agriculture

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Diversity and Pathogenicity of *Botryosphaeriaceae* and *Phytophthora* Species Associated with Emerging Olive Diseases in Italy

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Abstract: Extensive collar rot, sunken and bleeding cankers, shoot blight, and fruit rot symptoms on olive trees have recently been observed in several orchards in Italy. Since there is little information about the etiology of these diseases and given the high economic relevance of this iconic crop, a study was conducted from autumn 2017 to summer 2022, in four Italian regions, to define the occurrence, distribution and impact of the main pathogens involved. A total of 1064 symptomatic olive samples were collected and processed. Based on colony appearance, micromorphological analysis and DNA sequence data, thirty-eight species, including eighteen *Botryosphaeriaceae* species belonging to five genera and fifteen *Phytophthora* species, were isolated and identified, thirteen of which, *Diplodia africana*, *D. fraxini*, *D. subglobosa*, *Dothiorella omnivora*, *Do. sarmentorum*, *Do. sempervirentis*, *Sardiniella urbana* (*Botryosphaeriaceae*), *Phytophthora cactorum*, *P. cinnamomi*, *P. citricola*, *P. crassamura*, *P. niederhauserii* and *P. pseudocryptogea*, are reported here for the first time in olive trees. Pathogenicity tests performed on unripe drupes and on potted olive seedlings completed Koch postulates and highlighted that several species of *Botryosphaeriaceae* and *Phytophthora* represent a growing threat to olive trees.

Keywords: invasive pathogens; drupe rot; branch dieback; root rot

Citation: Linaldeddu, B.T.; Rossetto, G.; Maddau, L.; Vatrano, T.; Bregant, C. Diversity and Pathogenicity of *Botryosphaeriaceae* and *Phytophthora* Species Associated with Emerging Olive Diseases in Italy. *Agriculture* **2023**, *13*, 1575. https://doi.org/10.3390/agriculture13081575

Academic Editors: Dalia Aiello and Giorgio Gusella

Received: 20 July 2023
Revised: 31 July 2023
Accepted: 3 August 2023
Published: 7 August 2023

1. Introduction

The olive tree (*Olea europaea* L. subsp. *europaea* var. *europaea*) is one of the first domesticated fruit crops and one of the most widespread species in Mediterranean agroecosystems [1]. Its domestication dates back to more than 5000 years ago [2], and it is supposed that current cultivars have originated from the wild Mediterranean olive (oleasters) [3,4]. Over time, human activities have largely contributed to its dissemination from Mediterranean countries to new cultivation areas such as Australia, California, Chile, China, South Africa and New Zealand [5–9]. In Europe, olive production reaches more than 14 million tons, and Italy is the second producer country after Spain, with 2.4 million tons in 2022 [10,11].

In Italy, olive orchards are present in all regions with an overall growing area of 1,156,334 ha, of which 29% is located in Apulia, followed by Calabria (16%), Sicily (14%), Tuscany (7%), Lazio (7%), Campania (6%), Abruzzo and Sardinia (4%) and Umbria (2%) and the rest is in the other regions [11].

Pests and diseases remain the major limiting factor in olive production in both traditional and emerging producing countries [12–14]. Among fungal diseases, the olive anthracnose caused by *Colletotrichum* spp., olive leaf spot caused by the ascomycetous fungus *Venturia oleaginea*, and verticillium wilt caused by the soil-borne fungus *Verticillium*

dahliae are considered the most important olive diseases and the major limiting factor for olive oil production worldwide [15–18]. In addition to these well-known diseases, for which management strategies are available, since 2014, a complex wilting syndrome has been recorded in different olive orchards, initially in Veneto and Lombardy and subsequently in other Italian regions, including Sardinia and Calabria. Preliminary field surveys have highlighted a complex symptomatology characterized by the simultaneous presence of leaf chlorosis, shoot blight, progressive branch dieback, drop of unripe drupes, and root and collar rot. These diseases have a great impact on reducing plant productivity and vitality [19]. The symptoms observed on olive trees in northern Italy resemble those reported by Sánchez-Hernanádez et al. [20] in Spain, Úrbez-Torres et al. [14] in California and Carlucci et al. [21] in southern Italy, in which several species of *Phytophthora* and *Botryosphaeriaceae* were associated with declining olive trees.

Species in the family *Botryosphaeriaceae* are emerging as important olive pathogens causing leaf spot, fruit rot and branch dieback in different countries and continents [14,22]. In addition to *Botryosphaeria dothidea*, the causal agent of Dalmatian disease [23], *Neofusicoccum mediterraneum*, *N. luteum*, and *N. parvum* have recently been reported in Australia, California, Croatia, Italy, Spain, and Uruguay as emerging olive pathogens [21,24–28]. At the same time, over the last decade, reports of root and collar rot and sudden-death symptoms in young and mature olive trees caused by *Phytophthora* spp. have increased worldwide [20,29–31].

Therefore, given the growing expansion of the new wilting syndrome in various Italian olive orchards and the still limited information available about its etiology, a study was conducted in four Italian regions to isolate and identify the main species involved, as well as to evaluate their pathogenicity.

2. Materials and Methods

2.1. Field Surveys and Sampling

From summer 2017 to autumn 2022, the health status of olive trees belonging to 18 cultivars was monitored in thirty-eight orchards located in Veneto and Lombardy (northern Italy), Sardinia (center) and Calabria (south) (Table 1). The olive trees were checked for the presence of symptoms on fruits (necrotic depressed spot and rot), stem and branches (dieback, exudates and cankers) and at the collar and root system (exudates, necrosis and loss of fine roots). A total of 1064 symptomatic samples, including necrotic fruits (299 unripe and 266 ripe drupes), cankered branches (366) and necrotic roots, including the rhizosphere (133), were randomly chosen for laboratory diagnostic analysis (Table 1).

In order to estimate and measure the amount of plant disease symptoms [32], premature fruit drop was estimated in 2020 in an organic farm in Veneto, site 17. In particular, a field survey was conducted from May to September on eight mature olive trees chosen at random. Under the canopy of the selected trees, a square-shaped plot (1×1 m) was established. Every two weeks in the period from June to September, the drupes fallen into the plots were collected and counted, and the plot surface was cleaned. The occurrence and incidence of wilted branches with sunken cankers were monitored in the period from 2017 to 2021 on 160 olive trees in site 27 (Sardinia). The disease incidence was evaluated as the number of symptomatic trees among the total number of olive trees monitored [32]. The symptomatic branches were cut every year in February immediately after the disease-incidence survey.

Table 1. Details of the monitored orchards and number of symptomatic samples collected per site.

