Investigations on Fungi Isolated from Apple Trees with Die-Back Symptoms from Basilicata Region (Southern Italy)

Stefania Mirela Mang 1,*, Carmine Marcone 2, Aurel Maxim 3 and Ippolito Camele 1,*

1 School of Agricultural, Forestry, Food and Environmental Sciences (SAFE), University of Basilicata, Viale dell’Ateneo Lucano 10, 85100 Potenza, Italy
2 Department of Pharmacy, University of Salerno, Via Giovanni Paolo II 132, 84084 Salerno, Italy; cmarcone@unisa.it
3 Department of Engineering and Environmental Protection, Faculty of Agriculture, University of Agricultural Sciences and Veterinary Medicine, No. 3-5, Calea Manastur Street, 400372 Cluj-Napoca, Romania; aurel.maxim@usamvcluj.ro
* Correspondence: stefania.mang@unibas.it (S.M.M.); ippolito.camele@unibas.it (I.C.); Tel.: +39-0971205519 (S.M.M.); +39-0971205544 (I.C.)

Abstract: Val d’Agri is an important orchard area located in the Basilicata Region (Southern Italy). A phenomenon affecting cv. “Golden Delicious” apples which lead to tree death has been observed in the past several years in this area. This phenomenon has already been detected in about 20 hectares and is rapidly expanding. The symptoms observed were “scaly bark” and extensive cankers, mainly located in the lower part of the trunk, associated with wood decay. Dead plants ranged from 20% to 80% and, in many cases, trees were removed by farmers. In order to identify the causes of this phenomenon, investigations were started in autumn/winter 2019. In order to determine the possible causal agents, fungal and bacterial isolations, from symptomatic tissues, were performed in laboratory. Bacterial isolations gave negative results, whereas pure fungal cultures (PFCs) were obtained after 3–4 passages on potato dextrose agar (PDA) media. Genetic material was extracted from each PFC and amplified by PCR using three pairs of primers: ITS5/4, Bt2a/Bt2b and ACT-512F/ACT-783R. The amplicons were directly sequenced, and nucleotide sequences were compared with those already present in the NCBI GenBank nucleotide database. All isolated fungi were identified based on morphological features and multilocus molecular analyses. Neofusicoccum parvum, Diaporthe eros and Trametes versicolor were most frequently isolated, while Pestalotiopsis funerea, Phomopsis spp. and Diaporthe foeniculina were less frequently isolated. All nucleotide sequences obtained in this study have been deposited into the EMBL database. Pathogenicity tests showed that N. parvum was the most aggressive fungus, while Phomopsis sp. was demonstrated to be the less virulent one. All the investigated fungi were repeatedly reisolated from artificially inoculated twigs of 2-year-old apple trees, cv. “Golden Delicious”, and subsequently morphologically and molecularly identified. The role played by the above-mentioned fungi in the alterations observed in field is also discussed.

Keywords: apple die-back; canker; fungi; multi-loci phylogeny; wood decay

1. Introduction

Apple die-back syndrome is a complex disease initially characterized by a stunted appearance of the plants and the presence of chlorosis symptoms on leaves. As the disease develops, cracks and necrotic lesions of the cortex appear mostly at the base of the stem and at the grafting point. Subsequently, “scaly bark” and extensive cankers, generally located in the lower part of the trunk develop, which are also associated with wood decay, and the progressive death of the trees is registered. Many authors worldwide investigated the apple die-back syndrome, attributing it to different causal agents, such as various phytopathogens or other possible physiological causes [1–5]. Furthermore, in 2011, Cloete et al. [6] reported the presence of the die-back syndrome and cankers on apples and
pears in South Africa, from which they isolated several fungal pathogens belonging to the Diplodia, Phaeoacremonium, Phomopsis, Neofusicoccum and Eutypa genera. A very recent study by Di Francesco et al. [5], which characterized, in Brazil, apple cultivars’ susceptibility to Neofusicoccum parvum (Pennycook & Samuels) P.W. Crous, Slippers & A.J.L. Phillips, stated that due to climate change, this fungus is emerging as a new pathogen on species of the Rosaceae plant family. An apple tree die-back syndrome causing severe tree losses was also observed in the main apple producing regions in Tunisia, as described by Souli et al. [7]. The authors identified, both morphologically and molecularly, Phytophthora and Pythium species as being the causal agents and the factors that promoted apple tree die-back. They also showed that soil salinity contributed to increase the disease severity [7].

