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Characterization and Pathogenicity of Botryosphaeriaceae Species Associated with Gummosis, Dieback, Trunk and Branch Cankers of Almond Trees in Türkiye

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ABSTRACT
Members of Botryosphaeriaceae family with 25 genera and several species are spread over a wide range of lands and climates worldwide. They cause gummosis, decline, dieback and blight on many woody plants. The purpose of present study was to diagnose the pathogens linked to the aforementioned symptoms on almond trees in seven orchards of Yozgat province (Türkiye) with a DSb type climate (Hot humid continental - Köppen Geiger system of climatic classification). These trees displayed dieback, gummosis trunk and branch canker symptoms. They were identified by cultural and morphological characteristics, and compared by sequencing of the ITS regions, EF-1α and β-tubulin genes with those of other species in GenBank (NCBI). Based on the colony and conidial characteristics, 72 isolates were identified as *Diplodia seriata, Lasiodiplodia theobromae, Neofusicoccum parvum* and *Botryosphaeria dothidea*. Pathogenicity tests were succesfully realized on two-year-old almond cv: Ferradual seedlings using Koch's postulates. According to current data, *B. dothidea* was identified for the first time on almond trees in Türkiye. Accurate identification, prevalence and incidence of the pathogens are crucial for developing effective disease management strategies to prevent disease outbreaks in Türkiye.

Keywords: *Botryosphaeria*, Cultural characteristics, Molecular identification, *Prunus dulcis*

1. Introduction

The production and consumption of almonds (*Prunus dulcis* - family Rosaceae, Prunoideae, subfamily) has risen significantly during the last decade worldwide (Özdemir et al. 2022; Romero-Cuadrado et al. 2023a). Almond plant is more tolerant to different types of edaphic stresses; paradoxically it is more sensitive to stresses related to harsh climatic conditions (delayed spring frost and drought etc.) (Yeniay & Şık 2022). Therefore, it is desired to grow almond varieties that are resistant to these problems. (Freitas et al. 2023; Küçükyumuk & Suarez 2023). Türkiye is among the almond producing countries, ranking 4th in the world in 2022 (FAO 2022). Therefore, correct and effective disease management is inevitable to obtain the desired quantity and quality of almond production (Holland et al. 2021). Dieback in almond tree is usually caused by many biotic and abiotic stress factors (Ören et al. 2020; Goura et al. 2023). This requires correct identification of the problem (Avenot et al. 2022). Fungal pathogens present in soil and air, especially belonging to the Botryosphaeriaceae family is thought to have a major role in the dieback, gummosis, trunk or branch canker on almond trees (Olmo et al. 2016; Moral et al. 2019; Sohrabi et al. 2020). The previous studies reported dieback, gummosis and trunk or branch canker symptoms on almond trees in Türkiye (EFSA Panel on Plant Health et al. 2023) like Özer et al. (2022), who reported *Lasiodiplodia theobromae* and Kayım et al. (2015), who reported *Diplodia seriata* and *Neofusicoccum parvum* as fungal causal agents belonging to Botryosphaeriaceae family on almond trees. These are mostly prevalent in tropical, subtropical and temperate regions (Garcia et al. 2021; Silva-Valderrama et al. 2024). Grape (*Vitis vinifera*), peach (*Prunus persica* L.), plum (*Prunus salicina*), almond (*Prunus dulcis* (Mill.) and walnut (*Juglans regia*) are among some important hosts in this family responsible causing economic losses in Türkiye (Endes et al. 2016; Endes & Kayım 2022a; Çiftçi et al. 2023).

Field diagnosis of Botryosphaeriaceae species associated with on tree symptoms is frequently carried out by either producers or technical based on symptoms such as dieback, gummosis, trunk or branch canker, shoot, fruit and bud blight (Ezra et al. 2017; Moral et al. 2019; Antón-Domínguez et al. 2023). However, the above-mentioned symptoms fail to clearly distinguish the causal agents (Nouri et al. 2018; Holland et al. 2021). Therefore, asexual (anamorph) reproductive structures of these fungi are used for identification relying on cultural and morphological or physical characteristics of the mycelium formed by the respective fungus on agar solidified medium, to observe physical properties of the fungus such as colour, shapes of colony, the size and the structure of conidia (Avenot et al. 2022). This has risk of contamination with several anamorphic genera within the Botryosphaeriaceae

family along with the inadequacy of reliable morphological characterisation or overlapping them in some species as well as instability of morphological features, which often contribute to the potential misidentification (Ko et al. 2023). Since some of the Botryosphaeriaceae species do not produce anamorph and teleomorph structures on agar medium, species identification is difficult (Romero-Cuadrado et al. 2023a).

