

# Alternaria leaf spot on *Paeonia peregrina* and *Paeonia tenuifolia* in Serbia

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*Paeonia peregrina* Mill. and *Paeonia tenuifolia* L. are endangered species of herbaceous plants with high ornamental and medicinal values. There are a few natural habitats in Serbia where these plants spontaneously grow: Krivi Vir, Skrobnica, Golina, Pirot, Deliblato Sands, and Sokobanja, in the municipality of Knjaževac. In July 2021, the symptom of leaf spots was observed on *P. peregrina*, while spots on leaves, stems, and lower branches on *P. tenuifolia* in the localities of Pirot and Deliblato Sands. Disease incidence was estimated to 32% in Pirot and 25-35% in Deliblato Sands. Therefore, the aim of the study was to identify the causal agent of leaf spot on *P. peregrina* and *P. tenuifolia* in Serbia by molecular identification and characterization, and morphological characterization. For molecular identification and characterization, nuclear ribosomal internal transcribed spacer (ITS) region, partial translation elongation factor 1-alpha (*EF-1α*), beta-tubulin (*TUB2*) and histone 3 (*H3*) genes were amplified using primer pairs ITS1/ITS4, EF1-728F/EF1-986R, T1/Bt2b, and H3-1a/H3-1b, respectively. Morphological characterization of the representative isolates was done on potato dextrose agar (PDA) and potato carrot agar (PCA) media at 22 °C under an 8 h light:16 h dark regime. Based on the molecular and morphological characteristics of the obtained isolates, *Alternaria alternata* was identified as the causal agent of leaf spot on *P. peregrina* and *P. tenuifolia*. In Serbia, to the best of our knowledge, this is the first report of *A. alternata* causing a leaf spot of *P. tenuifolia*.

**Keywords:** *Alternaria alternata*; herbaceous peonies; molecular identification; molecular characterization; morphological characterization

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## ABBREVIATIONS

PDA – potato dextrose agar

PCA – potato carrot agar

ITS – nuclear ribosomal internal transcribed spacer

*EF-1α* – translation elongation factor 1-alpha gene

*TUB2* – beta-tubulin gene

*H3* – histone 3 gene

NaClO – 1% sodium hypochlorite solution

ML – Maximum likelihood tree

UV – ultraviolet

CTAB – cetyltrimethylammonium bromide

NCBI – the National Center for Biotechnology Information

## 1. INTRODUCTION

There are 34 species in the genus *Paeonia*, which are grouped into two subgenera (Moutan and herbaceous peonies) and seven sections (Hong, 2021). Herbaceous peonies make up one of five sections that comprise the subgenus *Paeonia* (Prijić et al., 2023). Temperate Asia, southern Europe, and western North America are habitats for *Paeonia peregrina*, known as the Balkan or Kosovo peony, and *Paeonia tenuifolia*, known as the fern leaf or steppy peony (The Plant List, 2023). In Serbia, their collection from nature is protected by law and requires permission from the Ministry of Environmental Protection of the Republic of Serbia.

*Alternaria* spp. can be found as saprophytic, endophytic, and pathogenic species in agricultural crops and products, soil, and organic matter (Neergaard, 1945; Pavón Moreno et al.,

2012; Polizzotto et al., 2012). The species are located in semi-arid and humid areas, and can affect leaves, stems, flowers, and fruits of several plant species (Deshpande, 2002). Also, *Alternaria* spp. are well-known as post-harvest pathogens that cause disease in over 100 host plants (Thomma, 2003). The species can survive on infected crop debris, seeds, and weeds in the form of mycelia or conidia, and can be transmitted by insects, wind, rain, irrigation, etc. Conidia are carried by air currents to the aerial parts of plants throughout the growing season (Battilani et al., 2009; Laemmlen, 2002). *Alternaria* spores are one of the most important airborne allergens (Thomma, 2003). The production of melanin, particularly in the spores, and the production of host-specific toxins in the case of pathogenic species are two distinguishing characteristics of *Alternaria* species (Thomma, 2003). The species of the genus, host plant, and combination of right temperature and humidity are important factors in the external environment for the development of infection. The spores penetrate the host's tissue through stomata, cuticle, or wounds. The first symptoms of infection appear after 2–3 days on the leaves and stems of the host plant. Black necrotic lesions surrounded by chlorotic halos are characteristic of leaf spots. Leaf spot can lead to reduced leafy crops. Additionally, it may cause the host to abscise leaves, thus lowering photosynthetic potential and crop yield indirectly (Armitage, 2013; Battilani et al., 2009).