In Italy, apple cultivation is mainly concentrated in the northeast part of the country, specifically in Trentino Alto Adige/Südtirol region. This area comprises about half of the harvested hectares and has an intensive growing system, producing almost 70% of the Italian apples. However, some other Italian regions such as Veneto, Emilia-Romagna, Piedmont, Campania and Basilicata also successfully cultivate apple trees with quite relevant productions. Overall, in 2019 in Italy, the surface cultivated with apple was about 58,000 hectares and the apple production reported in the same year was about 23 million quintals [8].

Due to the economic importance of this crop in Italy, many studies regarding its cultivation and phytosanitary status have been performed. The phytosanitary surveys on apples cultivated in Bolzano area, was investigated by Lindner [9]. The author reported the cortical damage of apple cvs. “Gala”, “Golden Delicious” and “Red Delicious”, in spring 2007, and alterations very much resembling the “blister bark” and “paper bark” symptoms often associated the with withering and drying of the branches were also described. Nevertheless, the author reported that they did not isolate any bacterial or fungal pathogen from the plants, concluding that the cause of the observed symptoms was probably to be found among particularly unfavorable climatic conditions registered during the winter and spring periods. The same author also observed damages at the grafting point level and identified two fungi, known as canker agents, namely Phomopsis mali and Diplodia malorum.

The apple tree die-back syndrome was reported during 2008–2009 in many apple orchards in the north of Italy and since that period many other trees have become infected, especially young plants.

The phytosanitary status of the apples from Trentino region (Northern Italy) was investigated by Prodorutti et al. [10]. The authors reported an increase in the die-back symptoms on apple trees, showing that plants were usually stunted with cracking and necrosis in the lower part of the trunk and on the graft union site. The trees died during the growing season. Furthermore, the same authors reported that the incidence of the disease was, in some cases, very high (reaching almost 80%), and that the most affected trees were the youngest ones of about 2–5 years old that had been subjected to various types of stress. One bacterium, Pseudomonas syringae pv. syringae, and a few fungi, such as Phomopsis spp., Neonectria spp. And some Botryosphaeriaceae, were isolated from trunk tissues, taken from symptomatic trees. Their role in the syndrome expression was also demonstrated, even if they appeared not to cause the death of the artificially inoculated branches [10].

The Val d’Agri area, located in the Basilicata Region (Southern Italy), has geographical and climatic conditions that favor apple cultivation. In particular, “Fuji” and “Golden Delicious” apple cultivars are mostly grown in the area. Apple orchards belong to private farmers and are kept as small-scale cultivation systems. During the autumn/winter 2019, in the Val d’Agri area, on about 20 hectares cultivated with apples, symptoms characterized by “scaly bark” and extensive cankers, mainly located in the lower part of the trunk and associated with wood decay, were observed. Additionally, dead plants ranged from 20 to 80%, and in many cases, trees were removed by farmers. In order to identify the causes of this phenomenon, investigations were started in 2019. It is to be mentioned that despite good prevention and control measures employed so far against the key diseases on fruit
trees in the south of Italy, including the Basilicata region, knowledge about the distribution and the pathogens involved in apple die-back syndrome is still missing. These data are very important since the presence of the die-back syndrome could economically affect the growers in the region. The identification and characterization of various fungal and bacterial pathogens attacking fruit trees, including apples, were initially based on only the morphological features of the pure cultures obtained in vitro [11,12]. However, over time, despite the ease of application, the morphological features proved to be inefficient to further classify fungal and bacterial pathogens. Therefore, other solutions, including molecular approaches, were investigated in order to identify and characterize the phytopathogens associated with the die-back symptoms [6,7,13,14]. Nowadays, several gene regions or genes, such as the Internal Transcribed Spacer (ITS) of the ribosomal DNA (rDNA), β-tubulin (TUB-2) and actin (ACT) protein-coding genes, are extensively utilized to identify and characterize phytopathogens [15–23].

