



SHORT COMMUNICATION

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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).

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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

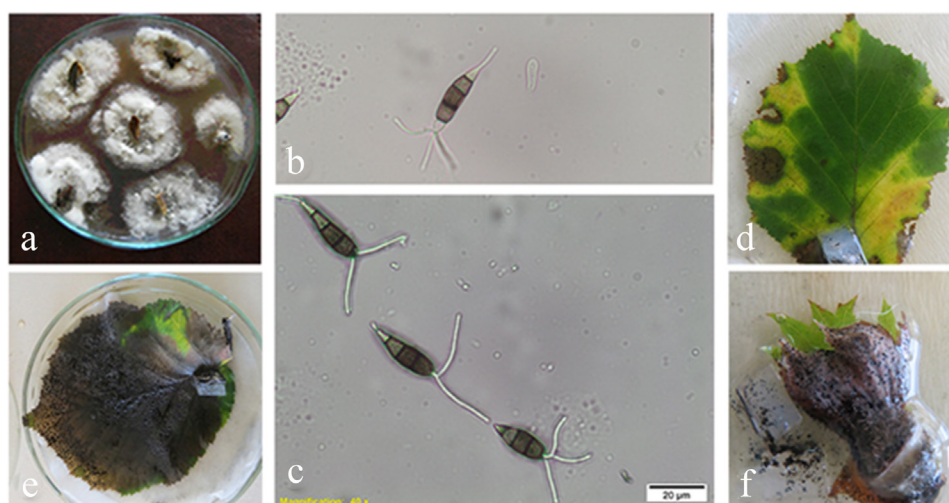


Figure 1. Colonies on PDA (a). Fusiform five-celled conidia (b, c). Leaf blight and necrosis on inoculated hazelnut leaves (d, e). Severe necrosis on the inoculated hazelnut fruit (f).

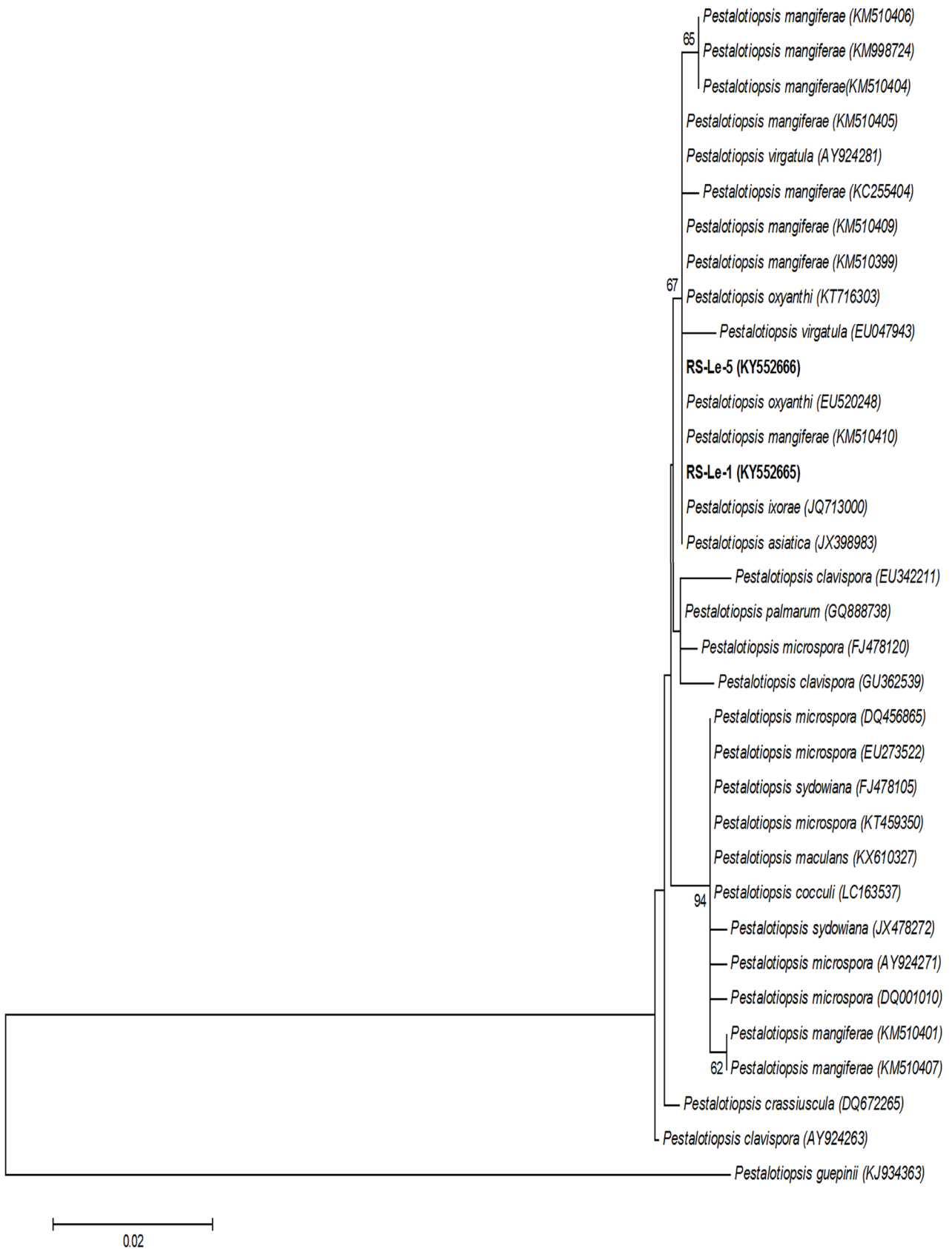


Figure 2. Neighbor-joining phylogenetic tree of 32 *Pestalotiopsis* spp. isolates reconstructed from ITS sequences. Only bootstrap values > 60% calculated from 1,000 replications are shown. Serbian sequences are shown in bold. Accession numbers of the isolates retrieved from GenBank are in parentheses.

