First report of black-foot disease, caused by *Cylindrocarpon destructans*, on ornamental marigold (*Tagetes minuta*) in Iran

Samad Jamali[1]*, *Zohreh Nasimi*[2]

[1] Department of Plant Protection, College of Agriculture and Natural Resource, Razi University, 67155-1158 Kermanshah, Iran
[2] Department of Plant Protection, School of Agriculture, Shiraz University, 71689-35936 Shiraz, Iran

Received: September 23, 2013
Accepted: April 30, 2014

Abstract: The ornamental *Tagetes minuta* is a herbaceous plant of the Asteraceae family. *T. minuta*, a species native to southern South America, is used as a condiment, as a refreshing beverage, and for medicinal purposes. In 2011, disease symptoms of yellowing, root and foot rot, drying of leaves, and plant death were observed in an ornamental marigold (*T. minuta*) greenhouse in Fars province. The infected plants were collected and transferred to a laboratory. Samples were washed, cut into small pieces, surface disinfested with a 0.5% NaClO solution, and cultured on Potato Dextrose Agar (PDA) acidified to pH 4.5 with 0.5% lactic acid. Based on morphological characters, the causal agent was identified as *Cylindrocarpon destructans*. To confirm morphological identification, DNA was extracted from isolates using a genomic DNA purification Kit. The region of internal transcribed spacers 1, 2, and 5.8S genes of rDNA were amplified using the ITS4 and ITS1 universal primer set. Fragments of 600 bp were recovered from PCR, purified, sequenced, edited, and deposited in GenBank. The isolates had a 100% identity with all the compared *C. destructans* sequences. The pathogenicity tests were done with a suspension of 1×10^6 conidia per ml homogenised in sterile water. The symptoms on inoculated plants were similar to those previously observed and the fungus was reisolated from the inoculated plants. This is the first documented report of *C. destructans* as a cause of root and foot rot disease on *T. minuta* in Iran.

Key words: black-foot disease, *Cylindrocarpon destructans*, Iran, molecular identification, *Tagetes minuta*

Introduction

Tagetes minuta, a species native to southern South America, is used as a condiment, as a refreshing beverage, and for medicinal purposes (Parodi 1959). *T. minuta* is commercially grown and harvested for its essential oils which are used in the flavor and perfume industry as "Tagetes Oil" (Craveiro *et al*. 1988). Essential oils are a source of bioactive compounds that are safe for human health and the environment (Souguir *et al*. 2013). There is evidence that the secondary compounds in *Tagetes*, through several different mechanisms, are effective deterrents of numerous organisms, including: fungi (Chan *et al*. 1975), pathenogenic fungi that can cause disease in humans (Camm *et al*. 1975), bacteria (Grover and Rao 1978), nematodes, and numerous insect pests. This plant does not have many pests and diseases. So far, only in the root-knot nematode, red spider mite has been reported from this plant (Steiner 1941). In September 2011, a disease of unknown origin was discovered in an ornamental marigold greenhouse in Fars province. The dying plants showed symptoms of wilt, and the dead ones had lost all their leaves. The roots and the crown of the dead plants were also affected. In the cross section of the crown there was discoloration. The present work was carried out with the purpose of establishing the identity of the microorganisms causing the described symptoms, and to evaluate their pathogenicity. Identification of root and foot rot pathogens by macroscopic and microscopic examinations are not only time-consuming but also challenging. Therefore, application of molecular detection procedures such as polymerase chain reaction (PCR) and sequencing analysis is critical for the accurate determination of these pathogens. There are advantages to using the PCR technique over traditional methods, for detection and diagnosis, because the fungi do not need to be cultured, prior to detection by PCR (Zhang *et al*. 2006). Recently, some diagnostic methods based on PCR have been developed for plant pathogen detection (Hamelin *et al*. 1996; Ippolito *et al*. 2004; Langrell 2005; Wang *et al*. 2008; Martin *et al*. 2009; Huang and Kang 2010).