The aim of the present study was to investigate the die-back syndrome on apple orchards from the Val d’Agri area (Basilicata region, Southern Italy). More precisely, the main objectives of the present study were to: (1) identify fungi or bacteria eventually associated with the die-back symptoms observed on apple trees; and (2) perform pathogenicity tests on apple trees in order to verify the involvement of the identified pathogens in the apple die-back disease observed in the Val d’Agri area.

2. Results

2.1. Pathogens Isolations

Pure culture fungal isolates on PDA media from die-back symptomatic material obtained in this study were selected for further characterization through morphological and cultural characteristics, DNA sequencing and phylogenetic analysis (Table 1).

From apple die-back symptomatic samples, the above-described fungi were isolated with different frequencies. Among the most frequently isolated fungi were the *N. parvum*, with a 55% isolation frequency (IF%), followed by *D. eres*, with a 15% IF, and *T. versicolor*, with a 14% IF. All the other fungi were less frequently isolated with an IF ranging from 10–12%, except for *Phomopsis* spp., which was very rarely isolated (<5% IF).

Despite repeated trials to isolate bacteria from symptomatic apple wood, no bacterial colonies were ever obtained. During the investigation for the identification of the apple die-back disease cause no symptom or damage of the root system were noticed. Furthermore, all isolation attempts, performed from roots taken from the symptomatic apple trees, gave negative results.

2.2. Morphological Identification

Based on their cultural and morphological features, pure fungal isolates were classified in five distinct genera: *Neofusicoccum* [24], *Diaporthe* [25–28], *Trametes* [29–33], *Pestalotiopsis* [34–36] and *Phomopsis* [6,37–40] (Table 1 and Figure 1). In particular, in the case of *Diaporthe* grayish or white colonies on PDA and alpha and beta conidia were observed; in the case of *Neofusicoccum*, grey-black colonies and fusiform conidia, nonseptate when young and bisepate ellipsoidal (partially light brown with a darker middle center) when old, were detected. In the case of *Phomopsis*, white colonies and alpha and beta conidia were observed. For *Pestalotiopsis*, reddish colonies and 4-septate conidia, fusiform to ellipsoid and straight to slightly curved, were noticed. *Trametes* genus was identified based on white colonies and the presence of clavate basidia with an inflated epibasidial segment, 4-spored, clamped at the base and basidiospore cylindrical in large spores with slightly inflated top, ellipsoid to ovoid.
Table 1. Fungal isolates obtained during this study with their respective GenBank accession numbers and percentage of identity when compared to reference nucleotide sequences for the same species from the NCBI nucleotide database.

| Isolate Name | Species        | GenBank Accession Number * | GenBank Accession Number * | Identity (%) * | Identity (%) * | Identity (%) * |
|--------------|----------------|----------------------------|----------------------------|----------------|----------------|----------------|
| Noefp1       | Neofusicoccum parvum | LR757960 OU022063 OU023206 | >99–100                    | >99            | 100            |
| Noefp2       | N. parvum       | LR757961 OU022064 OU023207 | -//-                       | -//-           | -//-           |
| Noefp3       | N. parvum       | LR757962 OU022065 OU023208 | -//-                       | -//-           | -//-           |
| Noefp4       | N. parvum       | LR757963 OU022066 OU023209 | -//-                       | -//-           | -//-           |
| Noefp5       | N. parvum       | LR757964 OU022067 OU023210 | -//-                       | -//-           | -//-           |
| Noefp6       | N. parvum       | LR757965 OU022068 OU023211 | -//-                       | -//-           | -//-           |
| Noefp7       | N. parvum       | LR757966 OU022069 OU023212 | -//-                       | -//-           | -//-           |
| Tramtv1      | Trametes versicolor | LR759930 - - | >99–100                    | -             |
| Tramtv2      | T. versicolor   | LR759931 - - | -//                        | -             |
| Tramtv3      | T. versicolor   | LR759932 - - | -//                        | -             |
| Tramtv4      | T. versicolor   | LR759933 - - | -//                        | -             |
| Tramtv5      | T. versicolor   | LR759934 - - | -//                        | -             |
| Tramtv6      | T. versicolor   | LR759935 - - | -//                        | -             |
| Diapore1     | Diaporthie res  | OU020696 OU022056 OU023199 | >99                       | >99–100       | >99            |
| Diapore2     | D. res          | OU020697 OU022057 OU023200 | -//-                      | -//-           | -//-           |
| Diapore3     | D. res          | OU020698 OU022058 OU023201 | -//-                      | -//-           | -//-           |
| Diapore4     | D. res          | OU020699 OU022059 OU023202 | -//-                      | -//-           | -//-           |
| Diaporf1     | Diaporthie foeniculina | OU020700 OU022060 OU023203 | >99–100       | 100            | >99            |
| Diaporf2     | D. foeniculina  | OU020701 OU022061 OU023204 | -//-                      | -//-           | -//-           |
| Diaporf3     | D. foeniculina  | OU020702 OU022062 OU023205 | -//-                      | -//-           | -//-           |
| Pestf1       | Pestalotiopsis funerea | OU020703 OU022070 | >99            | >99            |-             |
| Pestf2       | P. funerea      | OU020704 OU022071 - | -//                        | -//-           | -             |
| Pestf3       | P. funerea      | OU020705 OU022072 - | -//                        | -//-           | -             |
| Phomp1       | Phomopsis sp.   | OU026160 OU220703 OU023204 | >99–100       | >99            |-             |
| Phomp2       | Phomopsis sp.   | OU026161 OU022057 | -//-                      | -//-           | -             |
| Phomp3       | Phomopsis sp.   | OU026162 OU022062 | -//-                      | -//-           | -             |