Challenges overcome with PCR-based molecular tools for diagnosing complex fungal species (Avenot et al. 2022; Romero-Cuadrado et al. 2023a). Significant advancements in molecular methods and associated phylogenetic analysis have had a profound impact on the systematics and taxonomy of significant plant pathogenic fungi including Botryosphaeriaceae family (Yang et al. 2017; Ko et al. 2023). Botryosphaeriaceae species could be accurately identified with sequencing of ITS region, EF1-α and β-tubulin gene (Holland et al. 2021; Özer et al. 2022; Ko et al. 2023). Consequently, the amplification of at least two or three of these genes for determination of nucleotide sequences can act as an effective diagnosis tool for identifying them using cultural and morphological characteristics (Goura et al. 2023; Romero-Cuadrado et al. 2023a).

In view of the above information, this study aimed to

I. diagnose the pathogens causing dieback, gummosis, trunk and branch cankers on almond trees in DSb type climates;
II. identify using molecular, cultural, and morphological characteristics of the pathogens, and
III. determine the virulence of pathogens through pathogenicity tests.

2. Material and Methods

2.1. Sampling and fungal isolation

Field studies were carried out in orchards established in DSb climates (Anonymous 2024) of Yozgat province, Türkiye in May 2022. The survey studies were conducted in seven almond orchards. The sampling was done randomly from ten trees each orchard, considering each tree as a replication taking 5 plant tissue samples from each replication exhibiting dieback, gummosis, trunk and branch cankers symptoms for further fungal isolation studies (Adesemoye et al. 2014). Fungal isolation from infected almond trees was performed according to the procedure described by Endes & Kayım (2022a). The isolates were purified using single spore technique following method procedure by Ko et al. (2023).

2.2. Morphological identification and characterization

The cultural (colony colour, aerial mycelium, mycelial growth rate, optimum temperature for growth) and conidial (conidial dimensions, shape, colour, the number of septum) characteristics of fungal isolates were determined comparatively with previous studies and fungal species were identified tentatively (Phillips et al. 2013).

The isolates were firstly grouped based on their cultural characteristics (Akgül et al. 2015; Endes & 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 4 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) and the average and standard deviations were calculated. In addition, the structure, shape, colour and septa or without septa of the conidia were documented using a light microscope supplied with digital camera (Leica, DFC 450).

2.3. Effect of temperature on mycelial growth

Three isolates of each Botryosphaeriaceae species were used for this experiment. The 4 mm diameter agar discs obtained from 10-day-old cultures of Botryosphaeriaceae isolates were placed on PDA Petri dishes. Petri plates were incubated for 4 days at 5 °C intervals at 5 °C to 35 °C in the dark (Olmo et al. 2016). The colony diameter in each Petri plate was measured daily along two perpendicular axes (Goura et al. 2023). The experiment was designed according to the randomized complete block. Five replicates of each isolate (each replicate a petri dish) were used at each temperature. Moreover, the optimum temperature for radial growth and optimum daily radial mycelial growth (mm day^{-1}) of each isolate was calculated using third-order polynomial equations adapted using regression curves. Data were analysed using the Kruskal-Wallis test and the differences among the means were compared with Dunn's test at the 5% significance level (Endes 2021; Endes & Kayım 2022b).

2.4. Molecular identification and phylogenetic analyses

Total genomic DNA extraction, PCR analyses, and electrophoresis of Botryosphaeriaceae isolates were performed using protocol described 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 & Donaldson 1995) and EF1-728F/EF1-986R (Carbone & Kohn 1999) were used, respectively. The resulting PCR products were sequenced 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 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). Firstly, individual phylogenetic trees of three different gene regions of the isolates were constructed. Subsequently, three different gene regions were aligned since there was no sharp difference between the topologies of the trees. Bootstrap values were evaluated using 1000 replicates to test branch strength. *Guignardia philoprina* was used as an outgroup for phylogenetic analyses.

2.5. Pathogenicity tests

Pathogenicity experiments were carried out on two-year-old almond cv. Ferradual seedlings and 25 cm long cut the one-year-old healthy branch cuttings (cv: Ferradual) obtained from almond orchards. The detached branch pathogenicity tests were conducted as described by Endes et al. (2016). The cuttings were immersed in 1% sodium hypochlorite (NaOCl) for 10 min. Immediately, the cuttings were rinsed with tap water and bark tissue was removed from the middle of the cuttings with the help of a 4 mm diameter cork borer without damaging the xylem tissue. Seven-day-old PDA cultures were inserted into this aliquot with the same-sized cork borer. The inoculation area was wrapped with parafilm. All inoculated branch cuttings were placed in a transparent plastic container wiped with 96% ethanol and incubated for 15 days in the controlled room (25 °C temperature, 65% relative humidity, and 500 µmol m^{-2} s^{-1} photosynthetic active radiation). While the disease symptoms after inoculation were controlled routinely, the lengths of lesions formed in the xylem tissue of the branches were recorded after a 15-day incubation.