Despite the increase in peony production and consumption, available information on peony diseases and their treatment is limited. *Alternaria* spp. has been found on herbaceous and tree peonies in Canada (Coulson, 1923), the United States (Anonymous, 1960), China (Zhang, 2003), Korea (Cho and Shin, 2004), Bulgaria (Bobev, 2009), and the Netherlands (Andersen et al., 2009). The Texas Plant Disease Handbook (Anonymous, n.d.) describes the leaf spots as irregularly shaped, purplish-brown to reddish-brown. Later, the leaves may turn yellow and fall off (Texas Plant Disease Handbook). Coulson (1923) described *Alternaria* spp. as causing yellow leaf spots, irregularly shaped with a clearly defined, dark margin. Leaf spots caused by *Alternaria suffruticosae*, *A. suffruticosicola*, and *A. tenuissima* were circular or irregularly shaped, brown to black (Zhang et al., 2008). Cheng et al. (2018) recorded diversity of fungi of *Aspergillus*, *Alternaria*, and *Penicillium* on the leaf, stem, and root of *Paeonia lactiflora* Pallas. *Alternaria* sp., especially *A. alternata*, showed the maximum frequency of colonization and isolation (10.95% and 31.68%), similar to the results of previous reports (Jennings et al., 2002; Lin et al., 2007; Xiao et al., 2013). In addition, *A. tenuissima* was identified as the causal agent of red leaf spot disease on *P. lactiflora* in China (Sun and Huang, 2017). The first symptoms on the leaves appeared as small, circular, reddish to dark brown, necrotic spots that gradually enlarged and became irregular. The spots grew together, and the leaves withered, dried up, and defoliated. This disease destroys the aesthetic value of the Chinese peony and potentially reduces the yield and quality of its medicinal properties, affecting the economic importance of this plant.

The identification of *Alternaria* species is difficult and at present, molecular and phylogenetic analyses are essential for recognizing species (Lawrence et al., 2015; Woudenberg et al., 2015). Different loci have been used for molecular identification and characterization, among which are the nuclear ribosomal internal transcribed spacer (ITS) region (Lawrence et al., 2015; Matic et al., 2019; Woudenberg et al., 2015), partial translation elongation factor 1-alpha (*EF-1 $\alpha$* ) (Lawrence et al., 2015; Siciliano et al., 2018; Woudenberg et al., 2015), beta-tubulin (*TUB2*) (Lawrence et al., 2015; Matic et al., 2019; Peever et al., 2004; Siciliano et al., 2018), and histone 3 gene (*H3*) (Garibaldi et al., 2018a; Matic et al., 2019; Wang et al.,

2019; Zhang et al., 2020; Zhao et al., 2016).

In Serbia, *A. alternata* has been reported on *P. peregrina* originating from the location of Bogovo Guvno (Mikić et al., 2023), while there isn't information about this pathogen on *P. tenuifolia* (Farr and Rossman, 2023).

## 2. MATERIALS AND METHODS

### 2.1. Plant collection and fungal isolation

In July 2021, leaf spot were observed on *P. peregrina* and *P. tenuifolia* in the natural habitats in Piroć (43° 07' N; 22° 26' E 666) and Deliblato Sands (44° 57' N; 21° 03' E 167) areas, Serbia. The spots appeared on leaves and sporadically on stems of *P. peregrina*, while identical spots, white mycelia and rot appeared on stems and lower branches of *P. tenuifolia*. The percentage of infected plants at a given locality were calculated by counting the numbers of healthy and symptomatic plants separately. In total, 197 and 100 individual plants of the single populations of *P. peregrina* and *P. tenuifolia* were found on about 0.5 ha. Sixty-three plants of *P. peregrina* and 50 plants of *P. tenuifolia* were symptomatic.

Symptomatic plants were transplanted into pots and transferred to the laboratory. Isolation of the pathogen was done from symptomatic leaves and stems. Leaf fragments (5x5 mm) of five symptomatic plants and stem fragments (5x5 mm) of ten symptomatic plants were cut from the margins of the lesions and the healthy tissue. Leaf and stems fragments were washed, surface disinfected with 75% ethanol for 1 min, 1% sodium hypochlorite solution (NaClO) for 2 min, rinsed three times in distilled water, dried on sterilized filter paper, and placed on potato dextrose agar (PDA) in Petri dishes (90 mm) (Sun et al., 2022). After seven days of incubation at 25 °C, developed colony fragments were transferred to PDA to obtain pure cultures.