Italian Region	Sites	Cultivar	Type of Samples			
			Unripe Drupes	Ripe Drupes	Stem/Branches	Collar/Roots
Veneto	1	Casaliva	6	-	10	12
	2	Grignano	-	-	15	3
	3	Grignano	3	-	15	-
	4	Grignano	2	-	13	-
	5	Grignano	-	-	19	-
	6	Grignano	-	-	51	-
	7	Grignano	-	-	4	2
	8	Grignano	-	4	13	2
	9	Leccino, frantoio, Trepp, favarol	-	-	21	-
	10	Leccino, grignano, trepp	16	35	17	20
	11	Grignano	-	-	14	2
	12	Grignano	-	-	8	-
	13	Grignano	12	-	7	5
	14	Grignano	-	-	6	-
	15	Arbequina, arbosana	10	-	3	16
	16	Rasara, leccino, matosso, itrana	-	15	7	2
	17	Rasara	50	50	75	7
	18	Frantoio, leccino	-	5	4	2
	19	Frantoio, leccino, Grignano	-	8	6	2
	20	Frantoio, leccino	-	10	5	-
	21	Leccino, grignano, Frantoio	-	3	3	2
	22	Frantoio, leccino, pendolino	-	10	5	-
	23	Leccino, frantoio, Grignano	-	10	7	3
	24	Frantoio, leccino	-	5	5	2
Lombardy	25	Frantoio	-	6	5	3
	26	Frantoio	-	5	3	2
Sardinia	27	Frantoio	100	100	10	10
	28	Bosana	-	-	10	-
	29	Semidana	-	-	5	7
	30	Pizz'e carroga	100	-	-	-
Calabria	31	Carolea	-	-	-	5
	32	Biancolilla	-	-	-	5
	33	Nocellara del Belice	-	-	-	5
	34	Frantoio	-	-	-	3
	35	Carolea	-	-	-	3
	36	Carolea	-	-	-	3
	37	Carolea	-	-	-	3
	38	Carolea	-	-	-	2

In site 10 (Veneto), all plants were visually checked for the occurrence of typical *Phytophthora* symptoms such as poor growth, leaf chlorosis, shoot blight, bleeding cankers, and collar and root rot. The disease incidence was estimated as the number of symptomatic trees out of the total number of trees. In addition, the same plants were monitored for the presence of branch canker and dieback, as well as symptoms on fruits.

2.2. Isolation of Fruit Rot Agents

In the laboratory, olive fruits were surface-disinfected with 70% ethanol for 30 s, rinsed in sterile distilled water, and then air-dried in an aseptic condition. Ten fragments (4 × 4 mm) of symptomatic tissues (epicarp and mesocarp) for fruits were cut aseptically, placed onto potato dextrose agar (PDA, Oxoid Ltd., Basingstoke, UK) in Petri dishes, and incubated at 25 °C in the dark for 7 days. In order to obtain pure cultures, hyphal tips

from emerging colonies were sub-cultured onto half-strength PDA dishes, labelled and incubated at 25 °C in the dark.

2.3. Isolation of Branch Canker Agents

Branch samples were initially disinfected with ethanol (70%) for 30 s, and then the outer bark was removed with a sterile scalpel. Longitudinal and cross sections were made to observe internal disease symptoms. Isolations were performed from ten small fragments (4 × 4 mm) of the inner bark and xylem cut aseptically from the margin of the necrotic lesions. All fragments were placed onto 90 mm Petri dishes containing PDA and incubated at 25 °C for 7 days in the dark. Pure cultures were obtained and stored as reported previously.

2.4. Isolation of Collar and Root Rot Agents

Phytophthora isolations were performed using the method described by Linaldeddu et al. [33]. In particular, collar, roots and rhizosphere samples were flooded separately in 1 L of distilled water in plastic boxes. Young cork oak and elder leaves were placed as baits on the clean water surface after 24 h. Boxes were maintained at 18 °C under natural daylight for 5 days. Leaves showing dark spots or *Phytophthora* sporangia were cut in small fragments (4 × 4 mm), air dried and then placed in Petri dishes containing PDA supplemented with 100 mL/L of fresh carrot juice, 0.013 g/L of pimaricin and 0.05 g/L of hymexazol (PDA+) [34].

Isolations were also performed from collar and root tissue samples taken from the margin of inner bark necrotic lesions. The small fragments obtained with a sterile scalpel were aseptically placed onto 90 mm Petri dishes containing PDA+. The dishes were incubated in the dark at 18 °C and examined every 12 h. Pure cultures obtained from hyphal tips were stored on PDA and carrot agar (CA) at 20 °C in the dark.

2.5. Identification of the Isolates

Fungal and *Phytophthora* isolates were initially grouped in morphotypes on the basis of colony growth characteristics, including surface and reverse colony appearance observed after 7 days of incubation on PDA and CA at 20 °C and 25 °C in the dark. In addition, morphometric data of conidia and sporangia recorded using the software Motic Images Plus 3.0 paired with a Moticam 10+ camera connected to a Motic BA410E microscope (Motic, Wetzlar, Germania) were used for the identification of the isolates. To enhance sporangia production, the CA plugs of each isolate were placed in Petri dishes containing unsterile pond water and fine root samples of oaks, whereas the production of conidia was enhanced through the exposure of the fungal colonies to natural sunlight on the laboratory bench for one month.

Furthermore, DNA sequence data analysis was used to confirm the identification of all isolates at species level. Genomic DNA from the mycelium of pure culture was extracted, as reported by Linaldeddu et al. [33]. For all isolates, the complete internal transcribed spacer (ITS) region of the rDNA was amplified and sequenced using the primers ITS1 and ITS4 [35]. Polymerase chain reactions (PCRs) were performed in 50 µL reaction mixtures using the GoTaq® Hot Start Green Master Mix (Promega, Milan, Italy) and a SimpliAmp Thermal Cycler (Thermo Fisher Scientific Inc, Waltham, MA, USA). Amplification conditions were as follows: an initial denaturation step at 94 °C for 1 min, followed by 35 cycles of denaturation at 94 °C for 25 s, annealing at 54 °C for 35 s and extension at 72 °C for 45 s, and a final elongation step of 8 min at 72 °C. PCR products were purified using the MonarchTM PCR & DNA Cleanup Kit (New England Biolabs, Ipswich, MA, USA) according to the manufacturer's instructions. Both strands were sequenced using their BMR Genomics DNA sequencing service (www.bmr-genomics.it, accessed on 31 July 2023). Sequences were edited with FinchTV v1.4.0 (Geospiza, Inc., http://www.geospiza.com/finchtv, accessed on 31 July 2023) and compared with sequences of ex-type culture deposited in GenBank

(http://blast.ncbi.nlm.nih.gov, accessed on 31 July 2023). New sequences were deposited and are available in GenBank.

2.6. Pathogenicity Assays

Pathogenicity trials were performed according to Koch's rules on asymptomatic unripe fruits (cv. Rasara) and on 3 year-old-seedlings (cv. Leccino) using pure cultures of one representative isolate of the main species obtained. Three different assays were performed.

