Spanish Journal of Agricultural Research
15 (3), e10SC02, 5 pages (2017)
eISSN: 2171-9292
https://doi.org/10.5424/sjar/2017153-11297
Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria, O.A., M.P. (INIA)

SHORT COMMUNICATION

OPEN ACCESS

Morphological description and molecular detection of *Pestalotiopsis* sp. on hazelnut in Serbia

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Abstract

In autumn 2015, hazelnut plants with leaf blight symptoms were noticed in a commercial plantation in the Province of Vojvodina, Serbia. Symptomatic samples were collected and submitted to laboratory analysis. Based on morphological characterization, the fungus isolated from the material was initially identified as *Pestalotiopsis* sp. Pathogenicity tests showed that two selected isolates infected hazelnut leaves and fruits that developed symptoms after artificial inoculation. The pathogen was re-isolated from diseased leaves and fruits, confirming Koch's postulates. Molecular identification was performed with sequence and phylogenetic analysis of ITS, EF1-α, and TUB genomic regions. Phylogenetic analysis confirmed the results of the morphological identification. The detection of *Pestalotiopsis* sp., a causal agent of leaf blight on hazelnut in Serbia, is one of a few reports of these pathogenic fungi on hazelnut.

Additional keywords: *Corylus avellana* L.; leaf blight; β-tubulin; ITS; EF1-α.

Abbreviation used: EF1-α (partial translation elongation factor 1-α); ITS (internal transcribed spacer); PDA (potato dextrose agar); TUB (5' end of the β-tubulin gene).

Authors' contributions: Performed the experiments and contributed reagents/materials/analysis tools: TV, DJ, SZ and VK. Technical support: AL and SA. Critically revised the manuscript: SP. Analyzed the data and wrote the paper: TV and DJ.

Supplementary figures: Supplementary material (Figures S1 and S2) accompanies the paper on SJAR's website.

Citation: Vasić, T.; Jevremović, D.; Krnjaja, V.; Leposavić, A.; Andjelković, S., Živković, S.; Paunović, S. (2017). Short communication: Morphological description and molecular detection of *Pestalotiopsis* sp. on hazelnut in Serbia. Spanish Journal of Agricultural Research, Volume 15, Issue 3, e10SC02. https://doi.org/10.5424/sjar/2017153-11297

Received: 23 Feb 2017. **Accepted:** 07 Jul 2017.

Funding: Fruit Research Institute and Institute for Forage Crops (Serbia).

Competing interests: The authors have declared that no competing interests exist.

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Introduction

Hazelnut (*Corylus avellana* L.) is an important nut tree cultivated in many countries in the World. Turkey is a leading producer, with a share of more than 70% of the global production, followed by Italy, Azerbaijan, Georgia, USA and Spain (INC, 2016). In Serbia, hazelnut is cultivated on more than 2,200 ha, but the domestic production cannot meet local demands (Keserović *et al.*, 2014), which have significantly increased in the past decade. New large-scale orchards are currently being planted and hazelnut production is becoming an important part of agriculture. However, a rapid expansion of hazelnut potentially favors the emergence or expansion of pests and diseases that may endanger the production.

More than 220 *Pestalotiopsis* species have been described so far. Of these, at least 23 species were reported as endophytes and represent an important group of endophytic fungi (Liu *et al.*, 2007). Numerous *Pestalotiopsis* species are common plant pathogens in tropical and temperate climate conditions, causing leaf and twig blights in many plant species and some post-harvest diseases. In sensitive plant species and cultivars, they may reduce production and cause economic losses (Maharachchikumbura *et al.*, 2011). So far, *Pestalotiopsis* species on hazelnut have been reported in Iran, Chile and Turkey (Karaca & Erper, 2001; Arzanlou *et al.*, 2012).

The objective of our study was to identify the causal agent of leaf blight on hazelnut in Serbia using morphology and molecular-based methods.