Materials and Methods

Isolation

Isolation was made from symptomatic roots and crowns. After washing the material with running tap water for 1 h, small pieces, approximately 4 mm in size were taken from the interface of healthy and diseased tissues. Then, the pieces were surface disinfected by immersing them in 1.5% solution on NaOCl for 30 sec, rinsed three times in sterile distilled water, and plated on Potato Dextrose Agar

*Corresponding address:
jamali454@yahoo.com

(PDA) and Malt Extract Agar (MEA) amended with chloramphenicole (25 μg/ml). Plates were incubated at 25–27°C for 3 to 5 days to allow the fungi to grow. For fungal identification, during the incubation period, plates were observed daily for the appearance of fungal colonies.

Morphological characteristics

Morphological characters of isolates in the monoconidial culture were evaluated. The size and form of phialides, microconidia, macroconidia, and chlamydospores were examined for microscopic characteristics. Fifty measurements of each type of structure were made using BioloMICSMeasure software. Radial growth of the isolates was measured on MEA and PDA after 7 days at 25°C. The identification was carried out by comparing the information registered with those published in the specialised literature (Booth 1967; Brayford *et al*. 2004).

Pathogenicity tests

For the inoculation tests with the purified culture, 10 ornamental marigold seedlings which were 10 cm tall were used. The plants, were inoculated by dipping the roots (Scheck *et al*. 1998) in a 1×10^6 conidial suspension for 30 min. The control plants were dipped in sterile water. Inoculated plants were planted individually in pots containing sterilised soil (2 : 1 sand and soil) and placed in a glasshouse at 24°C in a completely randomised design. Starting 3 days after inoculation, the seedlings were examined periodically for damage to the roots and crown.

DNA extraction and amplification

For DNA extraction, isolates were grown on PDA for 10 to 15 days at 25°C in the dark. Fungal mycelium from pure cultures were scraped and mechanically disrupted by grinding the mycelium with a mortar and pestle to a fine powder under nitrogen. Total genomic DNA was extracted using a genomic DNA purification Kit (Fermentas, UK) according to the manufacturer's instructions. The Internal Transcribed Spacer (ITS) regions of nuclear rDNA were amplified with the universal ITS1 (5'-TCC GTA GGT GAA CCT GCG G-3') and ITS4 (5'-TCC TCC GCT TAT TGA TAT G-3') primers (White *et al*. 1990) on a CORBETT RESEARCH model CG1-96 thermocycler. For ITS amplifications, the samples were prepared as follows: a reaction tube contained 12.5 μl of a diluted DNA sample (1 : 10 or 1 : 100 dilutions of the original extract), 2.5 μl of 10X PCR buffer, 20 pmol of each primer, 1.25 nmol of each deoxynucleotide, 1.5 mM of $MgCl_2$ and 0.5 U of *Taq* polymerase (CinnaGen, Iran) in a reaction volume of 25 μl. The thermocycle was carried out according to the following program: an initial denaturation step at 94°C for 3 min; then 30 cycles, consisting of denaturation (30 sec at 94°C), annealing (30 sec at 50°C), and extension (2 min at 72°C); and a final extension step of 10 min was allowed at 72°C before cooling or removing the tubes. Amplified fragments were visualised under UV light after electrophoresis on 1% agarose gels stained with ethidium bromide and run in 1X TBE buffer. The controls, with no DNA, were included in every set of amplification to check the DNA contamination in reagents and reaction buffers.

Sequencing of the amplified ITS regions

The amplification products of all specimens were purified with the GeneJET PCR purification Kit (Fermentas, UK) to remove excess primers and nucleotides. Sequencing reaction was performed on purified PCR products in forward and reverse orientation using the primers used for amplification (ITS1 or ITS4). All DNA sequences of the ITS regions are deposited at the National Center for Biotechnology Information GenBank (NCBI, http://www.ncbi.nlm.nih.gov/Entrez) (Bethesda, MD, USA).

Phylogenetic analysis

Sequences of the internal transcribed spacer regions including the 5.8S gene of rDNA were used to study phylogenetic relationships of the studied isolates. The internal transcribed spacer sequences of rDNA generated in this study were compared to those of other isolates obtained from GenBank. Multiple alignments were performed with CLUSTALW (Thompson *et al*. 1994) using default settings and were manually optimised with BIOEDIT v.7.0.9 (Hall 1999). Phylogenetic analyses were performed by means of distance and maximum parsimony (MP) methods. Other statistics, including tree length, consistency index (CI), retention index (RI), rescaled consistency index (RCI), informative consistency index (iCI) and informative retention index (iRI) were calculated.