In the first assay, artificial inoculations of asymptomatic unripe olive fruits (cv. Rasara) collected at the end of July from an organic orchard located in Veneto (Arquà Petrarca) were performed on 12 cm Petri dishes under controlled conditions. Forty fruits per fungal isolate were initially surface disinfected with 90% ethanol and then wounded with a sterile needle (depth of 1 mm) and inoculated with a 3-mm-diameter mycelium PDA plug taken from an active 5-day-old colony. Forty fruits wounded in the same way were inoculated with a sterile PDA plug and used as controls. Inoculated and control fruits were incubated in a climatic chamber at 25 °C in the dark with 100% relative humidity for 7 days. Four fungal species were used in this assay. At the end of the experimental period, the occurrence of a necrotic lesion was assessed, and the size of the area was measured using Assess 2.0 [36].

The second assay was conducted on 3 year-old-seedlings (cv. Leccino) using pure cultures of ten fungal species. Five branches per fungus were used in the artificial inoculations. The outer bark surface of each branch was initially disinfected with 90% ethanol and then inoculated with an agar-mycelium plug (3 × 3 mm) in the center of a wound, of the same size, made with a sterile scalpel. The inoculation site was protected with a moist cotton ball and wrapped with aluminum foil. Five branches, inoculated with a sterile PDA plug, were used as controls. The assay was conducted in the period from April to July 2019 in a cold greenhouse with the temperature ranging from 6.1 °C to 37.6 °C. At the end of the experimental period (122 days), the length of necrotic lesions caused by each fungal species was measured and recorded after the removal of the outer bark.

Finally, in the third assay, five olive seedlings of the same cultivar and age were inoculated with thirteen *Phytophthora* species. The assay was performed at the collar by placing a mycelial agar plug of 5 mm diameter on a wound of the same size made with a sterile cork borer. The inoculation point was protected as previously reported, and the seedlings were kept in the same experimental conditions. Seedlings were regularly watered, and after one hundred and twenty-two days, the presence, nature and size of the external and internal symptoms were detected on all inoculated seedlings.

For each bioassay, re-isolation of the fungal and *Phytophthora* species was performed from twenty small fragments of inner bark or epicarp tissues cut around the margin of the necrotic lesions and placed onto PDA (fungi) or PDA+ (*Phytophthora*). Growing colonies were sub-cultured onto PDA, incubated in the dark at 20 °C for 7 days, and identified on the basis of colony appearance and DNA sequence data.

2.7. Data Analysis

The results of the pathogenicity test were checked for normality and then subjected to analysis of variance (ANOVA). Significant differences among mean values were determined using Fisher's least significant differences multiple-range test (p = 0.05) after one-way ANOVA using XLSTAT 2008 software (Addinsoft, Paris, France).

3. Results

3.1. Field Surveys

In all orchards, the widespread presence of severe diseases with a strong impact on productivity and vitality of olive trees was observed. In addition to the sporadic presence of some common olive tree diseases, such as peacock's eye disease and Cercospora leaf spot, several plants, regardless of age or variety, showed a complex symptomatology on the drupes, branches and root system (Figures 1–3).

Figure 1. Overview of the symptoms observed on unripe and ripe drupes: necrotic lesions caused by *Botryospharia dothidea* on unripe fruits (**a**–**c**), necrosis that progressively expands up to the stalk (red arrow) (**d**–**f**), extensive fruit drop observed in July (**g**), drupe with the typical light brown soft rot caused by *Neofusicoccum parvum* (**h**), and reddish-brown mummified drupes with black pycnidia of *B. dothidea* (**i**). From the left to right colony, morphology of *B. dothidea*, *Diplodia mutila*, *Diplodia olivarum*, *Diplodia seriata* and *N. parvum* after 7 days growth at 25 °C on PDA in the dark.

An anomalous unripe fruit drop was found in June, July and early August. The symptomatic drupes were characterized by the initial presence of small sunken necrosis, which progressively expanded up to the stalk, causing an intense carpoptosis (Figure 1a–g). Disease incidence was estimated at up to 100% in several sites, mainly in 2017 and 2019; in the monitored plot (site 17), the number of fallen drupes per square meter varied from 115 ± 28 (19 June 2020) to 214 ± 107 (31 July 2020), with total loss of production in August. Symptoms on ripe fruits varied depending on the fungal species involved but can basically be separated into two main groups: light brown soft rot and reddish-brown rot (Figure 1h,i). In the latter case, the infected fruits wrinkled, mummified and remained attached to the tree. On the epicarp of the mummified fruits, the occurrence of black pycnidia of *B. dothidea,* which represents an inoculum source for the next spring infection, was constantly observed (Figure 1i).

Furthermore, a wilting syndrome was observed in all of the monitored sites (Figure 2). Seasonal dynamics of wilting symptoms on twigs and branches include several stages: initially, olive trees show irregularly scattered light-red necrotic areas of the canopy as the wilted leaves rapidly turn to a rusty color; subsequently the necrotic areas expand and merge, affecting large portions of the canopy. In autumn, the leaves fall, leaving the branches bare (Figure 2a,b). On symptomatic branches, extensive sunken cankers characterized by inner bark and internal wood necrosis were often observed. In the cross section, sunken cankers showed the typical V-shaped necrotic sector caused by *Botryosphaeriaceae* (Figure 2k). In some cases, cankers on the stem and at the collar showed red-brown exudations. At site 27, the incidence of olive trees with new branch dieback

symptoms was found to vary over the five years of investigation from a minimum of 11% in 2018 to a maximum of the 27% in 2020.

