MORPHOLOGICAL AND MOLECULAR
CHARACTERIZATION OF BOTRYOSPHAERIACEAE
SPECIES ASSOCIATED WITH DIEBACK
AND GUMMOSIS ON PLUM TREES IN TURKEY

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Abstract

Plum (*Prunus salicina*) is among the most important and common fruit species in Turkey. Worldwide, Botryosphaeriaceae family, including significant plant pathogens causes disease in several woody plants including plums. Botryosphaeriaceae-induced dieback and gummosis symptoms have recently been encountered in plum orchards in Adana and Mersin provinces of Turkey. In the present study, morphological and molecular characterization of Botryosphaeriaceae isolates were performed and the obtained isolates were identified as *Diplodia seriata*, *Neofusicoccum parvum* and *Lasiodiplodia pseudotheobromae*. Pathogenicity tests revealed the virulence of all three species. To the best of our knowledge, this is the first report of *D. seriata*, *N. parvum* and *L. pseudotheobromae* causing disease on plums in Turkey.

Key words: Botryosphaeriaceae, β -tubulin, ITS, plum, symptoms

Introduction. Plum (*Prunus salicina*), largely grown in Europe, Asia and America, is among the most important fruit species [1]. In 2019, about 0.31 million tons of plum were produced from 21058 ha land area in Turkey, which was placed into the first 10 countries of world plum production [2]. Two plum species

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are largely cultured in Turkey and Japanese plums are commonly produced in southern regions of Turkey.

Botryosphaeriaceae species are cosmopolite species which cause fruit rots, stem or trunk and branch cankers, gummosis and diebacks and reduce both the yields and tree life [3]. Number of studies about the diseases on fruit trees caused by Botryosphaeriaceae species is quite limited in Turkey, thus lack of knowledge about gummosis, branch cankers and diebacks on plum trees makes the assessment of severity and significance of this disease difficult in Turkey. Therefore, objectives of the present study were set as follows: (i) to identify Botryosphaeriaceae species related to gummosis and dieback symptoms in plum trees with the use of morphological and molecular methods, and (ii), to determine virulence of identified Botryosphaeriaceae species.

Materials and methods. Sample collection and fungal isolation. Dieback and gummosis symptoms on stem and branches were monitored in 30 plum orchards in Adana (19) and Mersin (11) provinces of Turkey throughout the years 2015 and 2016. From these orchards, 86 infected plant tissue samples were collected. Fungal isolation was performed with the use of the method specified in ENDES et al. [4] and Botryosphaeriaceae isolates were purified through single spore isolations beforehand to use them in molecular and morphological characterization and pathogenicity tests [5].

Molecular identification and phylogenetic analyses. Total genomic DNA extraction and PCR of Botryosphaeriaceae isolates were performed in accordance with the protocol specified by MCDONALD and ESKALEN [6]. ITS4/ITS5 and Bt2a/Bt2b primers were used for amplification of ITS (ITS1, ITS2 and 5.8S) regions and a part of β -tubulin (Bt) gene. Resultant PCR products were double-stranded synthesized in Genoks company (Ankara, Turkey). Sequences were cleaned to the peaking parts using BioEdit software (version 7.2).

Sequences of Botryosphaeriaceae isolates were compared with NCBI GenBank sequences of closely related species selected with the use of Blast software. *Diatrypella verruciformis* (UCROK856) was added as an out-group. CMW9081 (*N. parvum*), CBS 112555 (*D. seriata*) and CBS 116459 (*L. pseudotheobromae*) isolates were ex-type specimens obtained from NCBI GenBank. Clustal W sequence alignment and phylogenetic analyses [Maximum Parsimony (MP)] were performed using MEGA 7. Maximum parsimony for all analyses was performed using the heuristic search option (branch swapping NNI). Bootstrap values were evaluated using 1000 replicates to test branch strength. Tree length, consistency index (CI), retention index (RI), rescaled consistency index (RC), and homoplasy index (HI) were also recorded for all analyses.

Morphological characterization. Isolates were incubated on potato dextrose agar (PDA) at 25 °C and 12-hour photoperiods (fluorescent light/dark) for 2–4 weeks. Within the scope of this incubation period, colonial (colony colour and aerial mycelium status) and conidial (conidia size, colour, shape, septate sta-

tus) characteristics of the isolates were respectively determined with the use of macroscopic and light microscope (Olympus BX51). For each isolate, length and width measurements were performed on 50 conidia, then average and standard deviations were calculated.