Results

Characteristics of isolated fungus

Only a fungus was isolated from the roots and crowns of infected plants. Based on morphological characteristics in laboratory and greenhouses, the isolates were identified as *Cylindrocarpon*. The isolates formed microconidia, macroconidia, and chlamydospores. Based on morphology, type and size of asexual structures, isolates were identified as *C. destructans*. Colony diameter on PDA varied from 30 mm to 55 mm after 10 days of incubation at 25°C in darkness. Colony texture was cottony or felty with aerial mycelium in the centre or over the entire colony (Fig. 1A). The colony colour varied from white to cinnamon (Fig. 1A). Zonation was absent in PDA. But in MEA, all the cultures formed concentric zones. The colony margin was even in both media. Sporodochia were produced in both PDA and MEA. Conidiophores, unbranched or branched, arose abundantly from the aerial mycelium and the agar surface. The phialides measured 15–30 × 2.2–3 μm. The microconidia were hyaline, cylindrical, and 5–8 × 3–4.5 μm. The macroconidia were hyaline, cylindrical with rounded ends, and 1–3 septate (Fig. 1C).

Pathogenicity

None of the control plants died, while 80% of the plants inoculated with *C. destructans* died at two weeks and the rest died during the following three weeks (Fig. 1E, F).

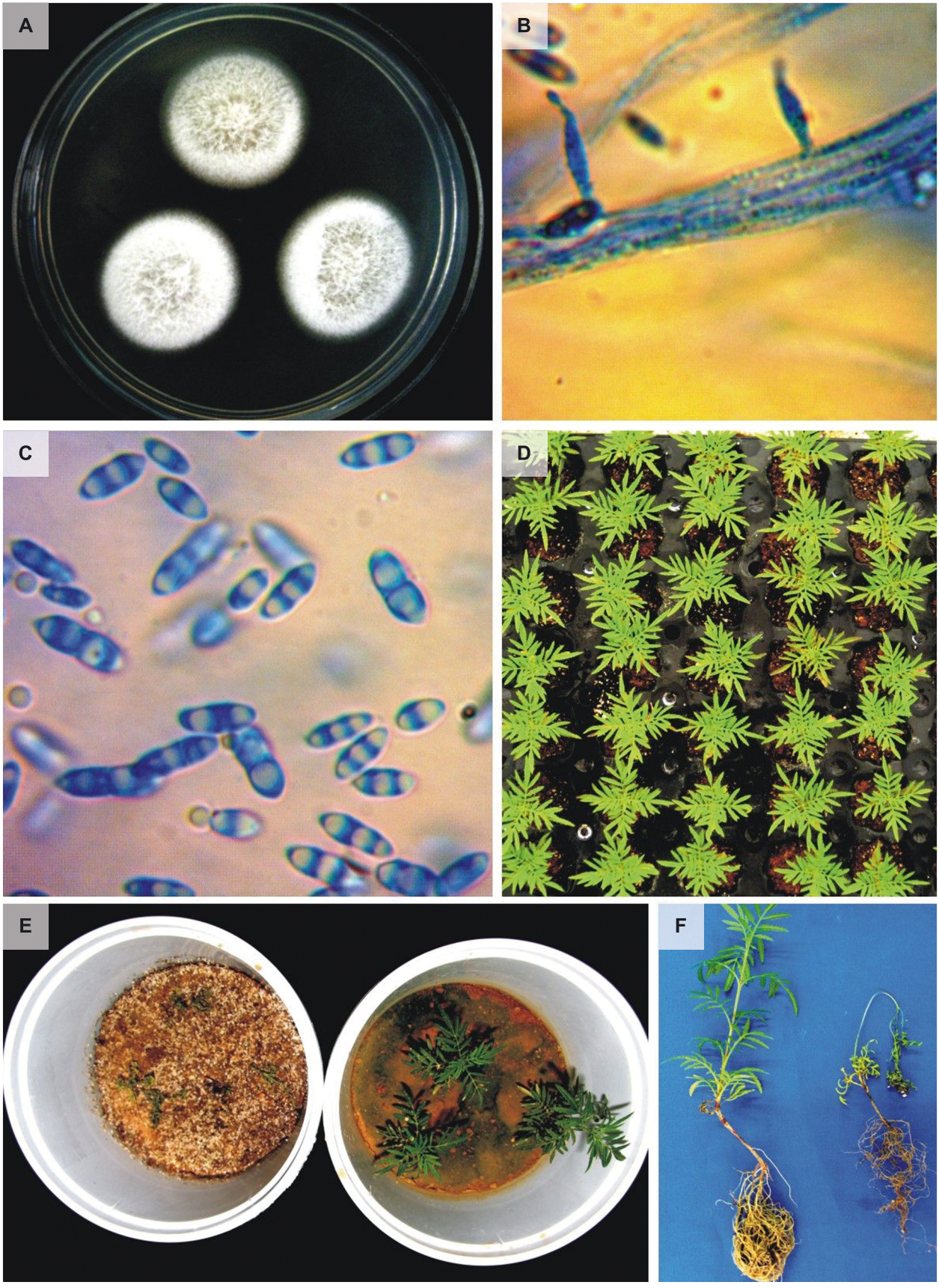

Fig. 1. Colonies of 14-day-old *C. destructans* on PDA (A), phialides (B), septate conidia (C), *T. minuta* plants (D), dead seedlings-left and control plants-right (E), foot rot symptom on *T. minuta* plant-left and the control plant-right (F)

