Association of First Report of *Botryosphaeria* Spp. With Almond Dieback and Gummosis in Türkiye

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Research Article

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Abstract

Türkiye is among the top-producing almond countries in the world. Almond is a precious nut in modern diets due to its bioactive compounds associated with health and disease treatment. However, the plant's yield is affected by factors, including pathogens. This study aimed to diagnose and identify biotic factors associated with decline, dieback, and gummosis in almond trees in Yozgat province with a temperate climate. Seven almond 10-year orchards were investigated and trees with symptoms were sampled. Isolated species were cultured in PDA medium to identify the morphological and cultural characterization. The results obtained with cultural and morphological characteristics along with Phylogenetic analysis of ITS, β-tubulin, and EF-1α sequence data, confirmed the presence of *Diplodia seriata*, *Lasiodiplodia theobromae*, *Neofusicoccum parvum, Botryosphaeria dothidea.* Among these species, *Botryosphaeria dothidea* was first reported from almond trees in Türkiye. Pathogenicity testing showed that although all species were virulent*, L. theobromae* and *N. parvum* isolates caused more gumming in the inoculation areas than *D. seriata* and B*. dothidea* isolates. These results confirm that multiple Botryosphaeriaceae species are associated with branch decline and dieback on almond in Türkiye, which agrees with similar studies on woody crops. Accurate diagnosis of fungal pathogens in almonds is vital for developing disease management strategies and may help improve horticultural practices in maintaining urban stands.

Introduction

Almond (*Prunus dulcis*), a member of Rosaceae subfamily Prunoideae, is mainly cultivated for its edible kernel. Considering the increasing interest in nut consumption, the production of almonds in the world has raised significantly during the last decade. Almond trees have adapted to severe climatic conditions, such as delayed spring frost and drought. Türkiye is one of the top-producing countries for almonds ranking 4th in 2021 with a leveled up compared to 2020 (Faostat 2021). However, proper disease management is inevitable to achieve desired quantity or quality of almond production. Yield losses caused by fungal diseases sometimes reach severe levels, even the death of hard-shell trees Endes and Kayım (2017a). Dieback or decline in woody plants is usually caused by biotic and abiotic stress factors. Among biotic factors, fungal pathogens in soil and air, such as members of the Botryosphaeriaceae family are the critical factors in the decline and death of almond trees (Sohrabi et al. 2020). *Lasiodiplodia theobromae* is the main factor of dieback on almonds cultivated in Turkey and first reported by Özer et al. 2022. Moreover, *Diplodia seriata* and *Neofusicoccum parvum* were isolated from almond trees in Çukurova Region of Türkiye (Kayım et al. 2015).

Members of Botryosphaeriaceae family (Botryosphaeriales*)* with approximately 24 genera are cosmopolitan and have a wide range of hosts worldwide mostly prevalent in tropical and subtropical climate regions (Wang et al. 2023). Pistachio (*Pistacia vera*), almond (*Prunus dulcis* (Mill.), walnut (*Juglans regia*), peach (*Prunus persica* L.), plum (*Prunus salicina*) and apricot (*Prunus armeniaca*) are among some economic hosts for pathogens belonging to Botryosphaeriaceae family (Chen et al. 2014, Endes et al. 2022a). Stem and branch canker, leaf, shoot, twig, fruit, and bud blight are symptoms caused

by Botryosphaeriaceae fungi (Olmo et al. 2016; Gusella et al. 2022). However, the precise diagnosis of disease agents in plants is necessary to control and manage the disease by estimating infection rates or disease prevalence. Since some species belonging to the Botryosphaeriaceae family rarely form sexual reproduction periods, asexual (anamorph) reproductive structures are used for identification (Elena et al. 2015). Therefore, cultural and morphological characteristics of the asexual mycelium formed by the fungus in agar media, including the color and shape of the colony, the size, color, shape and the number of divisions of the conidia, are the basis for the diagnosis of the pathogen. However, the presence of more than 20 anamorph genera of Botryosphaeriaceae species and the inadequacy of reliable morphological characters in some species, instability of morphological shapes often cause misdiagnosis. On the other hand, different fungal species within the same genus generally differ in nucleotide sequences in housekeeping genes such as ITS (ribosomal DNA; 18S, 5.8S, and 28S rRNA), β-tubulin, EF1-α despite stable sizes of these genes. Consequently, the amplification of at least two or three of these genes for determination of nucleotide sequences can be an effective diagnosis tool besides cultural and morphological characteristics (Inderbitzin et al. 2010; Olmo et al. 2016).

Therefore, this study aimed to (i) diagnose the pathogenes causing dieback, gummosis, stem and branch canker on almond trees in Türkiye, (ii) to identify molecular, cultural, and morphological characteristics of isolated pathogens, (iii) to determine the virulence of pathogens through pathogenicity tests.

Materials and Methods

Sampling and fungal isolation

Field studies were carried out in Yozgat, Türkiye, in May 2022. Survey studies were conducted in seven (7) almond orchards. The sampling was done randomly from ten (10) trees showing symptoms such as dieback, gummosis, and stem and branch cancer in five replicates per tree. The average disease incidence in selected orchards was %17.9, with the lowest %0 and the highest %37.1. The sampling was done randomly from ten trees showing symptoms such as dieback, gummosis, and stem and branch canker in five replicates per tree. The isolation of fungi from infected tissues was carried out according to Endes and Kayım (2022a). The obtained isolates were purified through single spore isolations according to the method specified by Choi et al. (1999).

Molecular identification and phylogenetic analyses

Total genomic DNA extraction, PCR analyses, and electrophoresis of Botryosphaeriaceae isolates were performed in accordance with the protocol specified by Olmo et al. (2016). To amplify ITS region of rDNA; a partial sequence of Beta-tubulin (β-tubulin) gene and a partial sequence of the elongation factor 1 alpha (TEF-1α), the primer ITS4/ITS5 (White et al. 1990); Bt2a/Bt2b (Glass and Donaldson 1995) and EF1-728F/EF1-986R (Carbone and Kohn 1999) were used, respectively. Resultant PCR products were synthesized by Molgentek Company (Adana, Türkiye). Sequences of Botryosphaeriaceae isolates were compared with NCBI GenBank sequences of closely related species selected with the use of Blastn software. Consequently, all of the isolates were identified at the species level. In addition, the phylogenetic

analyses [Maximum Parsimony (MP)] were performed using MEGA 11. 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. *Guignardia philoprina* was used as an outgroup for phylogenetic analyses.