### 2.2. Pathogenicity test

The pathogenicity was tested by artificial inoculation leaves of plant origin. Two wounded and two non-wounded detached leaves of healthy *P. peregrina* obtained from the natural habitat were used. The leaves were placed onto the filter paper moistened in Petri dishes, wounded with a sterile needle, and mycelial fragments (5 mm) of 7-day-old culture on PDA were placed on the adaxial surface of the leaves. Control leaves were treated the same way, but sterile PDA fragments were used (Fang et al., 2023). Petri dishes with inoculated leaves and control dishes were incubated at 21±2 °C under natural daylight conditions. The test was repeated once. Re-isolation of the pathogen was performed as described above.

### 2.3. DNA extraction, PCR amplification and amplicon sequencing

DNA extraction was performed by the cetyltrimethylammonium bromide (CTAB) protocol proposed by Day and Shattock (1997) from 7-day-old cultures grown on PDA. For molecular identification and characterization, the nuclear ribosomal internal transcribed spacer (ITS) region, partial translation elongation factor 1-alpha (*EF-1 $\alpha$* ), beta-tubulin (*TUB2*), and histone 3 gene (*H3*) were amplified using primer pairs ITS1/ITS4 (White et al., 1990), EF1-728F/EF1-986R (Carbone and Kohn, 1999), T1/Bt2b (Glass and Donaldson, 1995; O'Donnell and Cigelnik, 1997), and H3-1a/H3-1b (Glass and Donaldson, 1995), respectively. Each 25  $\mu$ L PCR mix contained 1  $\mu$ L of template DNA, 1xPCR Master Mix (Thermo Scientific, Vilnius, Lithuania) and 0.4  $\mu$ M of each primer. Conditions for amplification of ITS were: initial denaturation at 95 °C for 2 min, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s and elongation at 72 °C for 1 min. Conditions

for *EF-1 $\alpha$*  and *TUB2* amplifications were: initial denaturation at 95 °C for 8 min, followed by 35 cycles of denaturation at 95 °C for 15 s, annealing at 55 °C for 20 s and elongation at 72 °C for 1 min. Conditions for *H3* amplification were: initial denaturation at 94 °C for 2 min, denaturation at 94 °C for 60 s, annealing at 58 °C for 1 min and elongation 72 °C for 1 min; repeat protocol for 32 cycles. Final elongation occurred for 10 min at 72 °C. PCR products (5  $\mu$ L) were observed in 1.2% agarose gel, stained in ethidium bromide, and visualized with UV transilluminator. Amplified products were purified and commercially sequenced (Macrogen Inc., Seoul, South Korea) using the same reverse primers as for amplification. The obtained sequences were assembled using Pregap4 from the Staden programme package (Staden et al., 1998). For species identification, the obtained sequences were compared with those publicly available in NCBI's GenBank database using MegaBLAST (<http://www.ncbi.nlm.nih.gov/>). Sequences for each locus were aligned using Clustal X (Thompson, 1997), under MEGA version 7 (Kumar et al., 2016). The sequences of isolate were deposited in the National Center for Biotechnology Information (NCBI) GenBank.

## 2.4. Phylogenetic analyses

Evolutionary history was inferred based on combined analyses of the two (ITS and *EF-1 $\alpha$* ) loci of two representative isolates obtained in this study, reference isolates of *Alternaria* spp. and *Alternaria alternantherae* CBS 124392 as an outgroup, using Maximum likelihood (ML) method (MEGA X) (Kumar et al., 2018). Sequences used for phylogeny are presented in Table 1. For the ML, the best nucleotide substitution model was determined using the "find best model" option in MEGA X. For tree inference, the nearest-neighbor-interchange heuristic method was employed, with the initial trees being automatically generated by Neighbor-Join and BioN Jalgorithms. To estimate the statistical significance of the inferred clades, each tree was bootstrapped 1,000 times.