The second pathogenicity test, the most virulent isolates identified for each of the Botryosphaeriaceae species inoculated onto two-year-old almond seedlings under greenhouse conditions (Holland et al. 2021; Pouzoulet et al. 2022). Ten seedlings were used for each isolate. The inoculation was performed as described above. Disease symptoms were observed for three months' period, from July to September, and the length of xylem lesion in the tissue of the seedling stems was recorded.

2.6. Statistical analysis

Data obtained in the genetic study were analysed with Mega 11 software. All other data were analysed with SPSS v20 software. Prior to the statistical analyses, data of conidial dimensions, mycelial growth rates, and necrotic lesion lengths were checked for normality and homoscedasticity criteria. The dependent variables were analysed by one-way ANOVA. Posthoc test were performed using Tukey's Honestly Significant Difference test ($P<0.05$) to detect differences among the means.

3. Results and Discussion

3.1. Sampling and fungal isolation

Current study indicated that Botryosphaeriaceae species generally caused unilateral twig (Figure 1a) or entire canopy wilting in almond trees, subsequently leading to the drying of branches (Figure 1a) and trees (Figure 1b), especially towards the end of summer, before the falling of leaves from the trees. Gummosis and blight symptoms were observed on the trunks and main branches (Figure 1e-g) of the heavily infected trees (Figure 1c, d). Depending on the infection level, the colour of the bark tissue was darker (Figure 1h), and canker symptoms were clearly observed in the bark and woody tissues (Figure 1i, j) as well as gummosis in these areas. In addition, "V" shaped (Figure 1k-m) or similar necrotic lesions were observed on the xylem tissues when cross-sections were taken from the infected trunk and main branches.

Canopy wilting, dieback, gummosis, trunk and main branch cankers disease symptoms associated with Botryosphaeriaceae species on almond trees have been previously documented on almond trees in Spain (Olmo et al. 2016), Iran (Sohrabi et al. 2020), California (Holland et al. 2021), Türkiye (Özer et al. 2022) and Morocco (Goura et al. 2023). It was distinguished that the above mentioned disease symptoms associated with Botryosphaeriaceae cankers usually occurred on trunk cracks and near pruning wounds of 3-7 years old almond trees. These results were consistent with those obtained by Holland et al. (2021), who found the Botryosphaeriaceae species were associated with gummosis and V-shaped necrotic symptoms observed on 3-5 years old almond trees in California.

Figure 1- Disease symptoms caused by Botryosphaeriaceae species on almond trees in Yozgat, central Türkiye. a, b. Dieback and blight on the canopy; c, d. Gummosis (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 in the wood of trunk or scaffold branch

Isolation studies showed that 5 out of 7 almond orchards were infected with Botryosphaeriaceae species. Based on the colony and conidial characteristics, 72 Botryosphaeriaceae isolates were grouped under four species. Incidence of *D. seriata* was noted on 34.7% of all isolates, followed by *N. parvum* (31.9%), *Botryosphaeria dothidea* (18.1%), and *L. theobromae* (15.3%).

In the present study, symptoms associated with Botryosphaeriaceae pathogens were observed mostly on 3 to 7 years old almond trees. Botryosphaeriaceae symptoms were found in 71.4% of the total almond orchards. In contrast, Moral et al. (2019) reported that 40% of almond orchards including 10-80 years old almond trees on the island of Mallorca had disease symptoms related to Botryosphaeriaceae pathogens. The significant difference between these two studies can be explained by different tree ages in the almond orchards. In addition, Michalides et al. (2018) reported that young almond trees were more exposed to attack by Botryosphaeriaceae pathogens than older almond trees. Moreover, in this study, *D. seriata* was determined as the dominant species in agreement with those of Olmo et al. (2016) reporting that *L. theobromae* was the least prevalent species isolated. This can be explained that *D. seriata* causes natural infection more commonly than *L. theobromae* (Romero-Cuadrado et al. 2023b).

3.2. Molecular identification and phylogenetic analyses of Botryosphaeriaceae species

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

The sequence length of 16 isolates ranged 542 to 583 bp for ITS, 427 to 449 bp for β-tubulin, and 282 to 309 bp for TEF-1α. Phylogenetic trees of ITS, β-tubulin and TEF-1α (28 taxa, 331 characters) gene regions were constructed according to the maximum parsimony of each dataset. The combined ITS, β-tubulin, and TEF1-α dataset of Botryosphaeriaceae spp. contained 28 taxa and 1522 characters (including alignment gaps).

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

Species	*Isolates*[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}**	AY259094	DQ458856	AY573220
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	KP721698	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

The combined data consisted of 281 informative characters for parsimony. Using data in 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 analysis, without rooting, resulted in clustering of Botryosphaeriaceae isolates into two major clades. Each of the main clades were further subdivided into two sub-clades resulting in total of four distinct clusters in Botryosphaeriaceae isolates previously identified in the phylogenetic tree. The first major clade, *D. seriata* (YBUPd1, YBUPd2, YBUPd3, YBUPd4, YBUPd5) clustered with *L. theobromae* (YBUPd14, YBUPd15, YBUPd16); and the second major clade *N. parvum* (YBUPd6, YBUPd7, YBUPd8 YBUPd9, YBUPd10) clustered with *B. dothidea* (YBUPd11, YBUPd12, YBUPd13).