Morphological identification and characterization

The cultural and conidial characteristics of the obtained isolates were determined comparatively with previous studies and were identified tentatively (Alves et al. 2008, Phillips et al. 2013, Akgül et al. 2015, Endes and Kayım 2022a). Thereafter, to examine conidial morphology, cultures selected from the groups were incubated on Potato Dextrose Agar (PDA, Merck; 1.10130) and 3% Oat Meal Agar (OMA, 30 g oatmeal, 1000 mL distilled water) media at 25±1°C for four weeks under fluorescent light at 12-hour intervals to promote sporulation (Adesemoye et al. 2014). The length and width of 50 conidia for each isolate were measured by light microscopy (Leica, DM 750). In addition, the structure, shape, color, and division numbers of the conidia were photographed using a digital camera (Leica, DFC 450) combined with a light microscope.

Effect of temperature on mycelial growth

Three isolates from each of the four Botryosphaeriaceae species were used throughout this study (Table 1). The mycelial discs of four mm diameter obtained from 10-day-old cultures of Botryosphaeriaceae isolates were placed in Petri dishes containing PDA. Petri dishes were incubated for four days at 5℃ intervals at 5℃ to 35℃ in an incubator in the dark (Olmo et al. 2016). The effect of temperature on the mycelial growth of Botryosphaeriaceae species was determined according to Endes (2021).

Pathogenicity tests

Pathogenicity studies of Botryosphaeriaceae isolates were carried out on 2-year-old almond seedlings (Cultivar: Ferradual) and 25 cm long cut healthy branches obtained from 1-year-old healthy trees (Cultivar: Ferradual) in almond orchards. The detached branch Pathogenicity tests were conducted in accordance with the method described by Endes et al. (2016). The second pathogenicity test, the most virulent isolates identified for each of the four Botryosphaeriaceae species were inoculated into 2-year-old almond seedlings under greenhouse conditions. Ten seedlings were used for each isolate. Disease symptoms were observed during three months, from July to September, and the length of the wounds formed in the wood tissue of the seedling stems was recorded.

Results

Sampling and fungal isolation

The result of observations showed that Botryosphaeriaceae species generally caused unilateral twig (Figure 1a) or entire crown wilting in almond trees and later drying of branches (Figure 1a) and trees (Figure 1b), especially towards the end of summer. The leaves of dried trees remained on the branches

without falling. Gummosis and blight symptoms were observed on the heavily infected trees' trunks (Figure 1c, d) and main branches (Figure 1e-g). Depending on the severity of the infection, the color of the bark tissue became darker (Figure 1h), and cancerous tissues were observed in the bark and wood (Figure 1i-j) tissue as well as secretion of gum in these areas. In addition, “V” shaped (Figure 1k-m) or similarly shaped necrosis areas were formed in the wood tissue when cross-sections were taken from the infected trunk and main branches.

The results of isolation studies showed that five (5) out of seven (7) almond orchards were infected with Botryosphaeriaceae species. Based on the colony and conidial characteristics, 72 Botryosphaeriaceae isolates were grouped under four species. *Diplodia seriata* constituted 35% of all isolates, followed by *Neofusicoccum parvum* (32%), *Botryosphaeria dothida* (18%), and *Lasiodiplodia theobromae* (15%).

Phylogenetic analyses of species of Botryosphaeriaceae

Considering the cultural and conidial characteristics of isolates, a total of 16 out of 72 Botryosphaeriaceae isolates were sequenced three gene regions for phylogenetic analysis. The ITS, β-tubulin, and TEF-1α gene sequences of these isolates were stored in NCBI GenBank database accession numbers were taken (Table 1).

The sequence length of sixteen isolates ranged from 542 – 583 bp for ITS, 427 to 449 bp for β-tubulin, and 282 to 309 bp for TEF-1α. Phylogenetic trees of ITS (28 taxa, 727 characters), β-tubulin (28 taxa, 475 characters), and TEF-1α (28 taxa, 331 characters) gene regions were constructed according to the maximum parsimony of each dataset. Firstly, individual phylogenetic trees of three different gene regions of the isolates were constructed using Mega 11 program, and three different gene regions were aligned since there was no great difference between the topologies of the trees. The combined ITS, β-tubulin, and TEF1-α dataset of Botryosphaeriaceae spp. contained 28 taxa and 1522 characters (including alignment gaps). The combined data consisted of 281 informative characters for parsimony. Using data in the current study, one of the trees showed the most parsimony. As a result of maximum parsimony analysis, the tree length, consistency index, retention index, and composite index were identified as 350, 0.911, 0.973, and 0.887, respectively (Figure 2). Composite data of the most parsimonious tree without root clustered in two major clades in Botryosphaeriaceae isolates. Each of the main clades was clustered in two sub-clades. As a result, species in Botryosphaeriaceae isolates clustered with four previously identified Botryosphaeriaceae in the phylogenetic tree. The first major clade, *Diplodia seriata* (YBUPd1, YBUPd2, YBUPd3, YBUPd4, YBUPd5) clustered with *Lasiodiplodia theobromae* (YBUPd14, YBUPd15, YBUPd16); and the second major clade *Neofusicoccum parvum* (YBUPd6, YBUPd7, YBUPd8 YBUPd9, YBUPd10) clustered with *Botryosphaeria dothidea* (YBUPd11, YBUPd12, YBUPd13).

Culture and conidia morphological characterization

Botryosphaeriaceae isolates were also used for the examination of culture and conidia characteristics (Table 2, 3). All isolates produced anamorphic structures within 3 to 4 weeks on PDA, 3% OMA and autoclaved 20 mm long almond shoots onto the ½PDA medium. No ascospores were observed in the

studied cultures. Isolates were collected into four groups based on colony growth characteristics and conidial morphology.