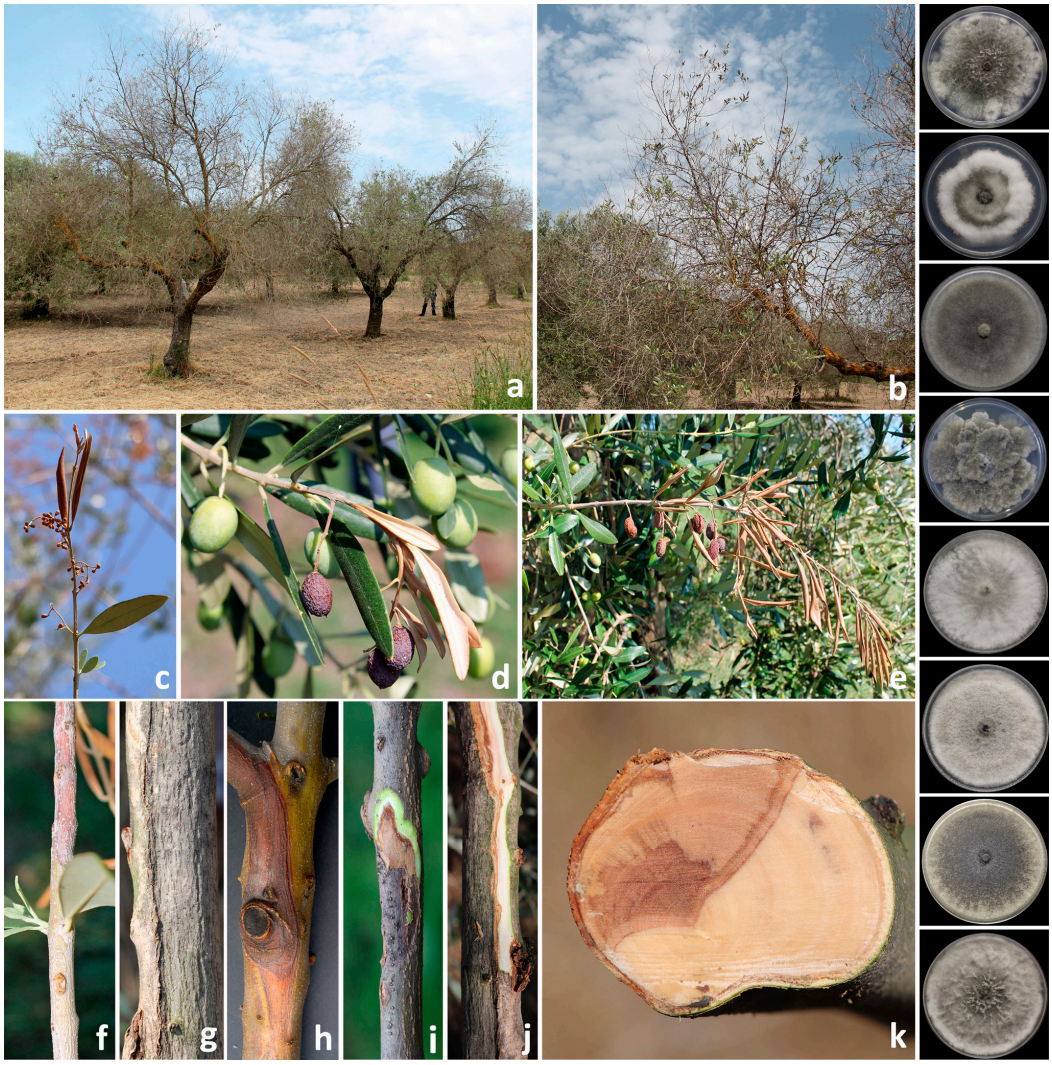

Figure 2. Overview of symptoms observed on the olive tree canopy: extensive dieback with phylloptosis (**a**,**b**), shoot blight (**c**), twig dieback (**d**,**e**), sunken cankers on twigs and branches (**f**–**j**), and V-shaped necrotic sector in cross section (**k**). From top to bottom, colony morphology of *Botryosphaeria dothidea*, *Diplodia fraxini*, *Diplodia mutila*, *Diplodia olivarum*, *Diplodia seriata*, *Diplodia subglobosa*, *Dothiorella sarmentorum* and *Neofusicoccum parvum* after 7 days growth at 25 °C on PDA in the dark.

Finally, in the monitored orchards, several plants showed leaf chlorosis, poor growth and often sudden-death symptoms. The latter symptom was particularly frequent in early autumn. Plants with sudden-death symptoms were easily recognizable as the dark yellow leaves remained attached to the branches even for an entire growing season. The plants characterized by sudden death showed large, necrotic, inner-bark lesions at the collar and main roots, as well as loss of fine roots (Figure 3). Disease incidence was very variable among sites. In site 10, 20% of olive trees showed these typical *Phytophthora* disease symptoms.

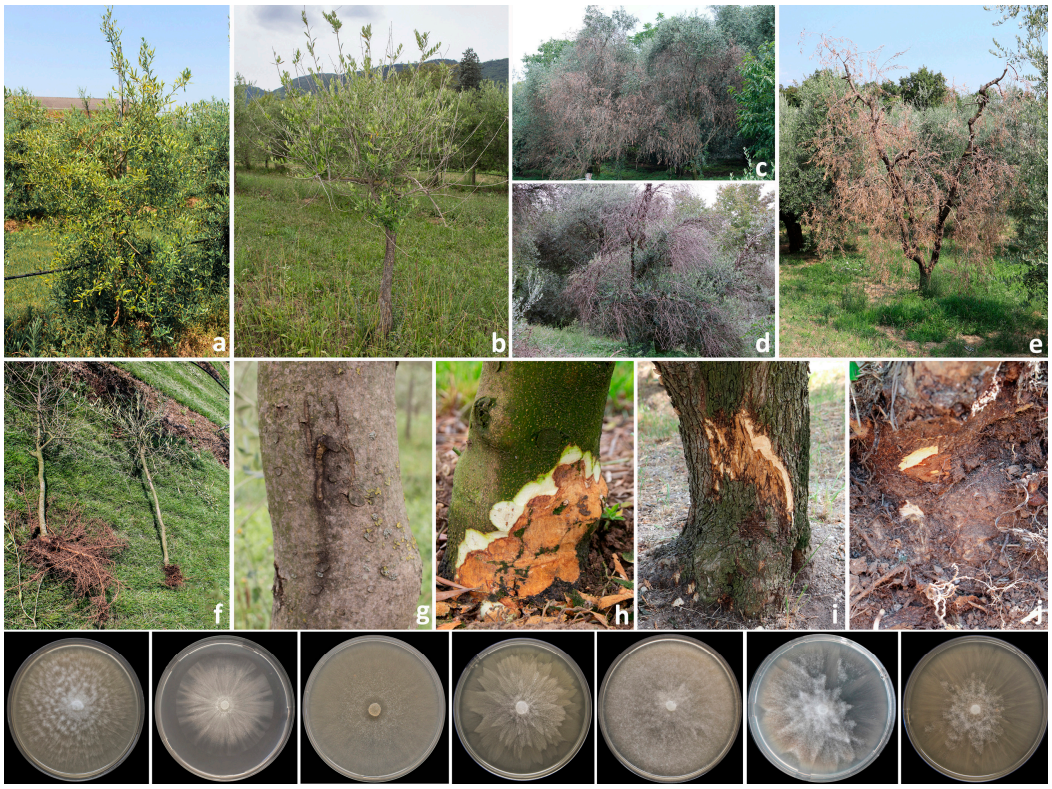

Figure 3. Overview of *Phytophthora*-related symptoms observed on olive trees: chlorosis and increased transparency of the canopy (**a**,**b**), trees with sudden death syndrome (**c**–**e**), root rot and loss of fine roots on 6-year-old plants (**f**), and stem canker and inner bark necrotic lesion at the collar (**g**–**j**). From left to right, the colony morphology of *Phytophthora acerina*, *P. bilorbang*, *P. crassamura*, *P. oleae*, *P. palmivora*, *P. pini* and *P. plurivora* after 7 days growth at 20 °C on CA in the dark.