Material and methods

Samples and fungal isolation

In autumn 2015, during a survey of hazelnut diseases, we noticed plants with leaf blight symptoms in a commercial

plantation in the Province of Vojvodina, Serbia. To isolate the pathogen, hazelnut branches were surface-sterilized with 5% sodium hypochlorite for 2 min, followed by three washes with sterile distilled water. Surface-sterilized tissue was transferred to sterile filter paper, placed on potato dextrose agar (PDA) containing streptomycin, and incubated at 24°C in the dark for 10 days. Individual germinating conidia were selected, transferred directly to PDA plates according to the procedures described by Choi *et al.* (1999), and stored on PDA in tubes at 4°C. Colony morphology (color, shape and growth rate) was determined after 7−10 days of incubation on PDA at 25°C in darkness. Dimensions of microscopic structures were calculated based on 30 measurements for conidial morphology (shape, color and cell number), size (length and width), and the presence and size of apical and basal appendages where possible. Images were captured by an Olympus SC100 color camera mounted on a BX31 microscope (Olympus, Japan).

Pathogenicity tests

Pathogenicity tests were performed on detached leaves and hazelnut fruits. Unhardened developed leaves and fruits were collected and surface-disinfected with 70% ethanol. Disinfected leaves and fruits were placed in glass Petri dishes containing moistened sterile filter paper. Plant tissue was inoculated using a 5-mm mycelial cube from an actively growing edge of the 10-day-old fungal culture. Samples were incubated for 14 days at 20−25°C with a 12 h photoperiod (Sezer & Dolar, 2015). Isolates RS-Le-1 and RS-Le-5 were tested and PDA cubes were used as negative control.

Molecular identification

For molecular analysis, fungal DNA was extracted from cultured mycelia with 2% CTAB buffer (Day & Shattock, 1997). Three separate PCR reactions were performed using ITS1/ITS4, EF1-728F/EF-2, and T1/Bt2b primer pairs, amplifying the fragments encompassing ITS1, ITS2, and 5.8S rDNA gene (ITS), partial translation elongation factor 1-α region (EF1-α), and 5' end of the β-tubulin gene (TUB), respectively (White *et al.*, 1990; Glass & Donaldson, 1995; O'Donnell & Cigelnik, 1997; O'Donnell *et al.*, 1998; Carbone & Kohn, 1999). The polymerase chain reactions (PCR) were carried out in a Tpersonal thermal cycler (Biometra, Germany). Amplified PCR products were analyzed by electrophoresis in 1.5% agarose gel, stained with ethidium bromide, and visualized under UV light with a Gel Doc EZ System (Biorad, USA). The PCR products of tested isolates obtained with all three primer pairs were purified and custom sequenced (Macrogen, the Netherlands). Sequences of the Serbian isolates were aligned and compared with closely related sequences retrieved from the GenBank. Multiple sequence alignments were carried out using the software package BioEdit v. 7.0.5.2 (Hall, 1999); phylogenetic analysis was performed with MEGA 6.0 (Tamura *et al.*, 2013).

Results and discussion

Isolation, morphological description of the agent, and pathogenicity test

Two isolates, RS-Le-1 and RS-Le-5, were isolated from the hazelnut branches. Isolates are maintained as

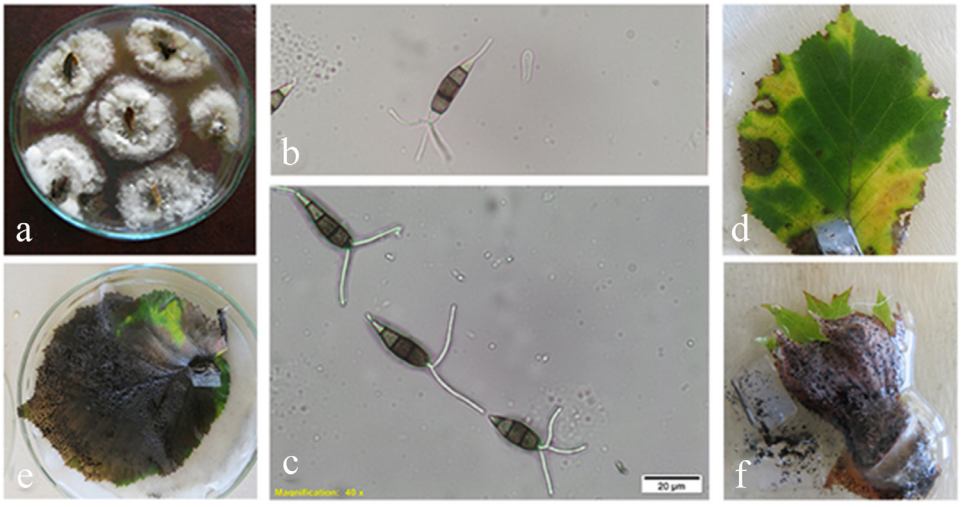

Figure 1. Colonies on PDA (a). Fusiform five-celled conidia (b, c). Leaf blight and necrosis on inoculated hazelnut leaves (d, e). Severe necrosis on the inoculated hazelnut fruit (f).