## 2.5. Morphological characterization

Macromorphological characteristics of two representative isolates (colony color, texture, and growth rate) were observed on PDA and PCA 40 cm below cool white fluorescent illumination at 23 °C for seven days (Blagojević, 2020; Simmons, 2007). The colony diameter was measured every day in two diagonal directions. Mean values were obtained for every day from three replicates with three plates each. The growth rate was calculated and expressed as the diameter of the colony after seven days. Micromorphological characteristics of selected isolates (length, width, shape, size, color of conidia, conidiophores, dimensions of the conical beak, and sporulation pattern) were observed on seven-day-old cultures grown on PCA at 22 °C under cool white fluorescent illumination and an 8/16 h light/dark regime (Simmons, 2007). Microscopic preparations were prepared in lactic acid and viewed under a microscope at 10x and 20x magnifications (Olympus CX43, Olympus, Hamburg, Germany). Photographs of conidiophore and conidia and measurements were obtained using the camera Axiocam ERc 5s, Zeiss, and software ZEN 2 (blue edition), Jena, Germany.

## 3. RESULTS AND DISCUSSION

### 3.1. Symptoms, isolates and pathogenicity

Circular or irregular, light to dark brown spots with defined dark margins appeared on the leaves and sporadically stems of *P. peregrina* during the stage of follicle formation, whereas on *P. tenuifolia* identical spots first appeared from the base at the bud stage and finally white mycelia and rot appeared at

the base of the stems and lower branches at the seed maturity stage (Figure 1). The spread of spots on leaves causing the plants to wilt and die. Disease incidences in the areas of Piroto and Deliblato Sands were 32% and 25-35%, respectively.

Following a seven-day incubation, five isolates of *P. peregrina* and five isolates of *P. tenuifolia* were obtained from symptomatic plants. In the pathogenicity test, on wound-inoculated leaves, leaf necrosis and mycelia appeared on the adaxial surface of the leaves, as well as necrosis along the leaf nerve, while non-wounded leaves and control leaves remained symptomless (Figure 2). The pathogen was reisolated from inoculated leaves and showed identical morphological characteristics as the original isolate, thus completing Koch's postulates. From each host, a representative isolate with proven pathogenicity was selected for further molecular and morphological characterization (isolate PpPili from *P. peregrina* and isolate SBDPst from *P. tenuifolia*).

### 3.2. Molecular identification

Following the amplification of ITS, *EF-1 $\alpha$* , *TUB2* and *H3*, PCR products of expected size (600, 300, 600 and 400 bp, respectively) were obtained for the two selected isolates. Comparison of the obtained sequences showed that isolates PpPili and SBDPst were 100% similar (identical) in ITS and *EF-1 $\alpha$* , but differed in 1 nt in *TUB2* and 1 nt in *H3* gene regions. BLAST analysis showed that both isolates were 100% similar with *A. alternata* ex-type CBS 916.96, established by Woudenberg et al. (2013) and Woudenberg et al. (2015), in ITS (AF347031) and *EF-1 $\alpha$*  (KC584634). Also, sequences of both regions of obtained isolates were identical with several corresponding sequences of *A. alternata* isolates (e.g. CBS 115188, CBS 113013, CBS 117143, CBS 119115, CBS 918.96, IT44, IT61) (Matić et al., 2020; Woudenberg et al., 2015). In *TUB2*, isolate PpPili was 100% similar with *A. alternata* ex-type CBS 916.96 (=EGS 34.016; MF070244) (Siciliano et al., 2018) and other published *A. alternata* sequences of *TUB2* region (Garibaldi et al., 2019; 2020), while isolate SBDPst was 99% similar with *A. alternata* ex-type CBS 916.96 and 100% similar with CBS 918.96 and CBS 115152 (Siciliano et al., 2018). In *H3*, isolate PpPili was 100% similar with *A. alternata* isolate IT44 (MG182429) (Garibaldi et al., 2018a), while isolate SBDPst was 100% similar with *A. alternata* isolate IT61 (MF997593) (Garibaldi et al., 2018b). Based on ITS, *EF-1 $\alpha$* , *TUB2* and *H3*, obtained isolates were identified as *A. alternata* which is in accordance with Woudenberg et al. (2015), Garibaldi et al. (2019; 2020; 2018a), Siciliano et al. (2018), and Matić et al. (2020).

### 3.3. Phylogeny

The phylogenetic analysis, based on Maximum likelihood method and Kimura 2-parameter model, of concatenated ITS and *EF-1 $\alpha$*  sequences of *Alternaria* spp. grouped two representative isolates, PpPili and SBDPst, with reference isolates of *A. alternata* (Figure 3), confirming the identification.