The first group, *Diplodia* spp. had aerial and fast-growing mycelium (Table 3), which were initially whitish-gray but dark olive-grey with age. Small pycnidia of the isolates were formed on PDA, 3% OMA, and almond shoots. Conidia were initially colorless and non-segmented, turning to dark brown over time, some rarely with a septum; they were oval, ellipsoid, or cylindrical, broad at the tip, rounded, and truncated at the base. The sizes of the conidia were given in Table 2. This fungal group was identified as *Diplodia seriata*.

The second group colonies formed initially white and fluffy mycelium but became dark olive green, and lastly were observed in black with age. No growth was observed in this group at 5℃ and 10℃. They produced larger and more abundant pycnidium onto the PDA, 3% OMA and on almond shoots than other groups. The conidia were oval, ellipsoid, thick-walled, colorless, and non-septate, while the conidia were dark brown as they matured. They were one-septate with a longitudinally straight appearance. The sizes of the conidia are given in Table 2. This group was identified as *Lasiodiplodia theobromae*.

The third group produced fast-growing and fluffy mycelium (Table 3). The colonies were initially white but turned pastel grey with age. None of the isolates produced pycnidium on PDA and 3% OMA agar. However, quite a few pycnidia were observed on almond shoots. The conidia were fusiform or ellipsoidal, colorless, and non-septate, while mature conidia were light brown, and usually contained one or two septate with age. The size of the conidia were given in Table 2. This group was identified as *Neofusicoccum parvum*.

The fourth group had very similar colony characteristics to the third group isolates. Similarly, the fourth group of isolates did not produce pycnidium on PDA and 3% OMA agar but produced very few pycnidia on almond shoots, but the fourth group colonies spread outward from the center of the petri dish in olive-grey color. Conidia were fusiform, ellipsoid, non-septate, and colorless. The sizes of the conidia were given in Table 2. This group was identified as *Botryosphaeria dothidea* (anamorph: *Fusicoccum aesculi*).

Effect of temperature on mycelial growth

None of the studied isolates grew on PDA culture at 5℃. *L. theobromae isolates* showed no mycelial growth at 10℃, while the other isolates grew at this temperature albeit limited. *L. theobromae* isolates had an average growth rate of 11.7 mm/day at 35℃, while other isolates showed limited growth. The optimum temperature for mycelial growth was in the range of 25.3- 29.9℃ (Table 3). Significant differences were found in the optimum growth temperature of the isolates ($P < 0.05$). The maximum radial growth temperature for *D. Seriata*, *N. Parvum, L. theobromae* and *B. dothidea* isolates was 26°C, 26°C 27°C, and 29°C, respectively (Table 3). The Kruskal-Wallis test also showed that the maximum growth rates of the isolates differed significantly ($P < 0.05$). For all isolates, the relationship between growth rate and temperature was best described by a third-order polynomial ($Y = aT^3 + bT^2 + cT + d$). In any case, the three regression coefficients were highly significant ($P < 0.05$), and the coefficient of

determination (R^2) ranged from 0.939 to 0.987 (Table 3). The isolates were statistically categorized into two groups. The first group consisted of *B. dothidea* isolates with a maximum growth rate of >28 mm/day, while the second group included all other isolates with a maximum growth rate of <28 mm/day (Table 3).

Pathogenicity test

All Botryosphaeriaceae isolates were re-isolated from inoculated cut almond branches at rates ranging from 80 to 100% at the end of the 15-day incubation period. No symptoms of disease were observed in the wood tissue of the branches used as control, and no pathogen was isolated (Table 4). *L. theobromae* and *N. parvum* isolates caused more gumming in the inoculation areas than *D. seriata* and *B. dothidea* isolates. The mean of lesion lengths formed in wood tissue by all Botryosphaeriaceae isolates was found to be statistically significantly different from the control ($F_{(16 - 153, 0.05)} = 911.72$; $P < 0.05$) (Table 4). All *N. parvum* isolates were statistically grouped into the same class and had significantly ($P < 0.05$) mean necrosis lengths longer than other Botryosphaeriaceae isolates. However, *D. seriata* isolates differed significantly from both the control and each other statistically.

D. seriata isolates were grouped into two classes based on virulence levels (Table 4). *L. theobromae* was the second species with the most virulent isolates with the most significant amount of gum formation in the inoculation areas. *B. dothidea* isolates were statistically grouped into a single class. *B. dothidea* isolates showed higher virulence levels than *D. seriata* isolates while they have lower virulence levels than *N. parvum* and *L. theobromae* isolates.

The statistically highly aggressive isolates selected by branch pathogenicity results were used for pathogenicity studies of seedlings, one isolate representative of each species (Figure 3). Similar results were obtained with cut branch pathogenicity tests at the end of the three-month incubation period. Gum exudates were observed at the inoculation points 2-3 weeks after Botryosphaeriaceae isolates were inoculated to the stems of 2-year-old almond seedlings. The average lengths of woody discoloration caused by Botryosphaeriaceae species were shown in Figure 3. All species differed significantly from each other in the length of lesions formed in the wood tissue of the trunk in almond seedlings and from the control treatment ($F_{(4 - 45, 0.05)} = 444.05$; $P < 0.05$) (Figure 3). *N. parvum* was the most virulent strain and had a mean lesion length (123.2 mm) that was significantly ($P < 0.05$) longer than the other strains, followed by *L. theobromae*, *B. dothidea,* and *D. seriata* (Figure 3). Cut branch pathogenicity test results show that *L. theobromae* and *N. parvum* caused more gum formation on seedling stems. All pathogenic Botryosphaeriaceae species were successfully (100%) re-isolated from the stems of almond saplings, thus confirming Koch's postulates. No pathogenic fungal organisms were isolated from control seedlings.