3.2. Fungal and Fungal-Like Species Associated with Symptomatic Samples

A total of 886 fungal and fungal-like isolates were obtained from the 1064 samples processed; in particular, *Botryosphaeriaceae* and *Peronosporaceae* (oomycetes) were the most abundant families, with 752 and 121 isolates, respectively. All fungal and *Phytophthora* isolates showed mycelial growth and conidia or sporangia production within 30 days. Overall, twenty-three fungi and fifteen *Phytophthora* species were identified based on morphological features and DNA sequence data. The ITS sequence of a representative isolate of each species was deposited in GenBank (Table 2). The complex symptomatology detected in the monitored orchards is due to the attack, often simultaneous, of various pathogens. In particular, the severe drop of unripe drupes is caused mainly by *B. dothidea*. The early attack of *B. dothidea* causes necrotic sunken lesions that progressively expand up to the stalk, determining the premature fall of drupes. In addition to *B. dothidea*, in the rot symptoms detected in autumn, thirteen other *Botryosphaeriaceae* species are involved, albeit with less frequency (Table 2). It is interesting to note that the assemblage of *Botryosphaeriaceae* species associated with symptomatic drupes changes during the growing season, with several species occurring on mature drupes, including the invasive species *N. australe* and *N. parvum* (October–November).

Table 2. Accession numbers deposited in GenBank and number of isolates of each species obtained from unripe drupes (UD), ripe drupes (RD), stem and branches (SB) and roots (R).

Fungal Species	Accession Numbers	Type of Samples				Sites
		UD	RD	SB	R	
Apiospora arundinis	OR284845	2	-	2	-	1, 9
Botryosphaeria dothidea	OR284846	208	73	16	-	6, 10, 15–23, 25–27, 30
Diplodia africana	OR284847	-	1	-	-	27
Diplodia fraxini	OR284848	2	-	15	-	10, 17
Diplodia mutila	OR284849	32	38	12	-	6, 9, 10, 12, 16–21, 27
Diplodia olivarum	OR284850	9	24	15	-	1, 6, 7, 9, 11, 12, 21, 24, 27, 30
Diplodia sapinea	OR284851	-	1	-	-	27
Diplodia seriata	OR284852	31	8	47	-	1–4, 6–8, 10, 11,13, 15–17, 27, 30
Diplodia subglobosa	OR284853	-	4	14	-	1–3, 9, 15, 17, 20
Dothiorella iberica	OR284854	1	-	2	-	10, 16
Dothiorella omnivora	OR284855	-	-	1	-	16
Dothiorella sarmentorum	OR284856	-	3	11	-	1–6, 17
Dothiorella sempervirentis	OR284857	-	-	1	-	6
Eutypa lata	OR284858	-	-	3	-	9, 16
Eutypa leptoplaca	OR284859	-	-	2	-	1, 9
Neofusicoccum australe	OR284860	-	29	1	-	27
Neofusicoccum cryptoaustrale	OR284861	-	11	4	-	27, 29
Neofusicoccum luteum	OR284862	2	5	-	-	27
Neofusicoccum mediterraneum	OR284863	3	5	7	-	27, 28
Neofusicoccum parvum	OR284864	7	18	90	-	1, 2, 4, 5, 6, 8–10, 15–28
Phaeoacremonium iranianum	OR284865	-	-	2	-	14
Phaeoacremonium scolyti	OR284866	-	-	2	-	9, 14
Sardiniella urbana	OR284867	-	1	-	-	17
Phytophthora acerina	OR284868	-	-	-	14	8, 10, 24, 27
Phytophthora bilorbang	OR284869	-	-	-	6	10, 15, 38
Phytophthora cactorum	OR284870	-	-	-	4	11, 17
Phytophthora cinnamomi	OR284871	-	-	-	2	19
Phytophthora citricola	OR284872	-	-	-	1	31
Phytophthora crassamura	OR284873	-	-	-	10	29, 31–33, 36
Phytophthora heterospora	OR284874	-	-	-	5	27, 29, 35
Phytophthora inundata	OR284875	-	-	-	4	29, 31, 32
Phytophthora nicotianae	OR284876	-	-	-	2	15
Phytophthora niederhauserii	OR284877	-	-	-	4	10, 27
Phytophthora oleae	OR284878	-	-	-	6	31, 32, 34
Phytophthora palmivora	OR284879	-	-	-	15	7, 25–27, 33, 34, 37
Phytophthora pini	OR284880	-	-	-	12	11, 15, 18
Phytophthora plurivora	OR284881	-	-	-	31	1, 6, 8, 12, 27
Phytophthora pseudocryptogea	OR284882	-	-	-	5	31, 33, 36, 37, 38

From the 366 branch samples with sunken canker and dieback symptoms, eighteen species of Botryosphaeriaceae (*Botryosphaeria dothidea*, *Diplodia africana*, *D. fraxini*, *D. mutila*, *D. olivarum*, *D. sapinea*, *D. seriata*, *D. subglobosa*, *Dothiorella iberica*, *Do. omnivora*, *Do. sarmentorum*, *Do. sempervirentis*, *Neofusicoccum australe*, *N. cryptoaustrale*, *N. luteum*, *N. mediterraneum*, *N. parvum* and *Sardiniella urbana*), two *Diatrypaceae* (*Eutypa lata* and *Eutypa leptoplaca*), two *Togniniaceae* (*Phaeoacremonium iranianum* and *Phaeoacremonium scolyti*) and one species of *Apiosporaceae* (*Apiospora arundinis*) were isolated. *Neofusicoccum parvum* was the dominant species associated with canker and dieback symptoms in Lombardy, Veneto and Sardinian olive orchards (Table 2). However, the etiology of woody samples was extremely complex, since from the symptomatic branch tissues, several other species were constantly isolated, such as *B. dothidea*, *Diplodia fraxini*, *D. olivarum*, *D. seriata*, *D. subglobosa* and *Dothiorella sarmentorum* (Table 2).

Isolation performed from 133 symptomatic collar and root samples yielded a total of 121 colonies belonging to fifteen *Phytophthora* species (Table 2). The most frequently isolated species were *Phytophthora plurivora* and *P. palmivora*, followed by *P. acerina* and *P. pini* (Table 2). *Phytophthora palmivora* was isolated in 7 out of 27 monitored orchards, including Veneto, Lombardy, Sardinia and Calabria, whereas the other dominant species were isolated in one or two regions. In particular, *P. plurivora* and *P. acerina* were widespread in Veneto and Lombardy, *P. plurivora* and *P. heterospora* in Sardinia and *P. crassamura*, *P. oleae* and *P. palmivora* in Calabria (Figure 4).