D. seriata has been reported as a pathogen of almond trees in many different countries (Olmo et al. 2016; Holland et al. 2021; Jiménez Luna et al. 2022). Moreover, *D. seriata* has also been recorded as aggressive pathogen from almond, plum, nectarine, and walnut by Kayım et al. (2015), Endes et al. (2016), Endes & Kayım (2022a) and Çiftçi et al. (2023) with the same sequence.

N. parvum was the most frequently isolated second species in this study and it has been previously reported to cause the largest cankers on trunks and limbs of almond trees in Iran (Sohrabi et al. 2020) and California (Jiménez Luna et al. 2022). This species was identified as a pathogen on almond trees by Kayım et al. (2015) along with identification of *N. parvum* as highly aggressive pathogen of other woody plant species like vineyard (Akgül et al. 2015) and plum (Endes & Kayım 2022a).

The other species, *B. dothidea* was also isolated from cankered tissues of almond trees in California (Michalides et al. 2018). However, there is no report showing that *B. dothidea* caused infection on almond trees in Türkiye. Therefore, it is assumed as first report on the subject.

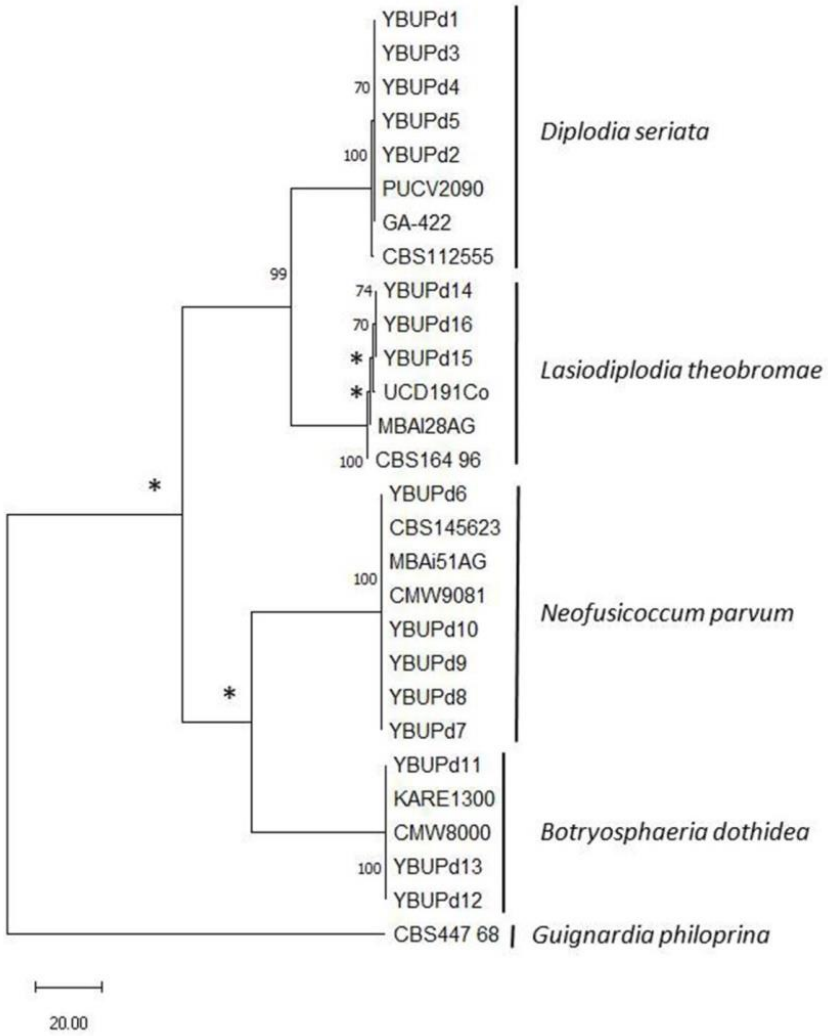

Figure 2- Most parsimonious unrooted tree based on internal transcribed spacer (ITS)1, 5.8S ribosomal DNA, ITS2, partial β-tubulin gene, and elongation factor 1-α sequences of Botryosphaeriaceae species inferred from maximum parsimony analysis using MEGA 11. Numbers on branches are bootstrap values >70% 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 = Türkiye isolates; GA-422 = Chinese isolate; PUCV2090 = Chile isolate. The other isolates were sequenced in this study

The last species, *L. theobromae* is a well-known as a serious pathogen that effects almond trees in agreement with the literature (Sohrabi et al. 2020; Goura et al.2023) and can cause a variety of disease symptoms, (Inderbitzin et al. 2010). The disease is frequently on fruit species like figs (Çeliker & Michailides 2012), strawberries (Yildiz et al. 2014), vineyards (Akgül et al. 2015), nectarine (Endes et al. 2016) and almond (Özer et al. 2022).