Discussion

The investigations of this study revealed four Botryosphaeria species associated with disease symptoms such as organ blight, dieback, and gummosis in almond fruit trees grown extensively in the Yozgat

Province of Türkiye. *D. seriata*, *L. theobroma, B. dothidea* and *N. parvum* isolates were characterized by combining morphological, pathological, and molecular data. In addition, these four species were detected for the first time in almond trees in Yozgat Province of Türkiye with this study as well as *B. dothidea* is the first report of this species in almond in Türkiye. Based on literature reports, these species have a cosmopolitan distribution and a wide host range (Gure et al. 2005; Slippers et al. 2007; Abdollahzadeh et al. 2010). In previous studies, *D. seriata* has been reported as a pathogen in woody plants such as peach (Endes et al. 2016), apricot (Smith and Stanosz 2006; Damm et al. 2007; Liu et al. 2015), plum (Phillips et al. 2012; Endes and Kayım 2022a), pear Kurbetli and Demirci (2014) and vineyard (Akgül et al. 2015), almond (Olmo et al. 2016; Gharbi et al. 2017; Holland et al. 2021). In this study, *D. seriata* was determined as the dominant species. Similarly, the isolation rate of *D. seriata* from host plants was reported to be higher than other Botryosphaeriaceae species (Slippers et al. 2007; Damm et al. 2007). Moreover, this species is quite common in walnuts (Chen et al. 2014) and almonds (Inderbitzin et al. 2010) in California and vineyards in Australia (Pitt et al. 2013). In contrast, *L. theobromae* was the least isolated species compared to the other three species. This can be explained that *L. theobromae* is more pathogenic in woody plants in tropical regions (Abdollahzedeh et al. 2010; Munirah et al. 2017). Although the Eastern Mediterranean Region of Türkiye generally has subtropical climatic conditions, *L. theobromae* was reported as a pathogen in peach orchards and plums in Adana Province of the Eastern Mediterranean Region (Endes et al. 2016; Endes and Kayım 2022a). However, in Türkiye's Aegean Region, which is warmer and closer to tropical climate conditions than the Eastern Mediterranean Region, highly aggressive isolates of *L. theobromae* were isolated from fruit species such as figs (Çeliker and Michailides 2012), vineyards (Akgül et al. 2014) and strawberries (Yildiz et al. 2014). Moreover, Burges et al. (2006) reported that *L. theobromae* is mostly pathogenic in tropical regions, and *N. parvum* is pathogenic in hot climate conditions. However, *L. theobromae* was also isolated from a temperate region such as Southern-east Anatolia (Özer et al. 2022) and Southern Iraq (Al-Saadoon et al. 2012). *N. Parvum*, obtained from infected almond trees, has been reported as a pathogen in peach, mango, pistachio, orange and horticultural crops such as vineyards in Australia (Cunnington et al. 2007); on *Syzygium cordatum* tree in South Africa (Pavlic et al. 2009); mango tree in Italy (Ismail et al. 2013); peaches in Greece (Thomidis et al. 2011); walnuts and olives in Spain (Moral et al. 2010); and in almond, vineyards, and plum in Türkiye (Kayım et al. 2015; Akgül et al. 2015; Endes and Kayım 2022a).

In morphological characterization studies, there was no overlap between the characters such as color, division, length, and width of the conidia as well as the mycelial development of the species. The optimum radial mycelial growth temperature was in the range of 26-29 °C for all species (Table 3). *L. theobromae* showed faster radial mycelial growth at 25 °C than the other three species. Similarly, Thomidis et al. (2011) reported that the temperature required for optimum radial mycelial growth of *N. parvum* was 25 °C and (Ismail et al. 2013) reported that *Neofusicoccum* isolates (*N. parvum* and *N. australe*) grow at a minimum of 10 °C, an optimum of 25 °C and a maximum of 35°C. Copes and Hendrix (2004) reported that the optimum growth temperature of *B. obtuse* was between 20°C and 26°C; although it grew in a temperature range of 8°C to 36°C and its development stopped at 4 °C; and *B. rhodina* grew from 15 °C to 35 °C, the optimum growth temperature was between 25 °C and 35°C; in 10 °C and 40 °C

the growth was stopped, or the growth was inconsiderable. In addition to these previous studies, Wang et al. (2011) found that *L. theobromea* had a faster radial mycelial growth rate than *D. seriata* at 25°C on PDA medium; Chen et al. (2014) reported that *N. parvum, D. seriata,* and *L. theobromea* showed optimum mycelial growth on PDA medium at 25°C and 30°C, respectively. The results obtained in this study were in accordance with mentioned literature.

In this study, all species produced specific colony morphology. *D. seriata* formed the only one colony with olive-gray color. Contrary, Moral et al. (2010) reported that *D. seriata isolates* formed two different groups based on their colony characteristics. The first group was initially pastel-gray but turned greenish-gray as it matured; the isolates in the second group produced abundant aerial mycelium and were initially whitish-gray but turned greenish-gray or black as they matured. Similarly, Akgül et al. (2015) reported that *D. seriata* isolates formed a single type of colony. However, in both studies, *D. seriata* isolates easily formed pycnidium on PDA medium. In this study, *D. seriata* produced oval, ellipsoid, or cylindrical-shaped brown conidia, some with septa (Gure et al. 2005; Endes et al. 2016). Reports from Akgül et al. (2015) and the data obtained in this study are quite similar. However, Wang et al. (2011) reported that this species produced abundant overhead hyphae with non-septa and dark brown mature conidia. The colony characteristic of *N. parvum* with aerial mycelium on whitish gray and bubble-shaped masses of hyphae, producing a very small amount of pycnidium compared to *L. theobromae* and *D. seriata* isolates (Amponsah et al. 2008); and fusoid shaped, colorless and non-septa conidia with 1-2 divisions turning brown after a long time (Ismail et al. 2013; Phillips et al. 2013; Akgül et al. 2015) showed that this species is culturally different from *L. theobromae* and *D. seriata* isolates. *L. theobromae* had the fastest radial mycelial growth on PDA medium at 25°C and formed large pycnidia, as well as ellipsoidal or cylindrical shaped, with septa, straight lines on brown mature conidia and paraphyses, a useful character in distinguishing *Lasiodiplodia* species from each other, making it easy to distinguish this species from *D. seriata* and *N. parvum* species (Burgess et al. 2006; Alves et al. 2008; Abdollahzadeh et al. 2010; Wang et al. 2011; Chen et al. 2014 Akgül et al. 2015; Endes et al.2016). *B. dothidea* showed the maximum temperature for optimum mycelial growth compared to the other three species. Similarly, the optimum temperature range for mycelial growth of *B. dothidea* was determined in the range of 25–32°C in previous studies (Luo et al. 2022; Nazerian et al. 2019).

