiello D., R. Faedda, A. Vitale, A. Pane and G. Polizzi, 2011. First report of Phytophthora foliar blight on Florida hopbush (*Dodonaea viscosa*) in Italy. *Journal of Phytopathology* 159, 697–699.

Aiello D., V. Guarnaccia, P.T. Formica, M. Hyachumachi and G. Polizzi, 2017. Occurrence and characterisation of *Rhizoctonia* species causing diseases of ornamental plants in Italy. *European Journal of Plant Pathology* 148, 967–982.

Blair J.E., M.D. Coffey, S.Y. Park, D.M. Geiser and S. Kang, 2008. A multi-locus phylogeny for *Phytophthora* utilizing markers derived from complete genome sequences. *Fungal Genetics and Biology* 45, 266–277.

Cacciola S.O., A.M. Pennisi, G.E. Agosteo and G. Magnano di San Lio, 2003. First report of *Phytophthora palmivora* on *Grevillea* spp. in Italy. *Plant Disease* 87, 1006.

Cacciola S.O., A. Pane, P. Martini, G.E. Agosteo, F. Raudino and G. Magnano di San Lio, 2008. Recovery of *Phythophthora* species from potted ornamentals in commercial nurseries in Italy. *Journal of Plant Pathology* 90(S2), 185.

Cacciola S.O., S. Scibetta, A. Pane, R. Faedda and C. Rizza, 2009. *Callistemon citrinus* and *Cistus salvifolius*, two new hosts of *Phytophthora* taxon *niederhauserii* in Italy. *Plant Disease* 93, 1075.

Cook R.P. and A.J. Dubé, 1989. Host-pathogen index of plant diseases in South Australia. South Australian Department of Agriculture, 1–142.

Cooke D.E.L., A. Drenth, J.M. Duncan, G. Wagels and C.M. Brasier, 2000. A molecular phylogeny of *Phytophthora* and related oomycetes. *Fungal Genetics and Biology* 30, 17–32.

Donahoo R.S. and K.H. Lamour, 2008. Characterization of *Phytophthora* species from leaves of nursery woody ornamentals in Tennessee. *Hortscience* 43(6),1833–1837.

Dunn A.R., M.G. Milgroom, J.C. Meitz, A. McLeod, W.E. Fry, M.T. McGrath, H.R. Dillard and C.D. Smart, 2010. Population structure and resistance to mefenoxam of *Phytophthora capsici* in New York State. *Plant Disease* 94, 1461–1468.

Erwin D.C. and O.K. Ribeiro, 1996. Phytophthora Diseases Worldwide. American Phytopathological Society, St. Paul, MN, USA.

Ferguson A.J. and S.N. Jeffers, 1999. Detecting multiple species of *Phytophthora* in container mixes from ornamental crop nurseries. *Plant Disease* 83, 1129–1136.

Guarnaccia V., D. Aiello, G. Polizzi, G. Perrone, G. Stea and A. Vitale, 2014. Emergence of prochloraz-resistant populations of *Calonectria pauciramosa* and *Calonectria polizzii* in ornamental nurseries of Southern Italy. *Plant Disease* 98, 344–350.

Guarnaccia V., Z.R. Hansen, D. Aiello, C.D. Smart and G. Polizzi, 2015. First report of root rot and foliar blight on Pittosporum (*Pittosporum tenuifolium*) caused by *Pythium irregulare* in Italy. *Journal of Phytopathology* 163, 411–414.

Hardy G.E. and K. Sivasithamparam, 1988. *Phytophthora* spp. associated with container- grown plants in nurseries in Western Australia. *Plant Disease* 72, 435–437.

Hong C.X. and G.W. Moorman, 2005. Plant pathogens in irrigation water: challenges and opportunities. *Critical Reviews in Plant Sciences* 24, 189–208.

Hu J.H., E.L. Stromberg, G.W. Moorman and C.X. Hong, 2005. Mefenoxam and propamocarb sensitivity of *Phytophthora cinnamomi* at ornamental plant nurseries in Virginia. (Abstr.) *Phytopathology* 95, S45.

Hu J.H., C.X. Hong, E.L. Stromberg and G.W. Moorman, 2008. Mefenoxam sensitivity and fitness analysis of *Phytophthora nicotianae* isolates from nurseries in Virginia, USA. *Plant Pathology* 57, 728–736.