No *Cylindrocarpon* was isolated from any of the control plants. In inoculation tests, *C. destructans* inoculated onto seedlings can cause the same symptoms as observed in the field. The only fungus consistently isolated from the experimentally infected seedlings was *C. destructans*, which demonstrates that it is the cause of black root rot and crowns of *T. minuta*.

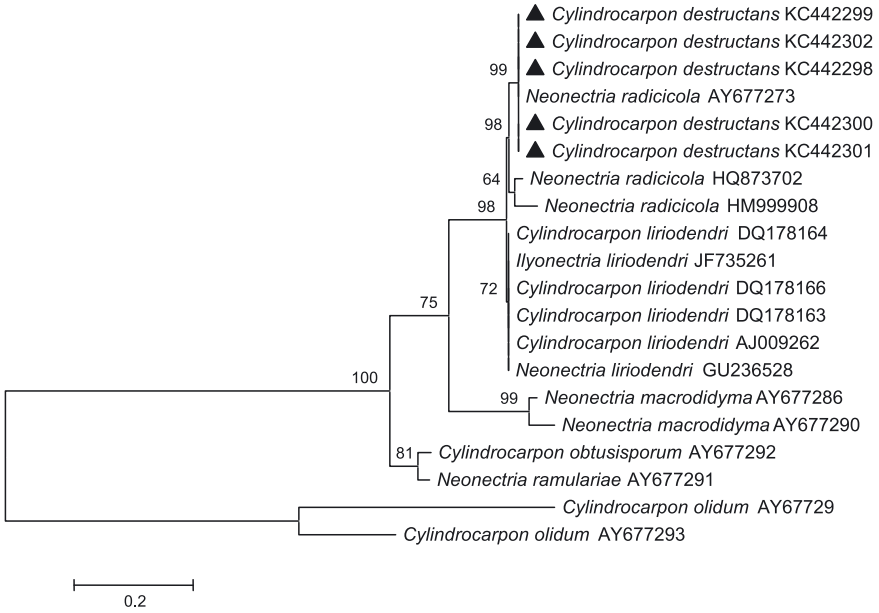

Fig. 2. Neighbor joining phylogram generated in Mega from the alignment of 20 combined ITS1, 5.8S subunit, and ITS2 regions of the genomic ribosomal RNA sequences of the *Cylindrocarpon* species, using the Kimura 2 parameter model with complete deletion gap handling and 1,000-replication bootstrapping. The shapes refer to isolates from Iran

Molecular study

All *Cylindrocarpon* isolates previously identified based on morphological and culture characters, were amplified using the primer pairs ITS1 and ITS4. All of the studied isolates gave strong amplification of a single PCR product of about 600 bp using primers ITS1 and ITS4. Through a Blast search in GenBank, all isolates were identified as *C. destructans.* All DNA sequences of *Cylindrocarpon* isolates (accession numbers: KC442298 to KC44302) showed 100% homology with valid sequences previously identified and deposited in GenBank. According to DNA sequence analyses and morphological characters, the isolates recovered from the wood of *T. minuta* showing black root rot symptoms, could be assigned to *C. destructans* (Fig. 2).