Recent PCR-based studies have shown that fungi of *Botryosphaeria* are generally associated with anamorph genera such as *Fusicoccum* and *Diplodia* (Crous et al. 2006; Slippers et al. 2007; Phillips et al. 2013). Studies proved significant differences between the morphological characters of these two genera of Botryosphaeriaceae (Wang et al. 2011). Slippers and Wingfield (2007) reported that fusicoccum-like species form hyaline (colorless), narrow (10 µm) conidia and have thin conidia walls (0.5 µm); Diplodia-like species, on the other hand, have wider conidia (10 µm) and thicker conidia walls (0.5 – 2 µm), with colored conidia over time in mature form, the other important anamorphic genus of *Botryosphaeria* fungi, *Lasiodiplodia*, have always been grouped separately from these two genera. In characterization studies similar to this study, the morphology of colonies and conidia of the species were supported by Phylogenetic studies. Fusicoccum-like species generally form colorless and fusoid-shaped conidia, and Diplodia-like species, which form brown oval, ellipsoid, and cylindrical-shaped conidia, were

grouped into two distinct clades (*N. parvum* and *Diplodia*-like species). Subsequently, *Diplodia*-like species forming colored conidia grouped in two different clades within themselves, one containing isolates of *D. seriata* and the other isolates of *L. theobromae*. Pathogenicity tests (Table 4; figure 3) determined *D. seriata*, *L. theobromae* and *N. parvum* as pathogens in almond trees in the Eastern Mediterranean Region. The results of cut branch pathogenicity and sapling pathogenicity overlap entirely with each other. However, in cut branch pathogenicity studies, while no isolate formed gum in the bark tissue of 25 cm long almond branches, it formed gum in the inoculation areas of the trunk of almond saplings. The pathogenicity test results of all isolates, *L. theobromae* was determined as the most pathogenic species by forming longer lesion length and more abundant gum than the other two species, which is in accordance with other studies (Britton and Hendrix 1989; Wang et al. 2011). In the cut-branch pathogenicity studies of *D. seriata* isolates, virulence levels were determined to be statistically different (Laundon 1973; Brown-Rytlewski and McManus 2000; Úrbez-Torres and Gubler 2009). This difference can be explained by the host plant's multiple resistance mechanisms against each isolate in species with a wide host range, such as *D. seriata* (Lv et al. 2012; Li et al. 2014). In addition, *D. seriata* produced less gum but longer lesions in trunk tissue than *N. parvum* compared to the other two species. However, Akgül et al. (2015) reported that *N. parvum* was the most pathogenic species among the Botryosphaeriaceae species isolated from the Vineyards in the Aegean Region of Türkiye. Similar to their study, in this study, it was demonstrated that *N. parvum* is at least as important as *L. theobromae* and *D. seriata* in the Eastern Mediterranean Region, considering the length of the lesion formed in the trunk as well as exuding gum from bark tissue. As far as our knowledge, this is the first study that deals with field survey, morphology, phylogeny, and pathogenicity of *L. theobromae*, *D. seriata* and *N. parvum* that cause wilt, gummosis, trunk and branch canker of almond trees in Türkiye.

This study also draws attention to the development of effective control strategies for these three species that cause wilt, gum disease, and dead tissue on the trunk and branches of apricot trees. Because these three species are among the potential risk factors for citrus, vineyard, pome, and stone fruit trees in the Eastern Mediterranean Region, which is one of the most important fruit production centers of Türkiye, therefore, to avoid or prevent diseases caused by *Botryosphaeria* species on almonds and other host plants, good care of fruit trees as well as the application of protective fungicide, especially after pruning, can be a good prevention approach.

Declarations

Authors' contributions AE carried out the survey analysis and sampling. AE developed laboratory analyses and performed mapping analyses and data interpretation. AE prepared the manuscript, and all authors read and approved the manuscript.

Data availability Data generated from this study is published within this article. Further materials can be provided on request from the corresponding author.

Competing interests The author declare no competing interests.

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Tables

Table 1. Isolates sequenced in this study and from GenBank included in the phylogenetic analyses

Species	Isolate[a]	GenBank accession number[b]		
		ITS	TUB2	TEF1
Diplodia seriata	YBUPd1	OP419496	OP819565	OQ053499
D. seriata	YBUPd2	OP419497	OP973766	OQ053500
D. seriata	YBUPd3	OP419498	OP973767	OQ053501
D. seriata	YBUPd4	OP419499	OP973768	OQ053502
D. seriata	YBUPd5	OP419500	OP973769	OQ053503
D. seriata	**CBS 112555^T**	AY259093	DQ458850	AY573219
D. seriata	**PUCV2090**	MT023558	MT063125	MT120819
D. seriata	**GA-422**	HQ660463	HQ660477	HQ660489
Neofusicoccum parvum	YBUPd6	OP419501	OP973770	OQ053504
N. parvum	YBUPd7	OP419502	OP973771	OQ053505
N. parvum	YBUPd8	OP419503	OP973772	OQ053506
N. parvum	YBUPd9	OP419504	OP973773	OQ053507
N. parvum	YBUPd10	OP419505	OP973774	OQ053508
N. parvum	**CMW9081^T**	AY236943	AY236917	AY236888
N. parvum	**CBS 145623**	MN611180	MN623344	MN623347
N. parvum	**MBAi51AG**	KJ921840	KP721702	KP721664
Botryosphaeria dothidea	YBUPd11	OP419506	OP973775	OQ053509
B. dothidea	YBUPd12	OP419507	OP973776	OQ053510
B. dothidea	YBUPd13	OP419508	OP973777	OQ053511
B. dothidea	**CMW8000^T**	AY236949	AY236927	AY236898
B. dothidea	**KARE1300**	MN166016	MN318117	MN318089
Lasiodiplodia theobromae	YBUPd14	OP419509	OP973778	OQ053512
L. theobromae	YBUPd15	OP419510	OP973779	OQ053513
L. theobromae	YBUPd16	OP419511	OP973780	OQ053514
L. theobromae	**CBS 164.96^T**	AY640255	EU673110	AY640258
L. theobromae	**UCD191Co**	DQ008308	DQ008331	EU012397
L. theobromae	**MBAI28AG**	KF182331	KF721698	KP721660
Guignardia philoprina*	**CBS447.68**	FJ824768	FJ824779	FJ824773

a = Isolates of species in bold were generated from GenBank. T = Isolates are ex-type specimens.
b = Sequences were registered in the gene bank according to three different gene regions. ITS = Internal Transcribed Spacer, TUB2 = β-tubulin-2, TEF1 = Translation Elongation Factor 1-α gene regions.
Asterisk (*) represented the out-group.