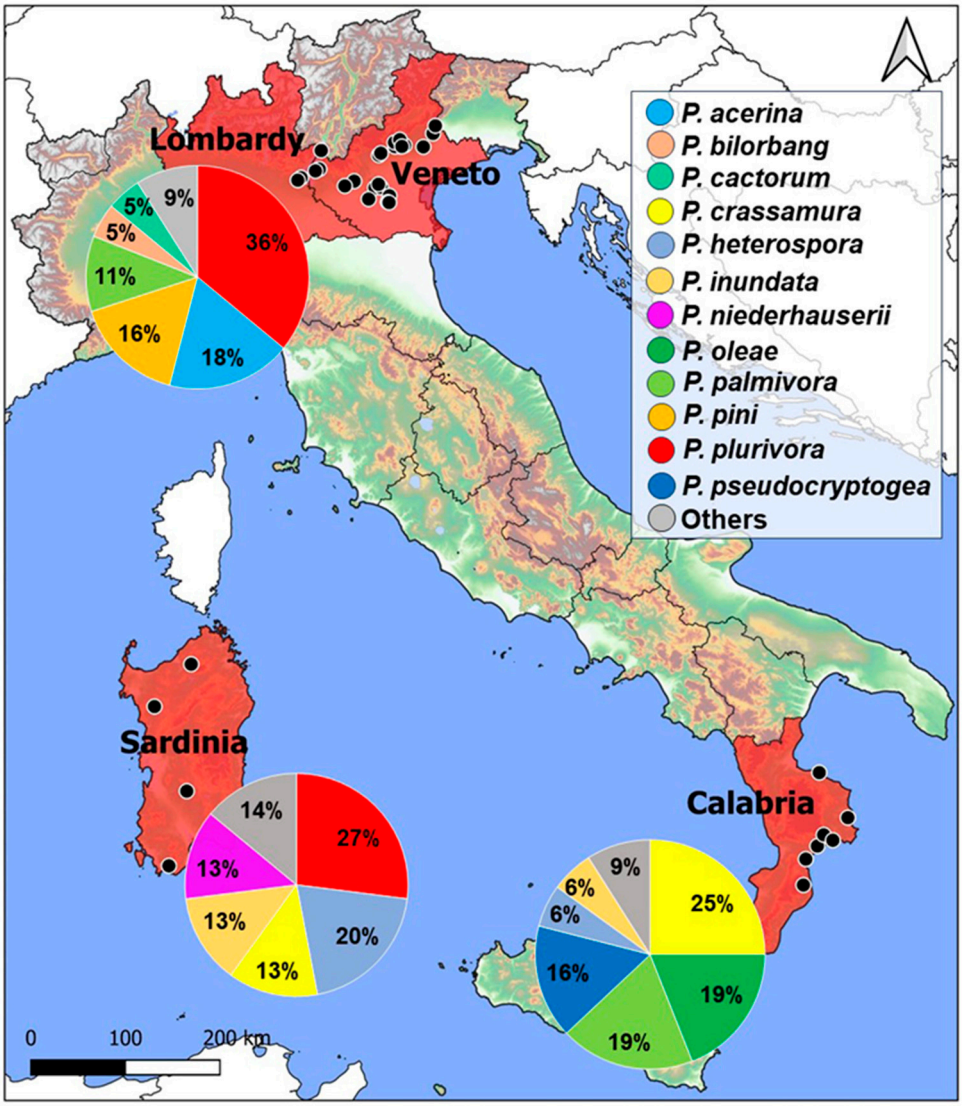

Figure 4. Isolation frequency and distribution of the 12 most common *Phytophthora* species isolated in this study.

3.3. Pathogenicity

In the first assay, all four *Botryosphaeriaceae* species tested were shown to be pathogenic on unripe olive fruits (cv Rasara) under laboratory conditions. A dark-brown, necrotic lesion surrounding the inoculation point was observed 48 h after inoculation, while 7 days later, the necrotic area in some cases extended over the entire surface. Control drupes remained asymptomatic. *Neofusicoccum parvum* produced the most extensive lesions, followed by *D. olivarum*, *B. dothidea* and *D. mutila* (Table 3).

Table 3. Mean lesion size (cm^2) ± standard deviation caused by the four *Botryosphaeriaceae* species on unripe drupes and percentage of positive re-isolations.

Fungal Species	Drupe Lesion Size [1]	Positive Re-Isolation (%)
Botryosphaeria dothidea	2.4 ± 0.8 b	100
Diplodia mutila	2.3 ± 0.9 b	100
Diplodia olivarum	2.7 ± 2.1 b	100
Neofusicoccum parvum	6.3 ± 2.6 a	100
Control	0.0	-

[1] Values with the same letter do not differ significantly at $p = 0.05$, according to LSD multiple range test.

In the pathogenicity assay on branches, all inoculated species, except *Apiospora arundinis*, proved to be pathogenic on olive seedlings, though with significant differences in aggressiveness (Table 4). The most aggressive species was *N. parvum*; at 122 days after inoculation, the isolate N2 caused significantly longer lesions that were externally visible as sunken cankers similar to those observed in the field (Table 4). In addition, even the cankers caused by *D. fraxini*, *D. olivarum* and *D. subglobosa* were characterized by sunken bark lesions, a symptom congruent with field observations.

Table 4. Mean lesion length (mm) ± standard deviation caused by the ten fungal species on olive branches and the percentage of positive re-isolations.

Fungal Species	Length Necrotic Lesion [1]	Wilting Symptoms	Positive Re-Isolation (%)
Apiospora arundinis	1.2 ± 0.4 ef	no	20
Botryosphaeria dothidea	6.1 ± 1.2 de	no	100
Diplodia fraxini	33.5 ± 6.5 b	yes	100
Diplodia olivarum	14.6 ± 2.6 c	no	100
Diplodia seriata	6.2 ± 1.3 d	no	100
Diplodia subglobosa	13.6 ± 2.6 c	no	100
Eutypa lata	6.1 ± 1.2 de	no	80
Eutypa leptoplaca	5.8 ± 1.2 de	no	80
Neofusicoccum parvum	58.2 ± 10.1 a	yes	100
Phaeoacremonium iranianum	5.2 ± 1.2 de	no	60
Control	0.2 ± 0.4 f	no	-

[1] Values with the same letter do not differ significantly at $p = 0.05$, according to LSD multiple range test.

In the inoculation at the collar, the inner bark necrotic lesions caused by *P. pini*, *P. plurivora* and *P. acerina* were consistently larger compared with those of other species and controls, confirming the aggressiveness of these species on olive trees (Table 5). Also, *P. cactorum* and *P. palmivora* caused extensive lesions, whereas *P. inundata* caused only a small brown discoloration, which did not differ statistically from the control.

On all control seedlings, a very small brown discoloration was visible in correspondence to the inoculation point, but the wound had begun to heal.

All fungal and *Phytophthora* species were successfully re-isolated from symptomatic tissue, thus fulfilling Koch's postulates.

Table 5. Mean lesion length (mm) ± standard deviation caused by the thirteen *Phytophthora* species on olive seedlings and percentage of positive re-isolations.