### 3.4. Macromorphology and micromorphology

After seven days, isolate PpPili formed circular, cottony, light brown to brown colonies, with a white border on PDA (diameter 60.3 mm), and circular, grayish-brown colonies on PCA (diameter 83.7 mm). Conidia were brown, ovoid, or ellipsoid with three to four transverse and one to three longitudinal septa (16.01-27.74  $\times$  5.50-14.41  $\mu$ m; average 20.83  $\times$  9.41  $\mu$ m, n = 50). Conidia formed in chains of 2-7, mostly 5-6. Dimensions of the conical beak were 0.70-5.27  $\times$  1.53-4.57  $\mu$ m; average 2.55  $\times$  2.68  $\mu$ m, n = 50. Conidiophores were simple, dark brown, and unbranched (27.86-187.31  $\times$  1.91-7.73  $\mu$ m; average 59.88  $\times$  4.39  $\mu$ m, n = 50) (Figure 4).

**Table 1.** Fungal isolates used for phylogeny.

Isolate	Host/Location	GenBank accession numbers		Reference
		ITS	EF-1 $\alpha$	
<i>Alternaria alternata</i>				
PpPili	<i>Paeonia peregrina</i> /Pirot	OP562152	OP643688	This study
SBDPst	<i>Paeonia tenuifolia</i> /Deliblatska peščara	nd	nd	This study
CBS 916.96 <sup>T</sup>	<i>Arachis hypogaea</i> , India	AF347031	KC584634	(Woudenberg et al., 2015)
CBS 115188	<i>Citrus clementina</i> , South Africa	KP124349	KP125125	(Woudenberg et al., 2015)
CBS 113013	<i>Malus domestica</i> , South Africa	KP124341	KP125117	(Woudenberg et al., 2015)
CBS 117143	<i>Capsicum annuum</i> , Italy	KP124355	KP125131	(Woudenberg et al., 2015)
CBS 119115	<i>Prunus sp.</i> , Greece	KP124360	KP125136	(Woudenberg et al., 2015)
CBS 918.96 (ex <i>A. tenuissima</i> <sup>R</sup> )	<i>Dianthus chinensis</i> , UK	AF347032	KC584693	(Woudenberg et al., 2015)
CBS 115152	<i>Psychotria serpens</i> , China	KP124348	KP125124	(Woudenberg et al., 2015)
REIS 68	<i>Ceratostigma willmottianum</i> , Italy	MN565914	MN627329	(Matić et al., 2020)
IT44	<i>Echinacea purpurea</i> , Italy	MG182428	MN627319	(Matić et al., 2020)
IT61	<i>Mentha × piperita</i> , Italy	MF997592	MN627337	(Matić et al., 2020)
<i>Alternaria alstromeriae</i>				
CBS 118809 <sup>T</sup>	<i>Alstroemeria sp.</i> , Australia	KP124297	KP125072	(Woudenberg et al., 2015)
CBS 118808 <sup>R</sup>	<i>Alstroemeria sp.</i> , USA	KP124296	KP125071	(Woudenberg et al., 2015)
<i>Alternaria alternantherae</i>				
CBS 124392	<i>Solanum melongena</i> , China	KC584179	KC584633	(Woudenberg et al., 2015)
<i>Alternaria arborescens</i>				
CBS 102605 <sup>T</sup>	<i>Solanum lycopersicum</i> , USA	AF347033	KC584636	(Woudenberg et al., 2015)
CBS 109730	<i>Solanum lycopersicum</i> , USA	KP124399	KP125177	(Woudenberg et al., 2015)
<i>Alternaria gaisen</i>				
CBS 632.93 <sup>R</sup>	<i>Pyrus pyrifolia</i> , Japan	KC584197	KC584658	(Woudenberg et al., 2015)
CBS 118488 <sup>R</sup>	<i>Pyrus pyrifolia</i> , Japan	KP124427	KP125206	(Woudenberg et al., 2015)
<i>Alternaria longipes</i>				
CBS 540.94 <sup>R</sup>	<i>Nicotiana tabacum</i> , USA	AY278835	KC584667	(Woudenberg et al., 2015)
CBS 121332 <sup>R</sup>	<i>Nicotiana tabacum</i> , USA	KP124443	KP125222	(Woudenberg et al., 2015)

Legend: T - ex-type isolate; R - representative isolate; nd - not deposited.