Note: * The percentage of identity was established after comparing the nucleotide sequences from this study with at least two of the reference species existent in the database for each fungal species. ** ITS = Nuclear ribosomal internal transcribed spacer regions; TUB-2 = β-tubulin 2 gene; ACT = actin gene. "-" = no data were obtained/or exist in the GenBank nucleotide database. "-//" = identical values as those in the previous row are reported.
Figure 1. Pure fungal cultures on PDA obtained from samples of apples with dieback symptoms. 
(a,g) = *Trametes versicolor*; (b,h) = *Diaporthe eres*; (c,i) = *Diaporthe feoniculina*; (d,j) = *Pestalotiopsis funerea*; 
(e,k) = *Phomopsis* spp.; (f,l) = *Neofusicoccum parvum*.

2.3. Molecular Characterization

The PCR amplifications for each gene investigated yielded amplicons of expected sizes: ITS5/ITS4 (~700 bp), *tub-2* (~500 bp) and *ACT* (~300 bp), which, after direct sequencing in both directions, using the same primers as for the amplification, led to 26 nucleotide sequences (Table 1). A megablast search, excluding “uncultured/environmental sample sequences”, performed in the NCBI’s nucleotide database (www.ncbi.org, accessed on 12 January 2022) for all nucleotide sequences obtained in this study, identified at Genus level all fungal isolates (Table 1).

2.4. Phylogenetic Analysis

Single locus analysis gave consistent results for all three loci (ITS, *tub-2* and *ACT*), and the topology of trees was congruent in terms of species grouping. All sequences obtained in this study have been deposited in the European Molecular Biology Laboratory (EMBL-EBI) nucleotide database (www.ebi.ac.uk, accessed on 12 January 2022) and their GenBank accession numbers are presented in Table 1.

The final alignment dataset, for the ITS region, was composed of a total number of 658 characters. It contained 58 nucleotide sequences, including five outgroup species, namely: *Diaporthella corylina* (acc. no. KC343004) utilized for *Diaporthe* fungi; *Valsa japonica* (acc. no AF191185) for *Phomopsis* sp. fungi; *Sordaria alcina* (acc. no. AY681198) for the *Pestalotiopsis* sp. Fungal group; *Grifola frondosa* (acc. No. AY049140) utilized for *Trametes* sp. Fungi; and for the *Neofusicoccum parvum* group, *Diplodia seriata* (acc. no. MH221102). Phylogenetic analysis based on the ITS region variation showed that fungal isolates from the same species clustered together into the same clade and, as expected, outgroup species were placed separately from the other groups (Figure 2).
Figure 2. Molecular phylogenetic tree obtained through the neighbor-joining (NJ) method, based on the 58 ITS region sequences data (658 bp) from fungal isolates in the present study and published sequences. Five fungal species (Diaporthella corylina, Valsa japonica, Sordaria alcina, Grifola fondosa and Diplodia seriata) were used as outgroups in the analysis. The optimal tree with the sum of branch length = 1.38532669 is shown. The confidence probability estimated using the bootstrap test (1000 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Tajima–Nei method and are in the units of the number of base substitutions/site. Scientific names of the fungi along with collection place, isolate abbreviation and GenBank AC number are shown in the trees.
Overall, the 53 nucleotide sequences obtained in this study, based on the ITS region sequence analysis, clustered in two clades which contained all six fungal species. Two of these species belonged to *Diaporthe* genus, namely, *Diaporthe eres* Nitschke and *D. foeniculina* (Sacc.) Udayanga & Castl., and others have been identified as *N. parvum*, *Pestalotiopsis funerea* (Desm.) Steyaert, *Phomopsis* sp. Sacc. & Roum. and *T. versicolor* (L.) Lloyd (Figure 2). Within the phylogenetic tree, the first clade grouped together five of the species previously mentioned, while the second clade contained only one species, *Trametes versicolor*, clearly separated from the others. Additionally, the fungal species isolated and identified in this study were positioned close to similar reference species downloaded from the GenBank for each fungus, and their location was well supported by very high (97–100%) bootstrap values (Figure 2). The ITS data confirmed the previous preliminary fungal identification based on morphological features.