***Phytophthora* Species**	**Length Necrotic Lesion [1]**	**Positive Re-Isolation (%)**
Phytophthora acerina	27.7 ± 4.9 a	100
Phytophthora bilorbang	13.8 ± 5.9 c	100
Phytophthora cactorum	20.1 ± 5.8 b	100
Phytophthora cinnamomi	12.6 ± 2.1 c	100
Phytophthora citricola	7.3 ± 3.8 d	100
Phytophthora crassamura	4.8 ± 2.3 de	100
Phytophthora heterospora	11.3 ± 4.2 c	100
Phytophthora inundata	2.4 ± 0.5 e	80
Phytophthora oleae	7.2 ± 2.5 d	100
Phytophthora palmivora	19.1 ± 4.1 b	100
Phytophthora pini	29.5 ± 5.4 a	100
Phytophthora plurivora	28.7 ± 3.5 a	100
Phytophthora pseudocryptogea	13.1 ± 3.8 c	100
Control	1.6 ± 1.2 e	-

[1] Values with the same letter do not differ significantly at $p = 0.05$, according to LSD multiple range test.

4. Discussion

This study represents the most comprehensive investigation to date on *Botryosphaeriaceae* and *Phytophthora*-related diseases on olive trees in Italy. The results obtained have allowed us to clarify the complex etiology of the emerging diseases affecting olive trees in four Italian regions achieving new insights into the symptomatology caused by *Botryosphaeriaceae* and *Phytophthora* attacks on this host. Specifically, the symptoms caused by the main isolated species often overlapped at site level and in the same tree, making a clear separation often difficult. The often-synergic attack of *Botryosphaeriaceae* and *Phytophthora* species in some cases causes serious economic losses due to tree decline, mortality and fruit yield reduction.

Based on ITS sequence data, twenty-three fungal species belonging to eight genera, including *Apiospora*, *Botryosphaeria*, *Diplodia*, *Dothiorella*, *Eutypa*, *Neofusicoccum*, *Phaeoacremonium* and *Sardiniella*, were identified.

Amon the fungal species, *Botryosphaeriaceae* were the main species isolated from olive trees with branch cankers and fruit rots; this is in accordance with other recent studies in which members of this family were found to be the most aggressive species isolated from olive trees in California, Croatia, Italy, Spain, South Africa, Tunisia and New Zealand [14,21,25,28,37,38]. Field surveys and pathogenicity tests have shown that *N. parvum* and *D. olivarum* are the main pathogens involved in olive cankers and dieback in the monitored orchards, although several other species, and in particular, *D. fraxini*, *D. olivarum*, *D. seriata* and *D. subglobosa*, can play an important role in the etiology of the symptoms observed at a local scale. *Neofusicoccum parvum* and *Diplodia olivarum* have recently been detected as the key agents of a new wilting syndrome on oleaster trees in Italy [39]. Species within the genus *Neofusicoccum* and *Diplodia* have been reported as pathogens causing cankers, dieback and mortality on several woody hosts worldwide [40]. The diversity and pathogenicity of *Botryosphaeriaceae* species associated with branch dieback of olive trees detected in this study suggest that no one single agent is involved in the etiology but that it is the result of multiple infections of different *Botryosphaeriaceae* species, which are variable in assemblages among regions and sites. The co-occurrence of different *Botryosphaeriaceae* associated with cankers with a V-shaped necrotic sector and dieback symptoms is a recurring aspect in the pathosystems in which these pathogens are involved in both forestry and agriculture [41–45]. The complex etiology detected in this study is in accordance with the results obtained by Urbez-Torres et al. [14] in California. Among the species isolated from cankered branches, the presence of *D. fraxini* and *D. subglobosa*, two aggressive pathogens involved in the ash dieback etiology, emerges [46,47]. This is the first report of these two *Diplodia* species as olive pathogens.

Further investigations are needed to establish the pathogenicity of *S. urbana*, *Do. omnivora*, *Do. sarmentorum*, *Do. sempervirentis* and *D. africana* on olive trees. *Sardiniella urbana* is a rather rare pathogenic species known only on hackberry [48]. This represents the first report of *S. urbana* on olive. Given that the olive orchard sampled in Veneto was surrounded by hackberry, it is likely that high inoculum pressure resulted in a few infections of the olive trees by this pathogen. The genus *Dothiorella* was established by Saccardo [49] and currently encompasses several endophytic, saprobic and plant pathogenic species associated with a wide range of woody hosts worldwide, including olive [50]. This is the first report of *D. africana*, *Do. omnivora*, *Do. sarmentorum* and *Do. sempervirentis* on olives.

Until 2005, *B. dothidea* was the only *Botryosphaeriaceae* species known as the causal agent of olive drupe rot, "Dalmation disease" [23]. Lazzizera et al. [22], during an extensive survey of drupe rot in southern Italy, found that in addition to *B. dothidea,* other species of *Botryosphaeriaceae*, namely *D. olivarum*, *N. australe*, *N. vitifusiforme*, *N. parvum* and *N. mediterraneum*, cause the same symptoms on olive drupes. The most common species was *B. dothidea*, which was isolated from 34% of the rotted drupes. This finding agrees with the results obtained in this study. Field surveys conducted in this study showed that *B. dothidea* invades the drupes in the early stage of fruit setting, and new infection can occur during all olive fruit growth and developmental stages. On unripe fruits, *B. dothidea* was the dominant species, while in the last stages of drupe development, several *Botryosphaeriaceae* species can cause fruit rot. However, a clear difference in symptomatology was noted between attacks on drupes of *B. dothidea* and *N. parvum* (Figure 1h,i). These differences were also confirmed in pathogenicity assays. Unlike the results of Lazzizera et al. [22], on ripe drupes, we found almost exclusively pycnidia of *B. dothidea* and rarely those of *N. australe*. All *Botryosphaeriaceae* species isolated from drupes are known to produce bioactive secondary metabolites [51–54]. The identification of the compounds, including pigments that could cause serious risks for human health and significant loss in the commercial value of oil, have several practical implications for health care and the improvement in olive oil quality in Mediterranean countries.

Diatrypaceae and *Togniniaceae* are often reported as weak or opportunistic olive pathogens in different countries [55,56]. In the pathogenicity assay, *Eutypa lata*, *E. leptoplaca* and *Phaeoacremonium iranianum* have been confirmed to be pathogenic in olives, although their role in these emerging diseases appears marginal. *Eutypa leptoplaca* is reported here for the first time as an olive pathogen. This species has been reported as a grapevine pathogen in California [57].

Fifteen *Phytophthora* species belonging to six out the twelve major *Phytophthora* clades were identified. The genus *Phytophthora* over the last three decades has expanded rapidly to reach the current 220 species [58], of which 15 have been isolated in this study. The presence of the fifteen species in the Italian regions was discontinuous, suggesting that different *Phytophthora* species can cause the same symptoms on olive trees. In particular, in north Italy, three species within clade 2, *P. acerina*, *P. plurivora* and *P. pini*, were widespread. This confirms the results of a preliminary study conducted in Veneto (north Italy) [30]. *Phytophthora plurivora*, like some other species in clade 2, is a polyphagous pathogen whose host range comprises plants of different families, including *Betulaceae*, *Fagaceaea* and *Oleaceae* in forest ecosystems, horticulture, and less frequently in agriculture [30,59–62].