Fig. 1. Symptoms of leaf spot on natural infected plants of *Paeonia peregrina* (left) and *Paeonia tenuifolia* (right).

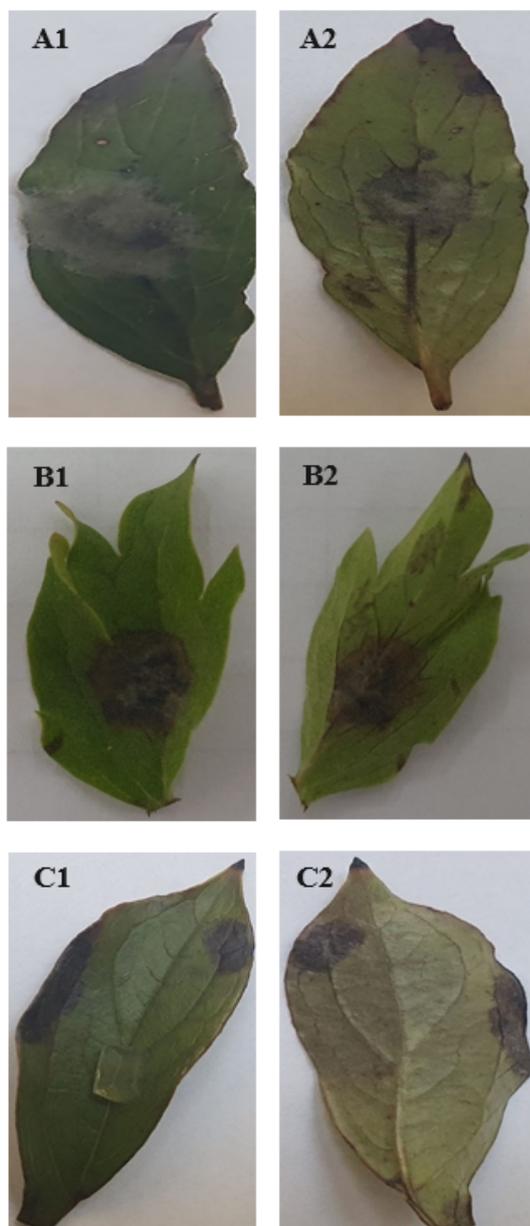


Fig. 2. Pathogenicity of obtained isolates on detached leaves of *Paeonia peregrina* (A and B) - the inoculated leaves with mycelial fragments (surface A1, B1; reverse A2, B2) and the control leaves of *Paeonia peregrina* with sterile PDA (surface C1 and reverse C2).