Since ITS alone does not provide sufficient resolution to exactly classify fungi at species level, other loci were considered for the phylogeny-based identification of the taxa investigated in this study. In particular, the β-tubulin (*tub-2*) gene, a very well-known molecular locus extensively used in phylogenetic studies of phytopathogenic fungi [35,38–40], was examined. A total number of 39 nucleotide sequences of the *tub-2* partial gene were obtained and employed along with the species *D. corylina*, *B. dothidea* and *S. alcina*, used as outgroups in phylogenetic analysis, which was carried out using the NJ method, as performed for the ITS (Figure 3).

Results from the phylogenetic analysis of the fungal species from this study, based on the *tub-2* gene, have shown that they grouped together with similar species from the GenBank database. A better separation within the clades and subclades compared to what obtained from the ITS was also observed (Figure 3). Two separate clades were obtained from the nucleotide sequences investigated in this study. In one, *Neofusicoccum* isolates grouped together into the same subgroup, which was very well supported (98% bootstrap support value), and were separated from the *Pestalotiopsis* subclade, which was also highly supported (99% bootstrap support value), whereas the *Diaporthe* isolates were all placed in a separate clade and were clearly distinguished in two subclades as species, e.g., *D. eres* and *D. foeniculina*, both sharing their vicinity with the same outgroup, *D. corylina* (Figure 3). All fungal species based the *tub-2* gene variation were grouped together with the same species from the GenBank with an elevated bootstrap support (98–99%). The separation of the fungal species within each clade or subclade, strongly supported by high bootstrap values, was 99% for *D. eres*, *D. foeniculina* and *N. parvum* and 98% in the case of *P. funerea* (Figure 3).

Regarding the third gene, namely actin, despite our repeated PCR trials, amplicons could not be obtained for all fungal species (Figure 4). Therefore, the alignment for the *ACT* gene used in phylogenetic analysis contained 298 characters and involved only 24 nucleotide sequences. Moreover, *ACT* gene analysis showed that the two *Diaporthe* species identified in this study along with their reference species from the GenBank were well separated from the *Neofusicoccum* sp. isolates and, thus, clustered in two different clades also supported by very high bootstrap values of 94% and 99%, respectively. In addition, *Neofusicoccum* isolates were grouped together with their reference species with a 99% bootstrap support value (Figure 4). The phylogenetic reconstruction based on the *ACT* gene reconfirmed the molecular identification based on other loci at species level for each fungal species analyzed and also was in concordance with the preliminary morphological characterization.
Figure 3. Molecular phylogenetic tree obtained through the neighbor-joining (NJ) method, based on the 39 tub-2 gene sequences data (500 bp) from fungal isolates in the present study and published sequences. The fungal species (D. corylina, B. dothidea and S. alcina) were used as outgroups in the analysis. The optimal tree with the sum of branch length = 1.08594872 is shown. The confidence probability estimated using the bootstrap test (1000 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Tajima–Nei method and are in the units of the number of base substitutions/site. Scientific names of the fungi along with collection place, isolate abbreviation and GenBank AC number are shown in the trees.
Figure 4. Molecular phylogenetic tree obtained through neighbor-joining (NJ) method based on the 24 ACT gene sequences data (298 bp) from fungal isolates in the present study and published sequences. The fungal species (*D. helianthi* and *B. dothidea*) were used as outgroups in the analysis. The optimal tree with the sum of branch length = 0.90526841 is shown. The confidence probability estimated using the bootstrap test (1000 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Tajima–Nei method and are in the units of the number of base substitutions/site. Scientific names of the fungi along with collection place, isolate abbreviation and GenBank AC number are shown in the trees.