3.3. Morphological identification of Botryosphaeriaceae species

Botryosphaeriaceae isolates were used to characterize culture and conidia (Table 2, 3). All isolates produced anamorphic structures within 3 to 4 weeks on PDA, 3% OMA and autoclaved 20 mm long almond shoots on the ½PDA medium. No ascospores were observed on different media. Morphological characterization studies, showed no overlap between the characters such as colour, septate, conidial dimensions as well as the colony development of the species. The isolates could be separated into four species based on the characteristics of colony growth and conidial morphology (Figure 3).

The first group, *Diplodia* spp. had aerial and fast-growing mycelium (Table 3), which were initially whitish-grey, later turned dark olive-grey with age. Small pycnidia of the isolates formed on PDA medium, 3% OMA, and almond shoots. Conidia were initially colourless and aseptate, turning to dark brown over time, some rarely with one septate. Conidia were oval, ellipsoid, or

cylindrical, broad at the tip, rounded, and truncated at the base. The sizes of the conidia are given in Table 2. This was identified as *D. seriata*. This pathogenic species was occasionally isolated from almond trees showing symptoms of dieback, gummosis and trunk cankers (Holland et al. 2021; Jiménez Luna et al. 2022). The findings of cultural and conidial characteristics in this study were in agreement with findings of Olmo et al. (2016) who identified *D. seriata* as the based on morphological characteristics on almond trees in Spain. Akgül et al. (2015) reporting that *D. seriata* isolates easily formed the colony with olive-grey colour on PDA medium and produced the conidia with one septate.

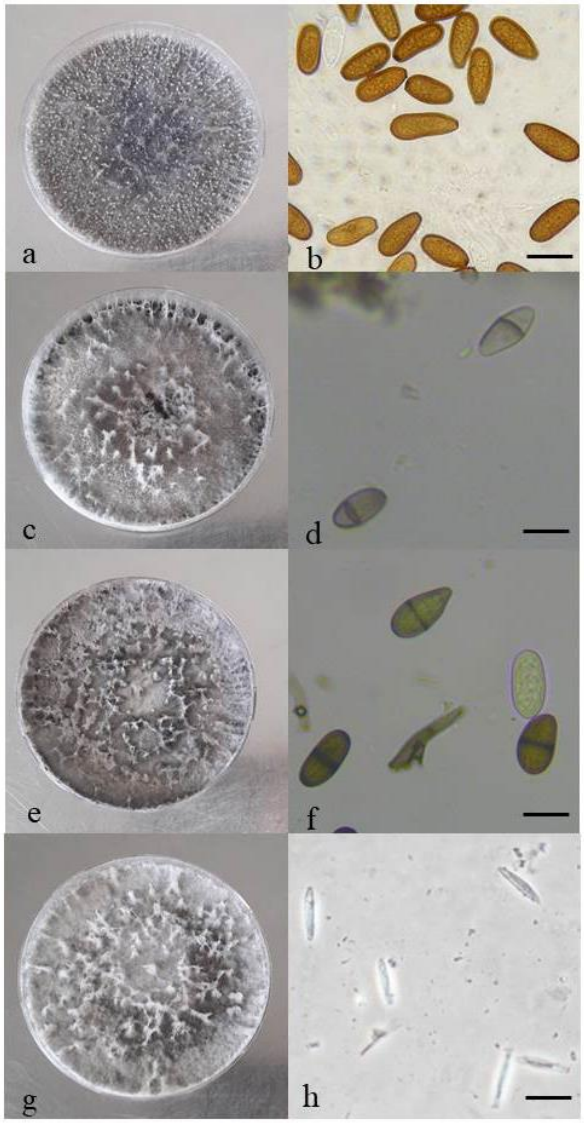

Figure 3- a–b, *Diplodia seriata*; c–d, *Neofusicoccum parvum*; e–f, *Lasiodiplodia theobromae* and g–h, *Botryosphaeria dothidea* (anamorph: *Fusicoccum aesculi*). conidial morphology. a, Colony morphology of 10-day-old *D. seriata*. b, mature brown conidia. c, Colony morphology of 10-day-old *N. parvum*. d, Pale-brown mature conidia. Central septum can be observed. e, Colony morphology of 10-day-old *L. theobromae*. f, Young colourless and mature brown conidia. g, Colony morphology of 10-day-old *B. dothidea*. h, Colourless and thin-walled conidia. Scale bars = 10 µm

The other fungal species produced fast-growing and fluffy mycelium (Table 3). The colonies were initially white, later turned pastel grey with age. None of the isolates produced pycnidia on PDA and 3% OMA agar. However, quite a few pycnidia were observed on almond shoots. The conidia were fusiform or ellipsoidal, colourless, and non-septate, while mature conidia were light brown, and usually contained one or two septate with age. Conidia dimensions are given in Table 2. This species was identified as *N. parvum*.