Table 2. Conidial size of Botryosphaeriaceae species from almond trunk and branch canker used in this study and comparison with previous studies.

Species	Isolate[a]	Conidial size (µm) (L × W)[b]	Mean ± SD (µm) (L × W)[c]	L/W ration[d]	Source of data
Diplodia seriata	YBUPd1	(17.5–)19.9–23.7(–27.0) × (8.5–)9.6–11.0(–11.8)	21.8 ± 1.9 × 10.3 ± 0.7	2.1	This study
D. seriata	YBUPd3	(18.8–)20.9–24.4(–25.0) × (8.8–)10.0–11.4(–11.8)	22.7 ± 1.8 × 10.7 ± 0.7	2.1	This study
D. seriata	YBUPd5	(18.8–)19.8–22.9(–25.0) × (9.5–)10.0–11.3(–12.0)	21.4 ± 1.5 × 10.7 ± 0.7	2.0	This study
D. seriata	**CBS 112555[T]**	**(21.5–)22–27(–28) × (11–)11.5–14.5(–15.5)**	**24.9 ± 1.9 × 12.9 ± 1.1**	**1.9**	**Phillips et al. 2007**
D. seriata	**BAL-10**	**(19.5–)20–26,5(–27) × (10.5–)11.5–14(–15)**	**23.6 ± 1.4 × 11.9 ± 0.8**	**2.0**	**Olmo et al. 2016**
Neofusicoccum parvum	YBUPd7	(11.3–)15.3–20.6(–23.8) × (3.8–)5.1–6.7(–8.0)	17.9 ± 2.6 × 5.9 ± 0.8	3.0	This study
N. parvum	YBUPd8	(14.8–)16.6–20.7(–23.8) × (4.5–)5.3–6.7(–8.0)	18.7 ± 2.0 × 6.0 ± 0.7	3.1	This study
N. parvum	YBUPd10	(12.5–)15.0–21.1(–28.3) × (3.8–)4.7–6.7(–7.5)	18.0 ± 3.1 × 5.7 ± 1.0	3.2	This study
N. parvum	**CMW9081[T]**	**(12–)15–19(–24) × 4–6**	**16.9 × 5.4**	**3.1**	**Slippers et al. 2004**
N. parvum	**BAL-42**	**(13.5–)16–20(–22.5) × (4.5–)5.5–6(–6.5)**	**17.9 ± 1.3 × 5.6 ± 0.7**	**3.2**	**Olmo et al. 2016**
Botryosphaeria dothidea	YBUPd11	(23.8–)25.0–27.6(–28.5)× (4.0–)4.6–5.7(–6.8)	26.3 ± 1.3 × 5.2 ± 0.6	5.1	This study
B. dothidea	YBUPd12	(24.0–)25.2–27.8(–28.8)× (4.5–)4.8–5.9(–6.8)	26.5 ± 1.3 × 5.4 ± 0.5	5.0	This study
B. dothidea	YBUPd13	(24.0–)25.2–27.5(–28.5)× (4.5–)4.8–5.6(–6.0)	26.3 ± 1.2 × 5.2 ± 0.4	5.1	This study
B. dothidea	**CMW8000[T]**	**(20–)23–27(–30)× 4–5(–6)**	**24.7 × 4.9**	**5**	**Slippers et al. 2004**
B. dothidea	**2E55**	**(22.5–)24–28.5(–32)× (4.5–)4.5–6.5(–7.5)**	**26.4 ± 2.4 × 5.5 ± 0.8**	**4.8**	**Chen et al. 2014**
Lasiodiplodia theobromae	YBUPd14	(21.3–)23.9–28.4(–31.3) × (11.3–)12.6–15.1(–16.3)	26.2 ± 2.3 × 13.8 ± 1.3	1.9	This study
L. theobromae	YBUPd15	(22.5–)24.7–28.1(–30.0) × (12.5–)13.3–15.3(–16.3)	26.4 ± 1.7 × 14.3 ± 1.0	1.9	This study
L. theobromae	YBUPd16	(19.3–)22.2–27.3(–29.3) × (10.8–)12.5–15.2(–16.3)	24.8 ± 2.6 × 13.8 ± 1.3	1.8	This study
L. theobromae	**CBS 164.96[T]**	**(19–)21–31(–32.5) × (12–)13–15.5(–18.5)**	**26.2 ± 2.6 × 14.2 ± 1.2**	**1.9**	**Alves et al. 2008**
L. theobromae	**7E87**	**(17.5–)20–24(–28) × (9–)11.5–13(–15.5)**	**22.0 ± 1.9 × 12.5 ± 0.4**	**1.8**	**Chen et al. 2014**

[a] Isolates of species in bold were generated from previous studies. T = isolates are ex-type or from samples that have been linked morphologically to type material of the species.

[b] L × W = length by width; (minimum–)average ± SD [Standard Deviation](–maximum).

[c] L × W = length by width.

[d] L/W = average length/average width.

Table 3. Temperature–growth relationship for Botryosphaeriaceae isolates*

Species	Isolate	Adjusted model[a]					Temperature (°C)[b]	Growth (mm/gün)[c]
		R^2	*a*	*b*	*c*	*d*		
Diplodia seriata	YBUPd1	0.987	-0.004	0.175	-1.018	0.666	26.4 b	23.9 b
D. seriata	YBUPd3	0.980	-0.004	0.178	-1.048	0.669	26.5 b	23.5 b
D. seriata	YBUPd5	0.985	-0.004	0.189	-1.226	1.503	26.4 b	23.5 b
Neofusicoccum parvum	YBUPd7	0.978	-0.004	0.170	-0.932	0.120	25.3 c	20.4 b
N. parvum	YBUPd8	0.981	-0.004	0.176	-1.042	0.583	26.1 bc	22.2 b
N. parvum	YBUPd10	0.980	-0.004	0.175	-1.040	0.569	26.0 bc	21.8 b
Botryosphaeria dothidea	YBUPd11	0.963	-0.004	0.208	-1.825	3.869	29.6 a	28.1 a
B. dothidea	YBUPd12	0.955	-0.004	0.213	-1.913	4.194	29.9 a	28.9 a
B. dothidea	YBUPd13	0.953	-0.004	0.210	-1.882	4.100	29.9 a	28.9 a
Lasiodiplodia theobromae	YBUPd14	0.946	-0.004	0.194	-1.593	2.623	27.7 b	22.5 b
L. theobromae	YBUPd15	0.939	-0.004	0.189	-1.518	2.317	26.9 b	20.6 b
L. theobromae	YBUPd16	0.939	-0.004	0.189	-1.519	2.329	26.9 b	20.4 b

* Data are the average of five replicates for each isolate. For each column, means with the same letter are not significantly different according to Kruskal-Wallis all pairwise comparisons test (P = 0.05).