Multilocus phylogenetic analyses for *Diaporthe*, *Neofusicoccum* and *Pestalotiopsis* spp. isolates showed that the topology of the trees was congruent in terms of grouping for all fungal species investigated, supporting the single locus phylogenetic outcomes (Figures S1–S3 in Supplementary Material).

2.5. Pathogenicity Trial

In the artificial inoculations test, on twigs of 2-year-old apple trees (cv. “Golden Delicious”), using the six fungi investigated in this study, the size of the observed lesions greatly varied among isolates ($F_{6,77} = 390$, $p < 0.001$). Furthermore, the tested fungal isolates produced lesions in the host that were always larger than those observed from the control (Tukey t-tests, $p < 0.001$ in all cases). Most of the fungal isolates (*D. eres*, *P. funerea*, *Phomopsis* spp. and *T. versicolor*) developed smaller lesions than *D. foeniculina* and *N. parvum*. No lesion developed after control treatment (Figure 5).
Among all fungi investigated, *N. parvum* produced the longest lesions (53.98 mm). It proved to be also the most pathogenic since the inoculated tree showed very strong die-back symptoms, such as reddish-brown cankers on the twigs, associated with internally brown necrosis. Finally, the death of all twigs and whole branches was observed at one month after artificial infection. *D. feoniculina* produced 25 mm length cankers on twigs with internally brown necrosis and death of some twigs and branches was also noticed. *D. eres*, *P. funerea* and *Phomopsis* formed similar lesions as described above but of a shorter length, which ranged between 8–14 mm. In the case of *T. versicolor*, symptoms of wood caries were also seen (Figure 5).

All the inoculated fungi were always reisolated from the lesions and based on molecular methods were identical to the cultures used for inoculation.

3. Discussion

This study is the first to address the presence of the die-back syndrome on apple orchards in the Val d’Agri region and to isolate and further characterize fungal species which could be involved in the observed disease through morphology, DNA sequencing and phylogenetic analysis. The search for the causal agent of the apple die-back syndrome contributed to in vitro isolation of six fungi, already known to be involved in different diseases in apple and other plant species. Both cultural and morphological features of the five phytopathogenic genera, namely *Neofusicoccum*, *Diaporthe*, *Trametes*, *Pestalotiopsis* and *Phomopsis*, identified in the present study were consistent and resembled the above-mentioned ones.

The morphological classification of fungi is an inexpensive and rapid tool but has also many limitations. As a consequence, current mycotoxonomy has changed a lot, now employing other methodological approaches, such as phylogeny, chemotaxonomy, genetics, ecology or molecular biology [35,41–46]. The preliminary identification of the fungi isolated from the apple plant, in the present study based on morphological features, was confirmed by molecular outcomes gained from the sequencing of the ITS region, (*tub-2* and actin (*ACT*) genes. A lesser variation was noticed over the ITS region, for all fungal species investigated, and only in the case of the reference sequences downloaded from the database, which was probably due to sequencing errors. It is widely accepted that
sometimes the sequences deposited in the GenBank are of poor quality and around 30% of the ITS sequences deposited may be associated with the wrong taxon [47].

ITS locus alone, despite its advantages and official recognition as a DNA barcoding marker [48–52], can be limited in providing enough resolution in the case of closely-related fungal species [53]. Considering all these limitations, for an accurate fungal species identification other loci like tub-2 and ACT were explored, showing that nucleotide variation was higher in ACT gene, followed by the tub-2 gene for all fungal species investigated.