Phytophthora acerina was originally described in 2014 on *Acer pseudoplatanus* in Lombardy (north Italy), and a few years later, in 2018, causing sudden death to olive trees in Veneto [30] and *Alnus glutinosa* in Sardinia in 2020 [63]. The high virulence displayed by *P. acerina* on inoculated olive seedlings, as well as on *Alnus* spp., proves it to be an extremely aggressive pathogen. The host and geographic range are still unknown; recently, it has been isolated in Slovenia along the Italian border (Bregant, Linaldeddu and Ogris unpublished data) and in the Marche region (central Italy) (Bregant, Linaldeddu and Murolo unpublished data).

Phytophthora pini was originally described as a new species in 1925 but without a Latin description (Leonian, 1925). In 2011, Hong et al. [64] examined ex-authentic cultures of

P. pini, proving that they were identical to *P. citricola* subgroup I. Therefore, *Phytophthora pini* has been resurrected to distinct species status and formally redescribed with a Latin description. Many aspects related to the ecology of this pathogen are unknown, and to properly evaluate its potential impact on olive and oil production in the Mediterranean region, further studies are necessary.

In addition to *P. plurivora*, in Sardinia, other two species *P. heterospora* and *P. crassamura* were frequently isolated. *Phytophthora heterospora* is a sister species of *P. palmivora,* formally described on olive trees and other hosts in 2021 [31]. In the compared pathogenicity assay, *P. heterospora* proved to be moderately aggressive on olives, although less so than *P. palmivora*. *Phytophthora heterospora* is common on nursery plants [65] but has recently also been isolated from declining oleaster and narrow-leaved mock-privet (*Phillyrea angustifolia*, family *Oleaceae*) in different natural ecosystems in Sardinia (Linaldeddu, unpublished data). *Phytophthora crassamura* was formally described as a distinct species in 2015 from different host species of the Mediterranean maquis in the archipelago of La Maddalena [66], but it is actually widespread. Recently, it has been reported in several restoration sites in California [67] and globe artichoke plants in the main growing area in Sardinia [68]. Almost all findings of *P. crassamura* came from geographic areas characterized by a Mediterranean climate. *Phytophthora crassamura* was the dominant species in the Calabrian olive groves. In this region, two other species, *P. oleae* and *P. bilorbang*, were constantly isolated from symptomatic trees.

Phytophthora oleae was originally reported, causing fruit rot in olive orchards in Italy and root rot in wild-olive trees in Spain [69,70]. In this study, *P. oleae* emerged as one of the most common pathogens involved in root rot symptoms. Its ability to infect roots, stem and also fruits highlights the risk posed by this species on olive trees.

Phytophthora bilorbang was formally described in Australia in 2012 [71], but it was previously informally designated as *Phytophthora* taxon oaksoil [72] and is known to occur in France [73] and Oregon [74]. In the present study, *P. bilorbang* was isolated in both Veneto and Calabria olive orchards. This is consistent with a previous record [75], in which this pathogen was reported on olive trees (cv. Nera di Gonnos) with leaf chlorosis, severe defoliation, and root rot symptoms in an experimental orchard in Calabria. *Phytophthora bilorbang*, like several other members of clade 6, has a prevalently aquatic lifestyle and is frequently recovered from streams and riparian ecosystems as an opportunistic pathogen [71]. Given its ecology and moderate pathogenicity, the innovative olive growing systems (intensive and super-intensive) could favor the attacks of this opportunistic species.

The other *Phytophthora* species were recovered less frequently. Among these, *Phytophthora cactorum*, *P. cinnamomi*, *P. citricola* and *P. niederhauserii* were detected for the first time on declining olive trees worldwide. The discovery of *P. niederhauserii* in Sardinian olive orchards is of particular concern due to its high virulence [76] and the recent discovery from the same oleaster trees showing the new wilting syndrome caused by *N. parvum* ([39]; Linaldeddu unpublished).

The current trend of discovering an increasing number of pathogenic *Phytophthora* species in agricultural crops [77] emphasizes how important efforts need to be made to develop appropriate management strategies to limit the impact on the quality and quantity of agricultural productions. Our finding has contributed to expanding knowledge on the biodiversity of *Phytophthora* species associated with olive trees in Italy, which currently include sixteen species.

Among the species evaluated in the pathogenicity tests, *Apiospora arundinis* (syn. *Arthrinium arundinis*) and *P. inundata* were the only species that did not cause any disease symptoms on inoculated seedlings, and both were re-isolated with a low frequency. *Apiospora arundinis* has been detected from branch cankers together with *N. parvum*, which in the pathogenicity test reproduced the symptoms observed in nature, suggesting an endophytic behavior of *A. arundinis* on olive trees. *Phytophthora inundata* is reported as an opportunistic pathogen of woody hosts in riparian ecosystems that is able to cause sporadic disease outbreaks on susceptible hosts after soil flooding or waterlogging [78].

5. Conclusions

In conclusion, in this study, the high diversity of *Botryosphaeriaceae* and *Phytophthora* species has been associated with olive tree diseases in Italy. The complexity of the etiology detected in the monitored orchards, together with the results from the pathogenicity assays, highlight that several *Botryosphaeriaceae* and *Phytophthora* species could represent a new emerging threat to the olive and oil industry in Italy. The overlap of symptoms caused by different pathogens suggests that only through a holistic diagnostic approach is it possible to identify the species involved.

Olive groves are long-term investments that require adaptive management strategies. The findings obtained in this study emphasize that new management strategies need to be developed to protect this iconic fruit crop. Currently, there are a few effective chemical products to prevent or cure the attacks of *Botryosphaeriaceae* and *Phytophthora* on olive trees.

Author Contributions: Conceptualization, B.T.L.; methodology, B.T.L. and C.B.; investigation, B.T.L., C.B., G.R., L.M. and T.V.; data curation, B.T.L., L.M. and C.B.; writing—original draft preparation, B.T.L.; writing—review and editing, B.T.L., C.B., G.R., L.M. and T.V.; funding acquisition, B.T.L. and L.M. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by Regione Veneto and Servizio Fitosanitario Regionale del Veneto, by the Land Environment Resources and Health (L.E.R.H.) doctoral course (University of Padova), and by Fondo di Ateneo 2020, an internal funding from the University of Sassari.

Institutional Review Board Statement: Not applicable.

Data Availability Statement: Not applicable.

Acknowledgments: The authors thank Chiara Padovan, Roberto Callegaro (Frantoio Evo del Borgo), Enzo Gambin (AIPO), Bruno Bernardi, and Sergio Carraro (Regione Veneto) for assistance during field surveys and sampling.

Conflicts of Interest: The authors declare no conflict of interest.

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