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 ($25 \pm 2^\circ\text{C}$). 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 $16.57\text{--}27.53 \times 5.05\text{--}9.13 \mu\text{m}$ (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 $2.95\text{--}12.38 \mu\text{m}$ (mean 7.49) long appendage. The apical cell had 2–3 appendages with the following dimensions: first $3.67\text{--}30.39 \mu\text{m}$ long (mean 17.14), median $5.73\text{--}27.88 \mu\text{m}$ long (mean 20.39), and third $10.12\text{--}33.49 \mu\text{m}$ 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 *Pestalotiopsis* 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.

References

- Arzanlou M, Torbati M, Khodaei S, Bakhshi M, 2012. Contribution to the knowledge of pestalotioid fungi of Iran. *Mycosphere* 3 (5): 871-878. <https://doi.org/10.5943/mycosphere/3/5/12>
- Carbone I, Kohn LM, 1999. A method for designing primer sets for speciation studies in filamentous ascomycetes. *Mycologia* 91: 553-556. <https://doi.org/10.2307/3761358>
- Choi YW, Hyde KD, Ho WWH, 1999. Single spore isolation of fungi. *Fungal Divers* 3: 29-38.
- Day JP, Shattock RC, 1997. Aggressiveness and other factors relating to displacement of populations of *Phytophthora infestans* in England and Wales. *Eur J Plant Pathol* 103: 379-391. <https://doi.org/10.1023/A:1008630522139>
- Glass NL, Donaldson GC, 1995. Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous ascomycetes. *Appl Environ Microbiol* 61: 1323-1330.
- Hall TA, 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucl Acids Symp Ser* 41: 95-98.
- Hu H, Jeewon R, Zhou D, Zhou T, Hyde KD, 2007. Phylogenetic diversity of endophytic *Pestalotiopsis* species in *Pinus armandii* and *Ribes* spp.: evidence from rDNA and β -tubulin gene phylogenies. *Fungal Divers* 24: 1-22.
- INC, 2016. Nuts and dried fruits. Global statistical review 2015/2016. International Nut and Dried Fruit Council. <https://goo.gl/A9AaUU> [17/04/2017].
- Karaca GH, Erper I, 2001. First report of *Pestalotiopsis guepinii* causing twig blight on hazelnut and walnut in Turkey. *Plant Pathol* 50: 415-415. <https://doi.org/10.1046/j.1365-3059.2001.00580.x>

- Keserović Z, Magazin N., Kurjakov A, Dorić M, Gošić J, 2014. Census of Agriculture 2012. Fruit growing. Statistical Office of the Republic of Serbia, Belgrade, Serbia. 94 pp.
- Liu AR, Xu T, Guo LD, 2007. Molecular and morphological description of *Pestalotiopsis hainanensis* sp. nov., a new endophyte from a tropical region of China. *Fungal Divers* 24: 23-36.
- Maharachchikumbura S, Guo L, Chukcatirote E, Bahkali A, Hyde K, 2011. *Pestalotiopsis* - morphology, phylogeny, biochemistry and diversity. *Fungal Divers* 50: 167-187. <https://doi.org/10.1007/s13225-011-0125-x>
- Maharachchikumbura SSN, Hyde KD, Groenewald JZ, Xu J, Crous PW, 2014. *Pestalotiopsis* revisited. *Stud Mycol* 79: 121-186. <https://doi.org/10.1016/j.simyco.2014.09.005>
- O'Donnell K, Cigelnik E, 1997. Two divergent intragenomic rDNA ITS2 types within a monophyletic lineage of the fungus *Fusarium* are nonorthologous. *Mol Phylogenet Evol* 7: 103-116. <https://doi.org/10.1006/mpev.1996.0376>
- O'Donnell K, Kistler HC, Cigelnik E, Ploetz RC, 1998. Multiple evolutionary origins of the fungus causing Panama disease of banana: concordant evidence from nuclear and mitochondrial gene genealogies. *Proc Natl Acad Sci of USA* 95: 2044-2049. <https://doi.org/10.1073/pnas.95.5.2044>
- Sezer A, Dolar FS, 2015. Determination of *Pestalotiopsis* sp. causing disease on fruit clusters in hazelnut growing areas of Ordu, Giresun and Trabzon provinces in Turkey. *Agriculture & Forestry* 61: 183-188. <https://doi.org/10.17707/agricultforest.61.1.23>
- Sutton BC, 1980. The Coelomycetes. Fungi imperfecti with pycnidia, acervuli and stromata. Commonwealth Mycological Institute, Kew, UK. 696 pp.
- Tamura K, Stecher G, Peterson D, Filipiński A, Kumar S, 2013. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. *Mol Biol Evol* 30: 2725-2729. <https://doi.org/10.1093/molbev/mst197>
- Wei JG, Xu T, Guo LD, Liu A-R, Zhang Y, Pan XH, 2007. Endophytic *Pestalotiopsis* species associated with plants of *Podocarpaceae*, *Theaceae* and *Taxaceae* in southern China. *Fungal Divers* 24: 55-74.
- White TJ, Bruns T, Lee S, Taylor J, 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: PCR protocols: a guide to methods and applications; Innis MA, Gelfand DH, Sninsky JJ, White TJ (eds), pp: 315-322. Acad. Press, NY, USA. <https://doi.org/10.1016/b978-0-12-372180-8.50042-1>