Table 2- Conidial size of Botryosphaeriaceae species obtained from current and 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

Similar to the current findings associated with the cultural and morphological characterisation of *N. parvum* isolates, Phillips et al. (2013) who described the diagnostic key of Botryosphaeriaceae species, that indicated their conidia, which become mature over time, are light brown in colour and have one or two septate. Paradoxically, *N. parvum* isolates cannot produce either young (Amponsah et al. 2008) or mature (Adesemoye et al. 2014) conidia on synthetic agar medium. This and previous study confirm that the production of conidia of Botryosphaeriaceae species is stimulated by the host plant tissues from which they are isolated. The third fungus species had very similar colony characteristics and showed similarities to the reported second fungus isolates which did not produce pycnidia on PDA and 3% OMA agar but produced very few pycnidia on almond shoots. These colonies spread outward from the centre of the Petri dish in olive-grey colour. Conidia were fusiform, ellipsoid, non-septate, and colourless. The sizes of the conidia are given in Table 2. This fungus was identified as *B. dothidea*. Zhang et al. (2013) reported that the conidia of *B. dothidea* were colorless, aseptate, thick-walled and fusoid-shaped, and their average conidia size was 20.5 × 5.5 (n = 50). This and previous studies, showed that *B. dothidea* appears to have longer conidia (Table 2). On the other hand, the findings were inagreement with those of Türkölmez et al. (2016), who reported that apple isolates of *B. dothidea* initially formed grey, but black colonies over time.

The fourth fungus colonies initially formed white and fluffy mycelium but became dark olive green, and lastly black with age. They produced larger and more abundant pycnidia onto the PDA, 3% OMA and on almond shoots compared to other fungus species. The conidia were oval, ellipsoid, thick-walled, colourless, and non-septate, while the conidia were dark brown as they matured. Conidia were one-septate with a longitudinally straight appearance. The sizes of the conidia are given in Table 2. This group was identified as *L. theobromae*. The findings of the cultural and morphological characteristics of this fungus are associated with *L. theobromae* in agreement with those by Goura et al. (2023), who reported that *L. theobromae* have aerial mycelium, white grey at the surface, olivaceous grey at the reverse plate, and became dark grey after two weeks of incubation on almond.

Table 3- Temperature–growth relationship for Botryosphaeriaceae isolates*

Species	*Isolate*	*Adjusted model*[a]					*Temperature (°C)*[b]	*Growth (mm day^{-1})*[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

The results of morphological studies of *L. theobromae* resemble with those mentioned by Endes et al. (2016), who firstly found *L. theobromae* as an aggressive pathogen on nectarine trees in Türkiye. The same authors reported that the conidia of *L. theobromae* were initially colourless, aseptate, ellipsoid or round, but with age they turned brown, one septate and longitudinally striped appearance.

Overall, the cultural and micro morphological characters as well as the PCR-based sequence information revealed that fungi of Botryosphaeriaceae species are generally associated with anamorphic genera like *Fusicoccum* and *Diplodia* (Phillips et al. 2013). Studies proved significant differences between the morphological characters of these two genera of Botryosphaeriaceae (Wang et al. 2011). Slippers & 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 format maturity. The other important anamorphic genus of Botryosphaeriaceae fungi, *Lasiodiplodia*, have always been simple grouped separately from these two genera because it contains longitudinal stripes on its conidia (Endes & Kayım 2022a; Özer et al. 2022; Goura et al. 2023).

3.4. Effect of temperature on mycelial growth

None of the isolates studied grew on PDA culture at 5 °C. *L. theobromae* isolates showed no mycelial growth at 10 °C, while the other isolates showed highly limited growth at same temperature. *L. theobromae* isolates had an average growth rate of 11.7 mm day^{-1} at 35 °C, while other isolates showed limited growth. The optimum temperature for mycelial growth was in the range of 25.3 - 29.9 °C (Table 3). Significant differences were found in the optimum growth temperature of the isolates ($P<0.05$). The maximum temperatures for growth were 26 °C, 26 °C, 27 °C, and 29 °C for *D. seriata*, *N. parvum*, *L. theobromae* and *B. dothidea* isolates respectively (Table 3).

The Kruskal-Wallis test also showed that the maximum growth rates of the isolates changed 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^{-1}, while the second group included rest of the isolates with a maximum growth rate of <28 mm day^{-1} (Table 3).