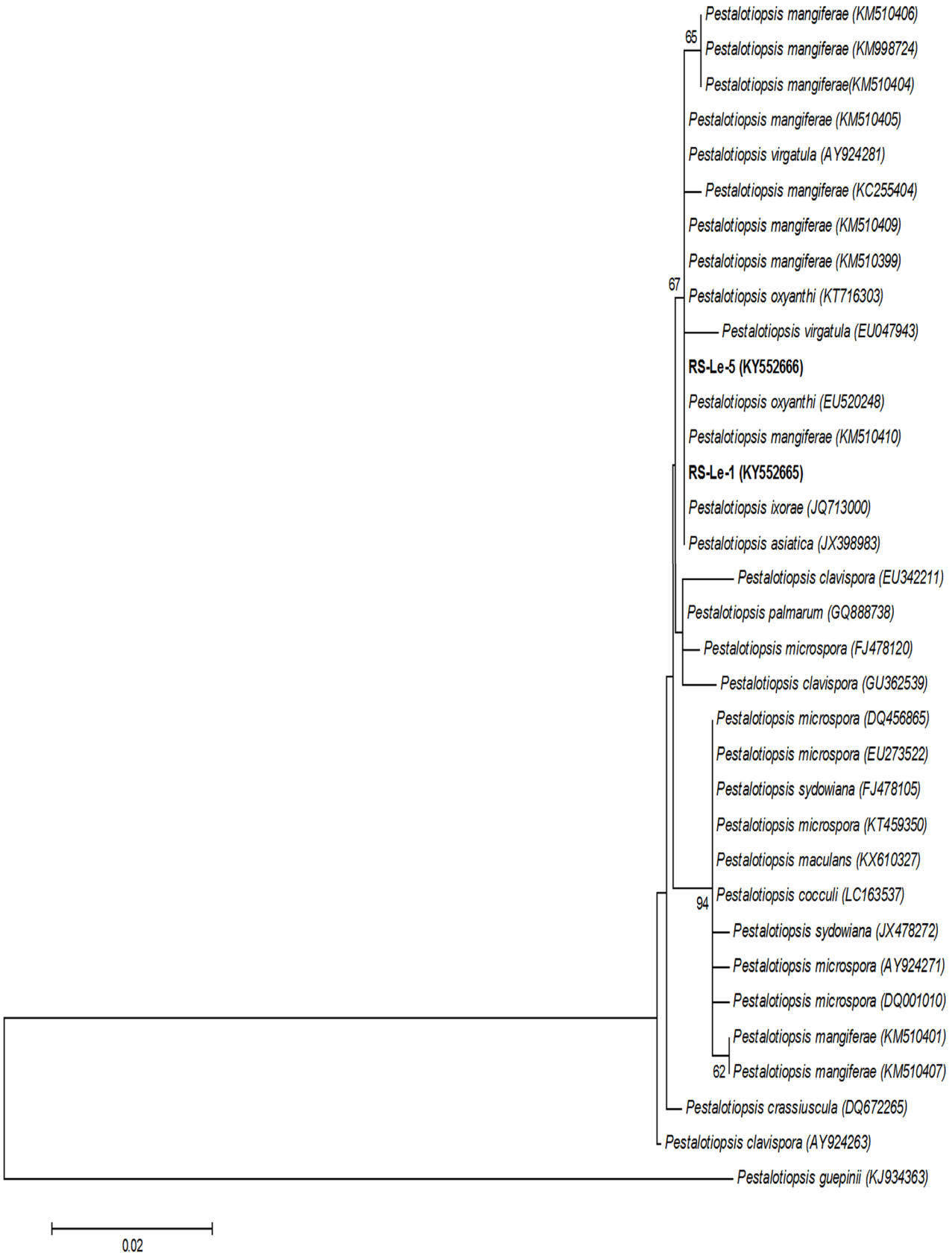

Figure 2. Neighbor-joining phylogenetic tree of 32 *Pestalotiopsis* spp. isolates reconstructed from ITS sequences. Only bootstrap values > 60% calculated from 1,000 replications are shown. Serbian sequences are shown in bold. Accession numbers of the isolates retrieved from GenBank are in parentheses.