Phylogenetic analysis

For the ITS data set, 20 sequences of ca. 600 bp covering the ITS1+5.8S+ITS2 regions were used. Both distance-based and cladistic methods were applied for phylogenetic reconstruction of 20 specimens. The ITS phylogenetic trees inferred by both distance-based (Fig. 2) and cladistic methods (data not shown) showed the same topology, although there were differences in percent bootstrapping. In the cladistic method, the tree length was 634 with CI of 0.83; RI = 0.83; RCI = 0.72 for all sites; iCI = 0.80 for parsimony informative sites and iRI = 0.83. With this, 72 trees were retained. Our isolates were clustered in a distinct monophyletic clade related to *Neonectria radicicola* from other authors (Fig. 2). *C. destructans* was a sister taxon of *C. liriodendri* [98% NJ (Neihbour joining) and 99% MP]. Our *C. destructans* isolates had 100% similarity between themselves and only an average of 98 with a range of 93–100% similarity between all *C. destructans* sequences analysed.

Discussion

Based on morphological characteristics, all isolates of recovered fungi from diseased ornamental marigold belonged to the genus *Cylindrocarpon*. A cosmopolitan, natural inhabitant of the soil, *C. destructans* is commonly associated with roots and residues of a wide variety of woody and herbaceous plants, especially in alkaline soils (Samuels 1990). This fungus is considered to be a necrotrophic and apportunistic pathogen (Brayfold 1991). However in this study, the recovered isolates of the *C. destructans* infected and rotted the roots of healthy, unstressed seedlings, indicating that it is a true pathogen of *T. minuta*. This plant does not have many pests and diseases and so far, only in the root-knot nematode, has the red spider mite been reported from this plant (Steiner 1941). Unfortunately, identification of *Cylindrocarpon* spp. by macroscopic and microscopic examinations, is not only time-consuming but also challenging. This is especially so with some *Cylindrocarpon* species that do not develop a teleomorphic stage *in vitro*. Therefore, application of molecular detection procedures, such as PCR and sequencing analysis, is critical for the accurate determination of the identity of the *Cylindrocarpon* species and helps to effectively treat and control *Cylindrocarpon* infections in nurseries and fields. Using PCR with the primers ITS1 and ITS4, a fragment of about 600 bp was obtained for *C. destructans* isolates. Based on ITS gene sequences, these isolates showed 100% homology with *C. destructans* isolates deposited in GenBank. Subsequently, both phenotypical and molecular data confirmed the identification of the *Cylindrocarpon* isolates as *C. destructans*.

Analysis of sequence alignment shows that, in the 20 *Cylindrocarpon* species compared, there are 722 potentially phylogenetic informative sites which are mainly comprised of substitutions, deletions and insertions. We applied both distance-based and maximum parsimony methods. The comparison of the two types of trees illustrates concordance between them and the results were broadly similar. Phylogenetic reconstruction of the combined ITS1, 5.8S subunit, and ITS2 regions of the genomic ribosomal RNA tandem gene repeat, which was applied for identification of species, could discriminate species and put them into their corresponding ITS clade. On the basis of the analysis of mitochondrial ribosomal DNA sequences, the species of *Nectria* with *Cylindrocarpon* anamorphs were placed in the genus *Neonectria*, including *Neonectria discophora* and *N. radicicola* (Brayford *et al*. 2004). Phylogenetic analysis of isolates based on internal transcribed spacers of rRNA genome put them into one monophyletic lineage. This group is comprised of our isolates which together with authentic isolates of *N. radicicola* constitute a monophyletic clade. The present study is the first report of *C. destructans* on *T. minuta* in Iran.

References

Booth C. 1967. *Nectria radicicola*. C.M.I. Descriptions of Pathogenic Fungi and Bacteria 148: 1–2.