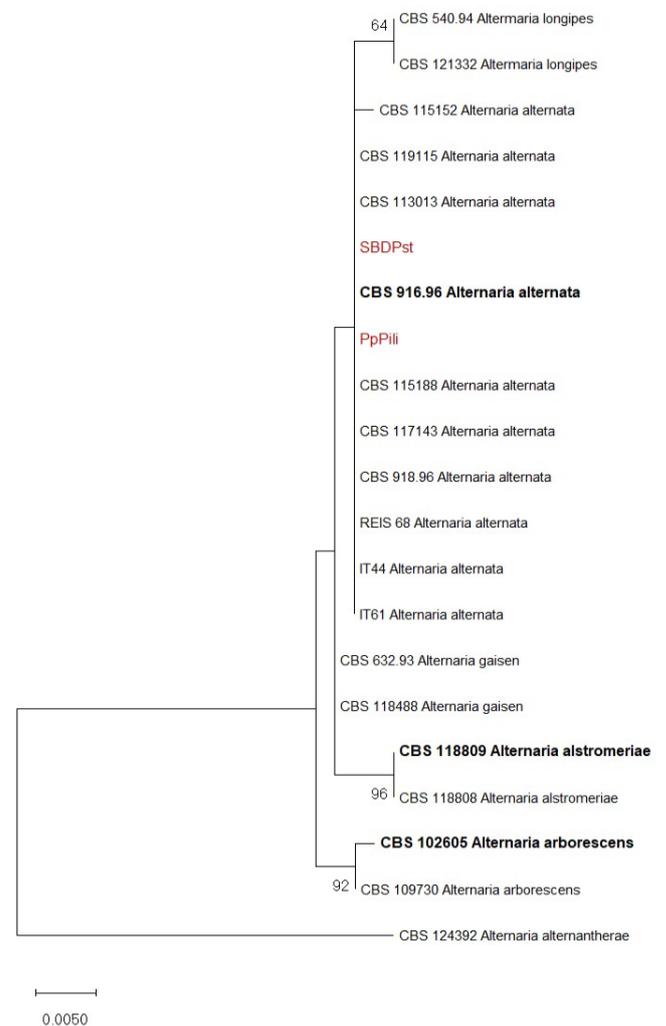
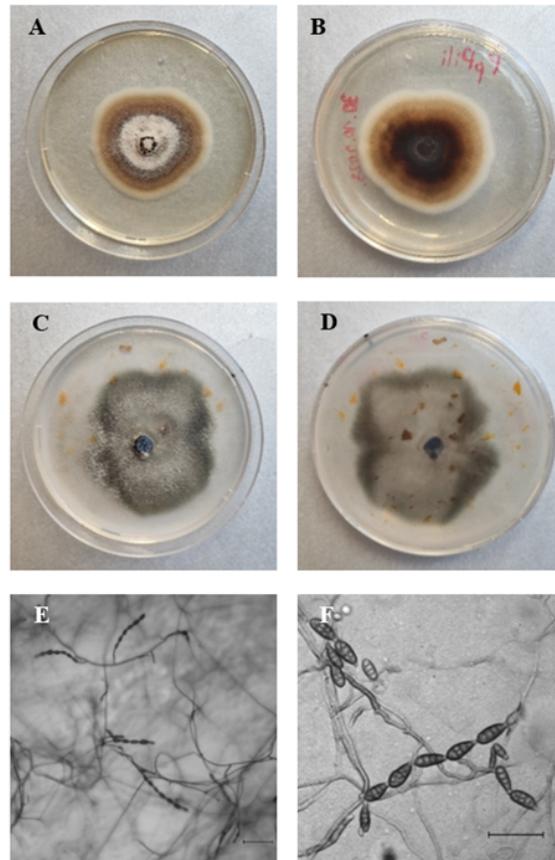


Fig. 3. Phylogenetic tree obtained from the maximum likelihood analysis of concatenated ITS and *EF-1 $\alpha$*  sequences of *Alternaria* spp. Numbers on the branches represent bootstrap values obtained for 1,000 replicates (only values above 60% are shown). Isolates obtained in this study are shown in red and ex-type isolates are shown in bold. The tree was rooted to *Alternaria alternantherae*. The scale bar represents the number of nucleotide substitutions per site.

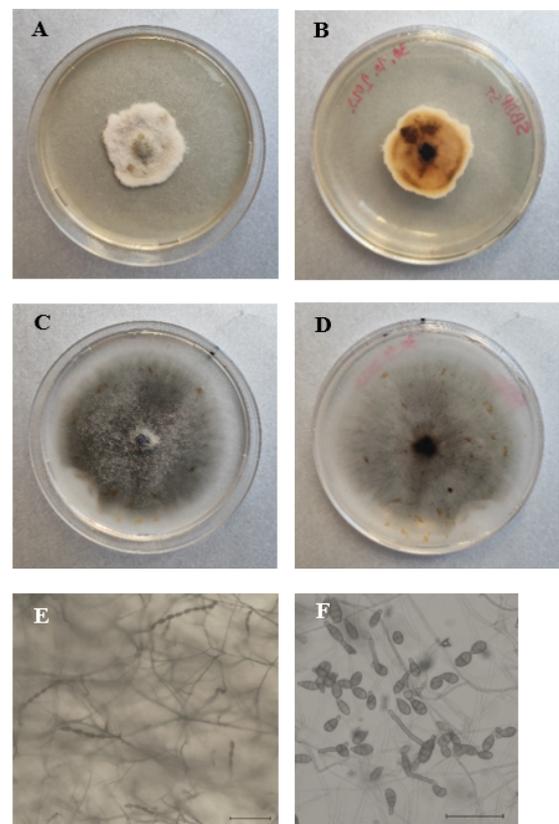


**Fig. 4.** Morphological characteristics of isolate PpPili from *Paeonia peregrina*: 7-day-old-culture on PDA (surface A, reverse B); and 7-day-old-culture on PCA (surface C, reverse D), sporulation pattern (E), conidia (F). In (E) scale bar 100  $\mu\text{m}$ , while in (F) scale bar 20  $\mu\text{m}$ .