[a] Mycelial growth on potato dextrose agar at 5 to 35°C was adjusted to a third-degree polynomial model: $Y = aT^3 + bT^2 + cT + d$ in which Y = mycelial growth (mm/day); a, b, c and d are the regression coefficients; and R^2 = coefficient of determination.

[b] Optimal temperature estimated by the adjusted model.

[c] Maximum growth rate estimated by the adjusted model.

Table 4. Average wood discoloration length on detached branches of *Prunus dulcis* cv. Ferradual, caused by mycelium plug inoculations with Botryosphaeriaceae species.

Species[a]	Isolate	Average wood discoloration length (mm)[b] ± SE		Gum exudation[c]	Reisolation[d]
Diplodia seriata	YBUPd1	39.5 ± 0.6	de	+	10
	YBUPd2	37.3 ± 1.1	e	n/a	9
	YBUPd3	40.8 ± 1.3	de	++	10
	YBUPd4	37.1 ± 1.1	e	n/a	10
	YBUPd5	42.2 ± 1.2	d	+	8
Neofusicoccum parvum	YBUPd6	98.8 ± 1.1	a	++	10
	YBUPd7	97.9 ± 0.8	a	+	10
	YBUPd8	99.5 ± 0.9	a	+++	9
	YBUPd9	98.6 ± 0.8	a	+++	9
	YBUPd10	98.9 ± 0.9	a	++	10
Botryosphaeria dothidea	YBUPd11	54.6 ± 1.0	c	+	8
	YBUPd12	53.6 ± 1.0	c	++	9
	YBUPd13	54.9 ± 0.6	c	+	9
Lasiodiplodia theobromae	YBUPd14	68.9 ± 0.7	b	+++	10
	YBUPd15	70.4 ± 0.9	b	++++	10
	YBUPd16	68.6 ± 1.0	b	+++	10
Control	—	6.4 ± 0.3	f	n/a	0

[a] Botryosphaeriaceae isolates were identified by morphological and molecular analyses

[b] Values followed by the same letters are not significantly different according to Tukey's HSD test ($P < 0.05$).

[c] n/a = not available, + = Poor, ++ = Moderate, +++ = Profuse, ++++ = Abundant

[d] Number of samples from which the fungus was reisolated out of 10 samples inoculated.

Figures

Figure 1

Disease symptoms caused by Botryosphaeriaceae species on almond trees in Yozgat province, central Türkiye. a, b. Dieback and blight of canopy; c, d. Gum exudation (band canker) on the trunk; e – g. Gummosis in scaffold branch; h – j. Wood discoloration and band canker tissue on root collar; k – m. Wedge-shaped and irregular vascular discoloration produced in the wood of trunk or scaffold branch