Brayford D., Honda B.R, Mantiri F.R., Samuels G.J. 2004. *Neonectria* and *Cylindrocarpon*: the *Nectria mammoidea* group and species lacking microconidia. Mycologia 96 (3): 572–597.

Camm E.L., Towers G.H.N., Mitchell J.C. 1975. UV-mediated antibiotic activity of some Compositeae species. Phytochemistry 14 (9): 2007–2011.

Chan G.F.Q., Towers G.H.N., Mitchell J.C. 1975. UV-mediated antibiotic activity of thiophene compounds of *Tagetes*. Phytochemistry 14 (10): 2295–2296.

Craveiro C.C., Matos F.J.A., Machado M.I.L., Alencar J.W. 1988. Essential oils of *Tagetes minuta* from Brazil. Perfum. Flavor. 13 (5): 35–36.

Grover G.S., Rao J.T. 1978. *In vitro* antimicrobial studies of the essential oil of *Tagetes erecta*. Perfum. Flavor. 3 (5): 28.

Hall T.A. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acid Symposium Series 41: 95–98.

Hamelin R.C., Berube P., Gignac M., Bourassa M. 1996. Identification of root rot fungi in nursery seedlings by nested multiplex PCR. Appl. Environ. Microbiol. 62 (11): 4026–4031.

Huang J.L., Kang Z.H. 2010. Detection of *Thielaviopsis basicola* in soil with real-time quantitative PCR assays. Microbiol. Res. 165 (5): 411–417.

Ippolito A., Schena L., Nigro F., Ligorio V.S., Yaseen T. 2004. Real-time detection of *Phytophthora nicotianae* and *P. citrophthora* in citrus roots and soil. Eur. J. Plant Pathol. 110 (8): 833–843.

Langrell R.H. 2005. Development of a nested PCR detection procedure for *Nectria fuckeliana* direct from Norway spruce bark extracts. FEMS Microbiol. Lett. 242 (1): 185–193.

Martin F.N., Coffey M.D., Zeller K., Hamelin R.C., Tooley P., Garbelotto M., Hughes K.J.D., Kubisiak T., Bilodeau G., Levy L., Blomquist C., Berger P.H. 2009. Evaluation of molecular markers for *Phytophthora ramorum* detection and identification: testing for specificity using a standardized library of isolates. Phytopathology 99 (4): 390–403.

Parodi L.R. 1959. Enciclopedia Argentina de Agricultura y Jardineria. Tomo II. Editorial Acme S.A.C.I., Buenos Aires, 845 pp.

Samuels G.J. 1990. Variation in *Nectria radicicola* and its anamorphs, *Cylindrocarpon destructans*. Mycol. Res. 94 (4): 433–442.

Scheck H.J., Vasquez S.J., Gubler W.D., Fogle D. 1998. First report of black-foot disease, caused by *Cylindrocarpon obtusisporum*, on grapevine in California. Plant Dis. 82 (4): 448.

Souguir S., Chaieb I., Chheikh Z.B., Laarif A. 2013. Insecticidal activities of essential oils from some cultivated aromatic plants against *Spodoptera littoralis* (Boisd). J. Plant Prot. Res. 53 (4): 388–391.

Steiner G. 1941. Nematodes parasitic on and associated with roots of marigolds (*Tagetes hybrids*). Proc. Biol. Soc. Wash. 54: 31–34.

Thompson J.D., Higgins D.G., 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. Nucl. Acid. Res. 22 (22): 4673–4680.

Wang X.J., Zheng W.M., Buchenauer H., Zhao J., Han Q.M., Huang L.L., Kang Z.S. 2008. The development of a PCR-based method for detecting *Puccinia striiformis* latent infections in wheat leaves. Eur. J. Plant Pathol. 120 (3): 241–247.

White T.J., Bruns T., Lee S., Taylor J. 1990. Amplification and direct sequencing of fungal ribosomal genes for phylogenetics. p. 315–322. In: "PCR Protocols: A Guide to Methods and Applications" (M. Innis, ed.). Academic Press, San Diego, 522 pp.

Zhang Z.G., Li Y.Q., Fan H., Wang Y.C., Zheng X.B. 2006. Molecular detection of *Phytophthora capsici* in infected plant tissues, soil and water. Plant Pathol. 55 (6): 770–775.