Pathogenicity tests. Pathogenicity tests of all Botryosphaeriaceae isolates (Table 1) were conducted in accordance with the method described by Endes et al. [4]. Variance analysis (ANOVA) for vascular colouration lengths on xylems of branches was conducted with the use of SPSS (IBM SPSS v20) software. Means were compared with the use of Least Significant Difference (LSD) multiple comparison test at 5% significance level [7].

Results and discussion. It was observed in infected plum orchards that leaves did not come down and remained on branches at the beginning of spring (Fig. 1a). Gummosis was encountered on branches especially in summer (Fig. 1b) and branches or the entire tree dried out later. Bark tissue colour gets darkened with the severity of the infection. In cross-sectional cuts of infected branches, V-shaped (Fig. 1c) or irregular-shaped (Fig. 1d) necroses were encountered on xylems [3,4,8]. Similar to the present findings, DAMM et al. [3] indicated that Botryosphaeriaceae species resulted in V-shaped necrosis in xylems of infected plum trees in South Africa and also reported wilt, dieback and gummosis in infected trees. Present findings are quite similar to the results of EZRA et al. [8] who worked on defoliating fruit species in Israel.

At the end of the isolation processes, a total of 15 Botryosphaeriaceae isolates were obtained; isolates, ITS and BT gene sequences were stored in NCBI GenBank database and accession numbers were taken (Table 1). ITS and β -tubulin sequences of *D. seriata*, *L. pseudotheobromae* and *N. parvum* from plum trees in Turkey were aligned with GenBank sequences of Botryosphaeriaceae spp. from different hosts and other countries (Fig. 2). The topology of the trees of ITS and β -tubulin gene regions was consistent without major conflict between them, indicating that the ITS and β -tubulin datasets could be combined in a single phylogenetic analysis. The combined dataset consisted of 1116 characters, of which 735 were constant, 335 were parsimony uninformative, and 138 were parsimony informative. The maximum parsimony analyses yielded one most parsimonious tree (length = 304, CI = 0.976, RI = 0.987, RC = 0.965, and HI = 0.024; Fig. 2). This tree contained two major clades, the first of which (100% bootstrap support) corresponded to *N. parvum* (AKER60, AYER39, AYER38, CMW9081, PL_NpSV, UCR1566). The second major clade (99% bootstrap support) resolved into two subclades.

The first subclade (100% bootstrap support) corresponded to *D. seriata* (ACER58, ASER6.1, ASER6.2, ASER6.3, ASER6.4, ASER6.5, ASER6.6, AYER241, ASER28, ASER29, CBS119049, CBS 112555, NeR1a-AYug). The second subclade (100% bootstrap support) corresponded to *L. pseudotheobromae* (MEYER129, MEYER130, CMW28517, CBS 116459, NeR1b-AYug).

T a b l e 1
Isolates of Botryosphaeriaceae studied

Species ^a	Isolates	GeneBank number		Vascular discolouration length (mm) ^b ± SE	Gum exudation ^c	Reisolation ^d
		ITS	β-tubulin			
<i>Lasiodiplodia pseudotheobromae</i>	MEYER130	KX244815	MG970286	109.0 ± 1.3 a	+++	10
<i>L. pseudotheobromae</i>	MEYER129	KX244814	MG970285	106.3 ± 1.2 a	+++	10
<i>Neofusicoccum parvum</i>	AKER60	KX244806	MG970282	92.7 ± 1.0 b	++	9
<i>N. parvum</i>	AYER39	KX244805	MG970281	90.8 ± 1.3 b	+++	10
<i>N. parvum</i>	AYER38	KX244804	MG970280	79.7 ± 1.0 c	+	10
<i>Diplodia seriata</i>	ACER58	KX244769	KX259152	74.2 ± 1.0 d	+++	10
<i>D. seriata</i>	ASER6.1	KX244763	KX259146	63.1 ± 1.1 e	++	10
<i>D. seriata</i>	ASER6.4	KX244766	KX259149	51.6 ± 1.0 f	++	10
<i>D. seriata</i>	ASER6.6	KX244768	KX259151	51.1 ± 0.8 f	++	9
<i>D. seriata</i>	ASER6.2	KX244764	KX259147	50.9 ± 1.0 f	+	9
<i>D. seriata</i>	ASER6.3	KX244765	KX259148	50.9 ± 0.9 f	+	10
<i>D. seriata</i>	ASER6.5	KX244767	KX259150	50.7 ± 0.9 fg	++	ph7
<i>D. seriata</i>	AYER241	KX244770	KX259153	47.9 ± 0.9 gh	+	10
<i>D. seriata</i>	ASER28	KX244771	KX259154	45.6 ± 1.4 hi	+	10
<i>D. seriata</i>	ASER29	KX244772	KX259155	43.5 ± 0.9 i	+	9
Control	—	—	—	5.3 ± 0.3 j		0
LSD ($\alpha = 0.05$)	—	—	—	2.9		