Ismail et al. (2013) showed that *Neofusicoccum* species (*N. parvum* and *N. australe*) developed at minimum temperature of 10 °C, optimum temperature of 25 °C, and maximum temperature of 35 °C. Also, Thomidis et al. (2011) has also mentioned that the optimum temperature for radial mycelial growing of *N. parvum* was the 25 °C. The findings of present study were in concordance with data of studies mentioned above.

Copes & Hendrix (2004) reported that *B. obtusa* (anamorph, *D. seriata*) can grow from 8 °C to 36 °C, but the optimum temperature for growth is between 20 °C and 26 °C, and mycelial growth did not observed at 4 °C. Furthermore, the same

researchers found that *L. theobromae* developed from 15 °C to 35 °C, with an optimum mycelial temperature for growth between 25 °C and 35 °C. Similarly, Wang et al. (2011) found that *L. theobromae* showed a faster mycelial growth percentage at 25 °C than *D. seriata*; Chen et al. (2014) found that *N. parvum*, *D. seriata* and *L. theobromae* exhibited optimum mycelial growth at 25 and 30 °C, respectively. In addition, *B. dothidea* showed the higher 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 °C to 32 °C (Luo et al. 2022). Moreover, Zhang et al. (2013) reported that the optimum temperature for radial mycelial development of *B. dothidea* was 30 °C, followed by 25 °C, 20 °C and 35 °C.

3.5. Pathogenicity test

All Botryosphaeriaceae isolates were re-isolated from inoculated almond branches at rates ranging from 80 to 100% after a 15-day incubation. No symptoms 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 gummosis in the inoculation sites compared to *D. seriata* and *B. dothidea* isolates. The mean of lesion lengths formed in wood tissue by all Botryosphaeriaceae isolates was significantly ($P<0.05$) different compared to the control (Table 4). All *N. parvum* isolates were statistically grouped into the same class and had significantly ($P<0.05$) different mean necrosis lengths, which were longer than other lesions by other isolates. However, *D. seriata* isolates differed significantly ($P<0.05$) among themselves and the control treatment.

D. seriata isolates were grouped into two classes based on virulence levels (Table 4). *L. theobromae* was the second species having the most virulent isolates with the most significant amount of gummosis formation in the inoculation sites (Li et al. 2014). *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.

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

Species[a]	Isolate	Average wood discoloration length (mm)[b] ± SE		Gummosis[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 at $P<0.05$ according to Tukey's HSD test ($P<0.05$). [c] n/a = not available, + = Poor, ++ = Moderate, +++ = Profuse, ++++ = Abundant. [d] Number of the samples from which the fungus was reisolated out of 10 inoculated samples each.

The statistically highly aggressive isolates, as determined by branch pathogenicity results were selected for pathogenicity studies of seedlings, one isolate representative of each species (Figure 4). Similar results were obtained with branch cuttings pathogenicity tests after a 90-day incubation. Gum exudates were observed at the inoculation points 2-3 weeks after Botryosphaeriaceae isolates were inoculated to the stems of two-year-old almond seedlings. The average lengths of woody discoloration caused by Botryosphaeriaceae species were shown in Figure 4.