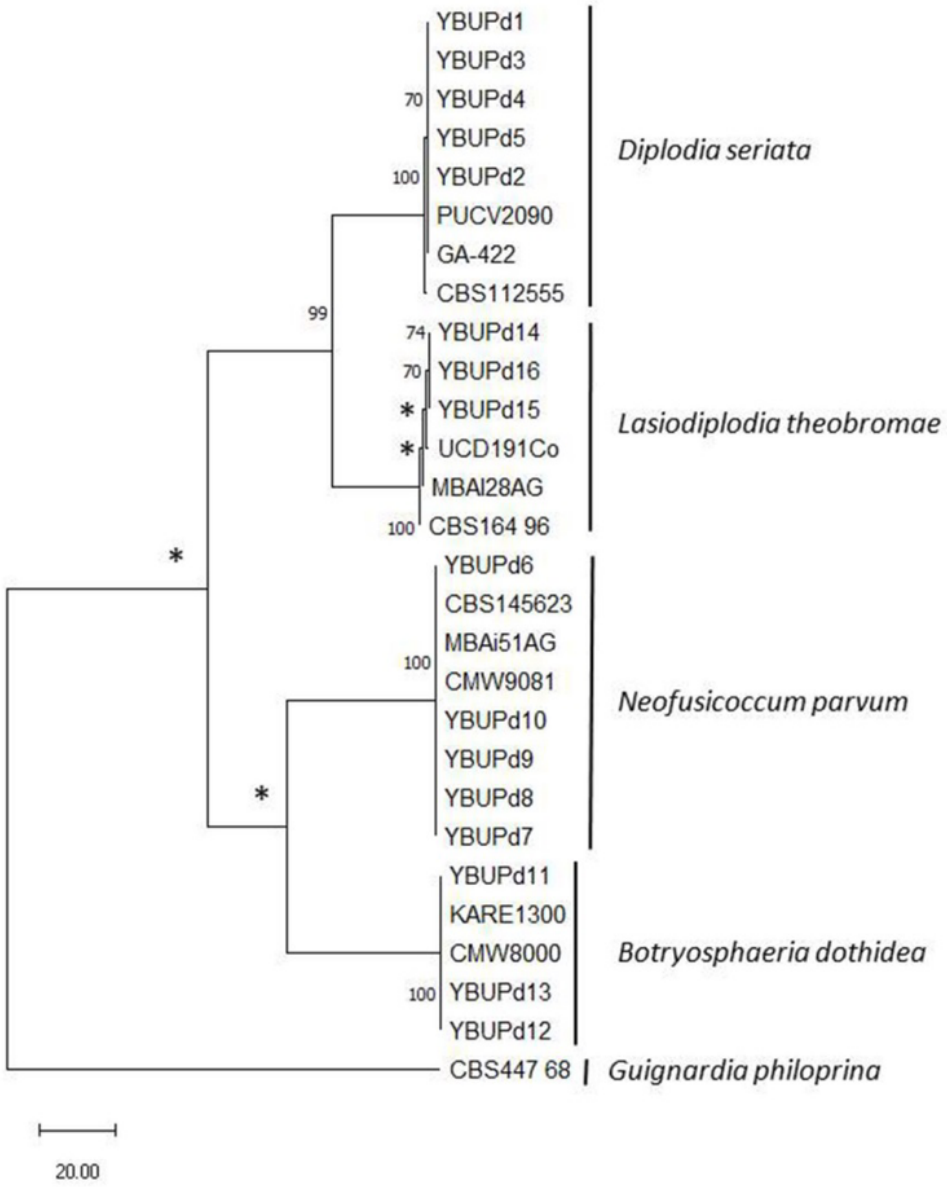

Figure 2

Most parsimonious unrooted tree based on internal transcribed spacer (ITS)1, 5.8S ribosomal DNA, ITS2, partial β-tubulin gene, and elongation factor 1-α sequences of isolates of species in the Botryosphaeriaceae family inferred from maximum parsimony analysis using MEGA 11. Numbers on branches are bootstrap values >70% in 1,000 replicates. Bootstrap values < 70% are indicated asterisk. Isolate CBS447 68 (Guignardia philoprina) was added as an outgroup. CBS = Centraalbureau

Schimmelcultures, Utrecht, The Netherlands; CMW = Culture Collection Forestry and Agricultural Biotechnology Institute, University of Pretoria, South Africa; UCD = University of California, Davis; UCR = University of California, Riverside; KARE = Kearney Agricultural Research and Extension; MBA = Turkish isolates; GA-422 = Chinese isolate; PUCV2090 = Chile isolate. The other isolates were sequenced in this study.

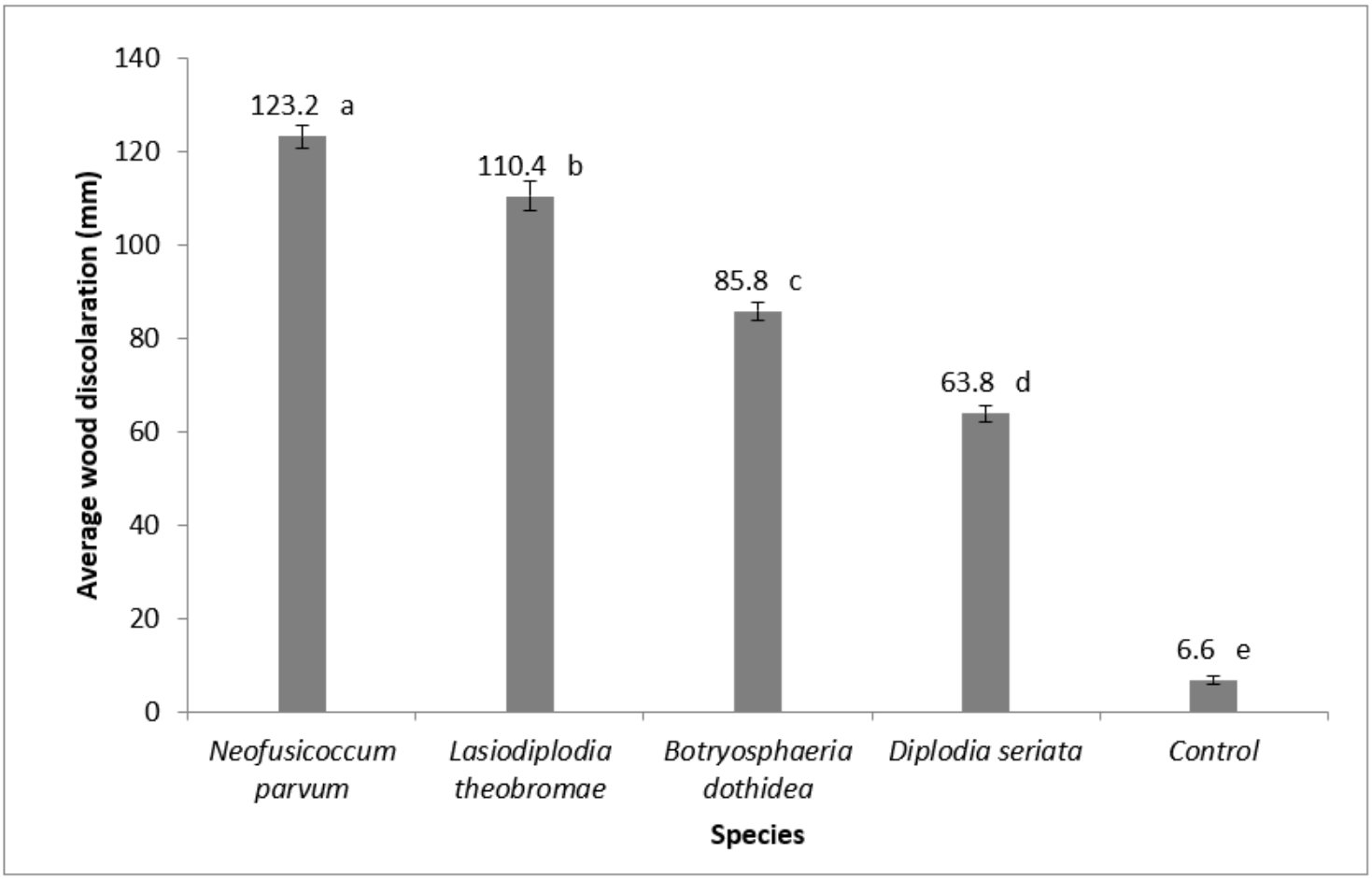

Figure 3

Average wood discoloration length (mm) on 2-years-old almond seedlings (cultivar: Ferradual) in 3 months after inoculation with a mycelium plugs of four Botryosphaeriaceae species. Bars topped with different letters indicate treatment means that are significantly different ($P < 0.05$) using the Tukey's HSD test. Vertical lines represent the standard errors of the means.