^a Botryosphaeriaceae isolates were identified by morphological and molecular analyses
^b Values followed by the same letters are not significantly different according to LSD test ($P < 0.05$)
^c + Poor, ++ Moderate, +++ Profuse, ++++ Abundant
^d Number of samples from which the fungus was reisolated out of 10 samples inoculated

Fig. 1. Symptoms of plum dieback and gummosis caused by Botryosphaeriaceae spp., and its colony and conidia morphology. (a) Dieback symptom of plum tree; (b) Fresh gum exudation from main limb; (c) V-shaped and (d) irregular necrosis of the xylem cuts made on gum-exudation limbs; (e) Colony and (h) conidia of *D. seriata*; (f) Colony and (i) conidia of *N. parvum*; (g) Colony and (j) conidia of *L. pseudotheobromae*

Botryosphaeriaceae isolates obtained in this study were also used for the examination of culture and conidia characteristics. The first species, *D. seriata*, developed a colony with pastel grey or greenish colour (Fig. 1e). Conidia were brown, oval or cylindrical, generally septate, but rarely non-septate with wide tip section, cut and oval base section and average size was measured as $24.1 \pm 2.6 \times 10.7 \pm 1.4 \mu\text{m}$ (Fig. 1h). The second species, *N. parvum*, developed a colony with pastel grey colour (Fig. 1f). Conidia were fusoid, ellipsoid, colourless and non-septate and average size was measured as $17.2 \pm 3.0 \times 5.6 \pm 1.0 \mu\text{m}$ (Fig. 1i). The last species, *L. pseudotheobromae*, developed more abundant colony (Fig. 1g) as

Fig. 2. Phylogenetic tree based on maximum parsimony analysis of combined ITS and partial β -tubulin gene sequences data. Numbers on branches are bootstrap values in 1000 replicates. Isolate UCROK856 (*Diatrypella verruciformis*) was added as an out-group. CBS – Centraalbureau Schimmelcultures, Utrecht, The Netherlands; CMW – Culture Collection Forestry and Agricultural Biotechnology Institute, University of Pretoria, South Africa; UCR – University of California, Riverside. NeR1a-AYug and NeR1b-AYug – Turkish isolates; PL_NpSV – French isolate; The other isolates were sequenced in this study. CMW9081, CBS 112555 and CBS 116459 were ex-type specimens

compared to the first two species and rapidly growing colony had light greenish grey colour. Conidia were initially oval or ellipsoid, thick-walled, colourless and non-septate, coloured with different tones of brown with the progress of growth, included a crosswise septation and lengthwise straight lines and average size was measured as $24.8 \pm 2.5 \times 14.2 \pm 1.3 \mu\text{m}$ (Fig. 1j).

Colony and morphological characteristics of the three species identified in this study were quite similar to the reports of earlier studies [9–11]. MORAL et al. [9] indicated that *D. seriata* isolates could be gathered under two different groups based on colony characteristics. The first group had initially pastel grey colour, then turned into greenish grey with the progress of growth; the second group isolates had initially whitish grey colour, then turned into black colonies as they grow older. Similar to the present study, AKGÜL et al. [10] reported that mature conidia of *D. seriata* isolates purified from grapevines had brown colour and some were non-septate. ISMAIL et al. [11] indicated that *N. parvum* isolates formed quite a small number of pycnidia and correspondingly conidia on PDA medium. Besides, mature conidia of *N. parvum* isolates were not microscopically observed during the incubation of isolates on PDA at 25 °C for a month [12,13]. However, PHILLIPS et al. [14] and OLMO et al. [15] indicated that conidia of this species turned into brown with the progress of growth and included one or two septations. *L. pseudotheobromae* was reported as a disease agent in English walnut [16], peach [4] and mango [17]. KWON et al. [17] indicated that *L. pseudotheobromae* isolates formed aerial mycelium on PDA at medium level. Contrarily, in the present study, *L. pseudotheobromae* isolates formed plenty of aerial mycelia.

Conclusions. To the best of our knowledge, this is the first report of plum dieback and gummosis caused by *D. seriata*, *N. parvum* and *L. pseudotheobromae* in South Turkey. This study has shown that the fungus associated with dieback, gummosis and cankers of plum trees may produce lesions in plum stem and branches. It is possible that these fungal pathogens could become established in the plum-growing areas of South Turkey. Control strategies must be explored to prevent further distribution of the pathogen.

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