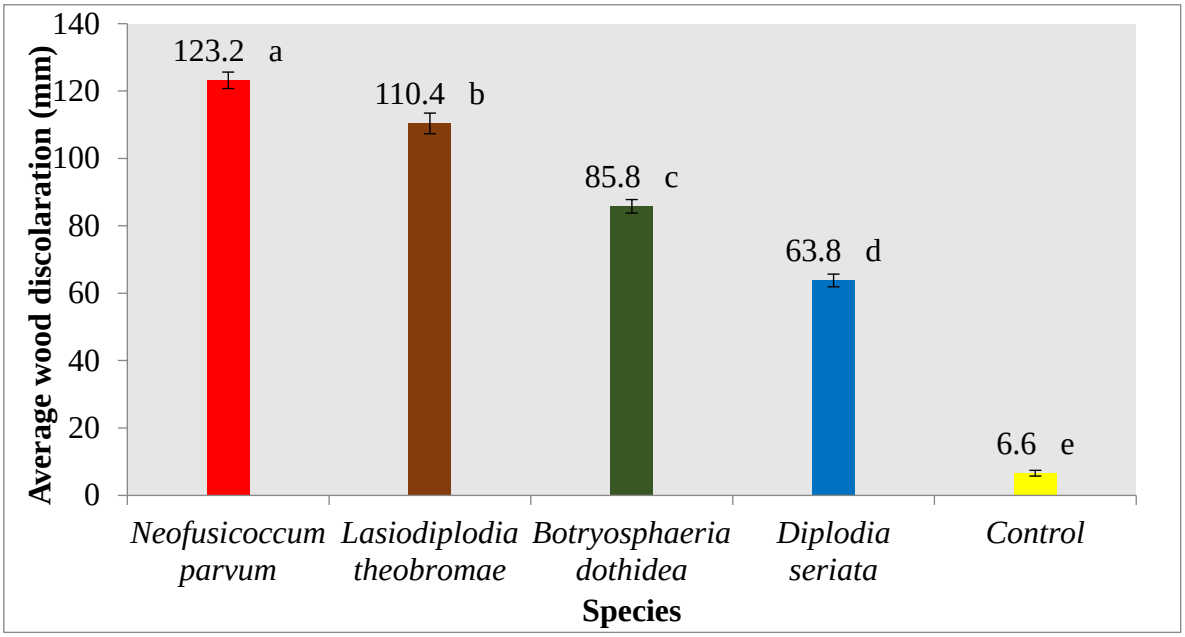

Figure 4- Average wood discoloration length (mm) on 2-years-old almond seedlings cv. Ferradual after a 90-day incubation with a mycelium plugs of four Botryosphaeriaceae species (*N. parvum* = YBUPd10; *B. dothidea* = YBUPd13; *L. theobromae* = YBUPd14; *D. seriata* = YBUPd5). Bars topped with different 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

All species differed significantly (P<0.05) among them in the length of lesions formed in the wood tissue of the trunk in almond seedlings (Figure 4). *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 4). Pathogenicity test results showed 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 seedlings, thus confirming Koch's postulates. No pathogenic fungal organisms were isolated from control seedlings.

Pathogenicity tests (Table 4; Figure 4) determined *D. seriata*, *N. parvum*, *B. dothidea* and *L. theobromae* as pathogens in almond trees in Spain (Olmo et al. 2016) California (Holland et al. 2021) and Morocco (Goura et al. 2023). The results of one-year-old branch cuttings and seedlings pathogenicity overlap entirely with each other. On branch cuttings with various isolates, lesions ranged in length from 37.1 mm to 99.5 mm on average, whereas no lesions were produced on control cuttings treated with PDA plugs devoid of mycelial fungi (controls).

In the second pathogenicity test, the isolates formed longer lesions on the trunks of almond seedlings. Pathogenicity tests also demonstrated that *N. parvum* exhibited the highest virulence on both branch cuttings and seedlings, resulting in longer lesions that averaged 98.7 mm and 123.2 mm, respectively. These results are consistent with findings of Inderbitzin et al. (2010), demonstrating that *N. parvum* and *N. nonquaesitum* species were found to be the most virulent when tested for virulence on almonds in California. In another study, Olmo et al (2016) reported that *Neofusicoccum* species were generally the most virulent in the almond cultivars tested, although there were differences between years in the pathogenicity test.

Furthermore, the pathogenicity tests revealed that the *L. theobromae* was determined to the second most virulent species on almond seedlings causing a longer lesion of an average of 110.4 mm. Özer et al. (2022) reported that *L. theobromae* was carried out the necrotic lesions with an average length of 60 to 80 mm on wood tissues of one-year-old almond seedlings were observed within 4 weeks in Türkiye. This result contrasts with that reported by Goura et al. (2023), who determined that *L. theobromae* was the most virulent strain, resulting in the longest necrotic lesion (285.17 mm) on almond twigs in Morroco. This difference can be explained by the different resistance mechanisms of host plant against each isolate in species with a wide host range such as *L. theobromae* (549 hosts), *B. dothidea* (514 hosts), *N. parvum* (295 hosts) and *D. seriata* (264 hosts) (Olmo et al. 2016; Silva-Valderrama et al. 2024).

In addition, *B. dothidea* produced less gum and lesions in trunk tissue than compared to the *N. parvum* and *L. theobromae*. Hovewer, *B. dothidea* species of the Botryosphaeriaceae is well known as a serious pathogen of almond trees globally (Olmo et al. 2016; Moral et al. 2019), and it is the Botryosphaeriaceae species most isolated from trunk and limb cankers on almond trees in California (Michailides et al. 2017).

In the present study, the different virulence levels were observed in artificial inoculation studies on almond seedlings and branch cuttings with *D. seriata* isolates. These results were similar to the pathogenicity test results of Inderbitzin et al. (2010) on

almond cultivars in California. Additionally, the pathogenic status of *D. seriata* in other hosts such as grapevine (Akgül et al. 2015) and plum (Endes & Kayım 2022a) is not very clear and still controversial among researchers. The findings in this study showed that *D. seriata* is a pathogenic species on almonds in Yozgat province, and *D. seriata* is one of the most isolated species of Botryosphaeriaceae family. Indeed, this pathogen is known to cause disease in a wide range of hosts (Olmo et al. 2016).

4. Conclusions

Current study provides the development of effective management strategies for these four species causing dieback, gummosis and trunk and limb cankers on woody plants. Because these species are among the potential risk factors for citrus, vineyard, pome, stone and nuts trees in the different region, which is one of the most important fruit production centres of Türkiye. Therefore, to avoid or prevent diseases caused by *Botryosphaeria*ceae 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.

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