After seven days, isolate SBDPst formed circular, cottony, white, or light brown colonies on PDA (diameter 45.3 mm). On PCA, colonies were circular, grayish-brown (diameter 85.5 mm), and brown, ovoid or ellipsoid conidia formed in chains of 3-10, mostly 3-8. Conidia had zero to three longitudinal septa, and were 13.56-24.27  $\times$  5.7-14.3  $\mu\text{m}$  in size (average 18.93  $\times$  9.67  $\mu\text{m}$ ,  $n = 50$ ), with a conical beak 1.00-5.52 in long (average 2.82)  $\times$  0.65-3.34 in wide (average 1.73)  $\mu\text{m}$  ( $n = 50$ ). Conidiophores were simple, dark brown, and unbranched, 12.64-53.15  $\times$  1.29-5.97  $\mu\text{m}$  in size (average 25.98-2.36,  $n = 50$ ) (Figure 5).

Observed morphological characteristics corresponded to the genus *Alternaria* (Simmons, 2007). According to the sporulation pattern the isolates could be identified as an *A. tenuissima* morphospecies, but with regards to a comprehensive revision by Woudenberg et al. (2015) and recently, Dettman and Eggerston (2021) which show lack of phylogenetic divergence in support of morphological differences and the fact that morphospecies are synonymized with *A. alternata*, the isolates were identified as *A. alternata*.

In this study, *Alternaria* leaf spot was described on two herbaceous peony species *P. peregrina* and *P. tenuifolia* in Serbia. Isolated fungi whose pathogenicity was proven, were identified as *A. alternata*. In Serbia, there has been a report of *Alternaria* leaf spot affecting wild herbaceous peony (Mikić et al., 2023), but other diseases have not been identified in these plant species. In the United States, Garfinkel and Chastagner (2019) described *Alternaria* leaf spot and other diseases on the herbaceous peony species *P. lactiflora* Pallas.. Also, Park et al. (1997) examined the effect of different pathogenic fungi (*Alternaria* sp., *Erysiphe aquilegiae*, and *Cronartium flaccidum*) on the growth and root yield of *P. lactiflora*; brown, dark-brown to black spots were observed on leaves, and finally the with-



**Fig. 5.** Morphological characteristics of isolate SBDPst from *Paeonia tenuifolia*: 7-day-old-culture on PDA (surface A, reverse B); and 7-day-old-culture on PCA (surface C, reverse D), sporulation pattern (E), conidia (F). In (E) scale bar 100  $\mu\text{m}$ , while in (F) scale bar 50  $\mu\text{m}$ .

ering of the above-ground part of the plant. The symptoms of *A. alternata* on tree peonies described by Shi et al. (2015) were similar with our findings of *A. alternata* on herbaceous *P. peregrina*. Zhang et al. (2008) reported *A. suffruticosae*, *A. suffruticosicola*, and *A. tenuissima* on woody peony – *Paeonia suffruticosa* cultivar Andrews.

#### 4. CONCLUSION

*Alternaria* spp. are present in humid and semi-arid regions, and can infect a wide range of plant crops through leaves, stems, flowers, and fruits. The fungus was isolated from infected plants collected at the localities Pirot and Deliblato Sands, and identified as *A. alternata* based on molecular characteristics. The ML phylogenetic tree of concatenated ITS and *EF-1 $\alpha$*  sequences, and ITS, *EF-1 $\alpha$* , and *TUB2* sequences of *Alternaria* spp., showed that two isolates obtained in this study clustered among *A. alternata*. After the pathogenicity test, the pathogen was reisolated from inoculated leaves and showed identical morphological characteristics as the original isolate, thus completing Koch's postulates. Based on the observed morphological characteristics and sporulation pattern the isolates can be identified as *A. tenuissima* morphospecies, but this morphospecies in recent taxonomy has been synonymized with *A. alternata*. In Serbia, to the best of our knowledge, this is the first report of *A. alternata* infecting herbaceous wild peony species. Further investigations of *A. alternata* should elucidate the importance of the disease it causes on peonies and propose possible control measures to preserve this important, rare, and protected plant species in its natural habitats in Serbia.

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#### CONFLICT OF INTEREST

The authors declare that they have no financial and commercial conflicts of interest.

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