Hwang J. and D.M. Benson, 2005. Identification, mefenoxam sensitivity, and compatibility type of *Phytophthora* spp. attacking floriculture crops in North Carolina. *Plant Disease* 89, 185–190.

Jung T., L. Orlikowski, B. Henricot, P. Abad-Campos, A.G. Aday, O. Aguin Casal, J. Bakonyi, S.O. Cacciola, T. Cech, D. Chavarriaga *et al*., 2016. Widespread *Phytophthora* infestations in European nurseries put forest, semi-natural and horticultural ecosystems at high risk of Phytophthora diseases. *Forest Pathology* 46, 134–163.

Kroon L.P., F.T. Bakker, G.B. van den Bosch, P.J. Bonants and W.G. Flier, 2004. Phylogenetic analysis of *Phytophthora* species based on mitochondrial and nuclear DNA sequences. *Fungal Genetics and Biology* 41, 766–782.

Kumar S., G. Stecher and K. Tamura, 2016. MEGA7: Molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Molecular Biology and Evolution* 33, 1870–1874.

Moralejo E., A.M. Pérez-Sierra, L.A. Álvarez, L. Belbahri, F. Lefort and E. Descals, 2009. Multiple alien *Phytophthora* taxa discovered on diseased ornamental plants in Spain. *Plant Pathology* 58, 100–110.

Orlikowski L.B. and G. Szkuta, 2006. Phytophthora rot of some orchids-new disease in Poland. *Phytopathology Polonica* 40, 57–61.

Parra G. and J.B. Ristaino, 2001. Resistance to mefenoxam and metalaxyl among field isolates of *Phytophthora capsici* causing Phytophthora blight of bell pepper. *Plant Disease* 85, 1069–1075.

Prigigallo M.I., S. Mosca, S.O. Cacciola, D.E.L. Cooke and L. Schena, 2015. Molecular analysis of *Phytophthora* diversity in nursery-grown ornamental and fruit plants. *Plant Pathology* 64, 1308–1319.

Robideau G.P., A.W. De Cock, M.D. Coffey, H. Voglmayr, H. Brouwer, K. Bala, D.W. Chitty, N. Désaulniers *et al*., 2011. DNA barcoding of oomycetes with cytochrome c oxidase subunit I and internal transcribed spacer. *Molecular Ecology Resources* 11, 1002–1011.

Schlenzig A., R. Campbell and J. Chard, 2014. *Phytophthora* Species Infecting Hardy Ornamentals in Nurseries and the Managed Environment in Scotland. *Journal of Phytopathology* 163, 686–689.

Schwingle B.W., J.A. Smith and R.A. Blanchette, 2007. *Phytophthora* species associated with diseased woody ornamentals in Minnesota nurseries. *Plant Disease* 91, 97–102.

Timmer L.W., J.H. Graham and S.E. Zitko, 1998. Metalaxyl-resistant isolates of *Phytophthora nicotianae*: Occurrence, sensitivity, and competitive parasitic ability on citrus. *Plant Disease* 82, 254–261.

Torres G.A., G.A. Sarria, G. Martinez, F. Varon, A. Drenth and D.I. Guest, 2016. Bud rot caused by *Phytophthora palmivora*: A destructive emerging disease of oil palm. *Phytopathology* 106, 320–329.

Uchida J.Y., 1994. Diseases of Orchids in Hawaii. *Plant Disease* 78, 220–224.

Werres S., R. Marwitz, W.A. Man In't Veld, A.W.A.M. De Cock, P.J.M. Bonants, M. De Weerdt, K. Themann, E. Ilieva and R.P. Baayen, 2001. *Phytophthora ramorum* sp. nov., a new pathogen on *Rhododendron* and *Viburnum*. *Mycological Research* 105, 1155–1165.

White T.J., T. Bruns, S. Lee and J.W. Taylor, 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: *PCR Protocols: A Guide to Methods and Applications* (M. Innis *et al*., ed.), Academic Press, San Diego, CA USA, 315–322.

Yamak F., T.L. Peever, G.G. Grove and R.J. Boal, 2002. Occurrence and identification of *Phytophthora* spp. pathogenic to pear fruit in irrigation water in the Wenatchee River valley of Washington state. *Phytopathology* 92, 1210–1217.

Yang X., B.M. Tyler and C. Hong, 2017. An expanded phylogeny for the genus *Phytophthora*. *IMA Fungus* 8(2), 355–384.

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