culture collections in the Institute for Forage Crops. On PDA, fungal colonies grew up to 55 mm in diameter within seven days at room temperature (25 ± 2°C). Colonies had a smooth, even to undulating, colorless margin. Aerial mycelium was cottony pure white (Fig. 1a). Acervuli formed on the aerial mycelium contained black, slimy conidial masses. Conidiophores were hyaline and branched. Conidiogenous cells were annellidic, hyaline and smooth. The conidia were fusiform, five-celled, straight or slightly curved (Fig. 1b, c). The cells comprised three colored median cells and apical and basal hyaline cells with appendages. Conidia measured 16.57−27.53 × 5.05−9.13 µm (mean 25.66 × 7.54), five-celled with three brown central cells, the first two darker than the third one. The basal cell had a single 2.95−12.38 µm (mean 7.49) long appendage. The apical cell had 2−3 appendages with the following dimensions: first 3.67−30.39 µm long (mean 17.14), median 5.73−27.88 µm long (mean 20.39), and third 10.12−33.49 µm long (mean 17.17). Based on these morphological characteristics, the isolates were initially identified as belonging to the genus *Pestalotiopsis* (Sutton, 1980; Maharachchikumbura *et al.*, 2014).

In pathogenicity tests, both tested isolates caused blight and necrosis on leaves and fruits (Figs. 1d,e,f). The pathogen was re-isolated from diseased leaves and fruits, confirming Koch's postulates.

Molecular detection

In PCR analysis, fragments of 548, 493, and 824 bp from ITS, EF1-α, and TUB regions were amplified in both isolates, respectively. The PCR products of the tested isolates were sequenced; the obtained nucleotide sequences were deposited in the GenBank under the accession numbers KY552665-552666 (ITS), KY568911-568912 (TUB), KY568913-568914 (EF1-α). Sequences of ITS, EF1-α, and TUB regions of the two Serbian isolates were identical. The ITS sequences of our isolates showed 100% nucleotide (nt) identity with the sequences of accessions KM510410 of *P. mangiferae* and AY924281 of *P. virgatula*. In the reconstructed neighbor-joining phylogenetic tree (Kimura 2-parameter model), Serbian isolates were grouped with *P. mangiferae*, *P. virgatula*, *P. oxyanthi*, *P. ixorae*, and *P. asiatica* isolates (Fig. 2). Based on the EF1-α sequences, Serbian isolates showed the highest nt identity (99.79%) with *P. asiatica* accession JX399049. The TUB sequences of Serbian isolates showed the highest nt identity (99.51%) with the accession JQ762258 of *P. ixorae*. Phylogenetic analysis based on EF1-α and TUB sequences did not yield more information and was sufficient for proper species identification of the examined isolates (Figs. S1 and S2 [suppl]). The ITS gene is often used for molecular identification of *Pestalotiopsis* isolates, but the analysis of at least two genes may be more informative (Hu *et al.*, 2007). In our study, phylogenetic analysis generated from three genes was not sufficient for species identification. A limited number of deposited EF1-α and/or TUB sequences of *Pestalotipsis* species in the GenBank impedes species identification. Previously, identification of *Pestalotiopsis* species was dependent on host association and on morphological and cultural characteristics (Wei *et al.*, 2007; Hu *et al.*, 2007). Phylogenetic analysis facilitated species identification and become a necessity in species description (Maharachchikumbura *et al.*, 2014). The detection of *Pestalotiopsis* sp. on hazelnut in Serbia is one of the several reports of these fungal species on this nut tree in the world. Our paper will provide valuable information for further study of *Pestalotiopsis* sp., reported here as a new causal agent of leaf blight on hazelnut in Serbia.

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