Typically, different wood-rotting fungi have been associated with the die-back syndrome in apple over the years as *Coriolus* spp., *Stereum* spp., *Schizophyllum commune* [1]. Apart from these, *Sphaeropsis pyripturensis* Xiao & J.D Dogers fungus was reported to cause cankers and twig die-back on apple and crabapple trees in the USA [54]. Cloete et al. [6] found that apple and pear trees in South Africa are the hosts of many fungi associated with the die-back symptoms, such as *Diplodia* spp., *Neofusicoccum* spp., *Phaeoacremonium* spp. and *Phomopsis* sp. Very recent studies of Jabiri et al. [55] reported symptoms of dieback disease, such as root rot, yellow leaves and wilting, caused by *Phytophthora vexans* on young apple trees (6–10 years old) of cv. “Golden Delicious” in Morocco.

The *Diaporthe* genus has also been associated to the shoot canker or fruit rot in pear [56,57]. Dissanayake et al. [28,58], based on molecular phylogenetic analysis, revealed seven new species, within the above-mentioned genus, in Italy. Among the *Diaporthe* species, *D. eves* has recently been reported to be linked to necrosis and stem cankers and caused the death of young apple rootstocks in Canada [59]. Moreover, *D. eves* is among the most serious phytopathogenic fungi affecting many plant species all over the world [26,28,56–63]. The outcomes from this study, showing the frequent isolation from apple with die-back symptoms of *D. eves*, agree with the previous studies by Sessa et al. [64] who reported the *D. eves* isolation from peach and apple with wood disease symptoms, such as wedge-shaped necrosis and canker. Additionally, the identification of more than one species of *Diaporthe*, namely *D. eves* and *D. neotheicola*, on the same plant species in our study is in agreement with the earlier works of van Niekerk et al. [40] and of Thompson et al. [65], who reported that the same host plant may be colonized by different *Diaporthe* spp. at the same time.

*Neofusicoccum parvum*, which recently emerged as a phytopathogen, was also among the most frequently isolated fungi in the present study. The *Neofusicoccum* Crous, Slippers and A.J. L. Phillips Genus was described by Crous et al. [24], aiming to include *Botryosphaeriaceae* with Fusicoccum-like anamorphs [66]. This fungus has been already reported to cause cankers on many cultivable plant species [67], including apple and pear [68], but it seems that it is expanding its host range, as demonstrated by the very recent study by Choi et al., in Korea [69]. Moreover, *N. parvum* was the most aggressive fungus in our study, as demonstrated by both natural and artificial inoculations on apple from Val d’Agri, also causing the death of the entire tree.

*Trametes versicolor* was among the wood fungi frequently isolated in the present investigation. Fungi from the Genus *Trametes* are white rot polypores. Nearly 60 species are known worldwide on many hosts, and some are used for medicinal purposes [70–72]. The taxonomy situation within *T. versicolor* is still complex, since unresolved phylogenies and unclear species boundaries exist [31,33]. The study of Kile [73], who examined host-pathogen relationships between the apple tree, *T. versicolor* and factors affecting host susceptibility, showed that the fungus was a facultative parasite which caused the white rot of the sapwood and the susceptibility of living wood to fungal decay increased with the age of the tree, due to a natural decline in host plant resistance. The frequent isolation of *T. versicolor* from apple trees showing die-back symptoms agree with the study of Darbyshire et al. [74], which associated the die-back of apple trees in Australia to the wood-rotting fungus *T. versicolor* and also showed that it is a low-sugar disease. An association between *Coriolus versicolor* (syn. *T. versicolor*) and the die-back disease of apples in Washington state, described by Dilley and Covey, supported the present study outcomes [75]. We can assume that frequent in vitro isolation of *T. versicolor* from apple trees obtained in our study and
the die-back symptoms observed after natural and/or artificial inoculation to this host can be associated with this disease.

Many studies reported that the Diaporthe (Phomopsis) fungi has been associated with shoot blight and canker, decay, wilting, necrosis of bark and fruit rot in several fruit tree species worldwide [27,37,76–78]. The isolation of Phomopsis sp. in this study agrees with the earlier studies by Pertot and Vindimian [79], who reported the diffusion of P. mali, causing the dieback of young apple trees in Trentino (Northern Italy). Cloete et al. [6] also found three Phomopsis sp. isolates from pear and apple exhibiting die-back symptoms and considered them as a possible inoculum source for grapevine trunk disease pathogens. Our outcomes concord with the results of Bai et al. [56], who identified the presence of these fungi on pear in China, and of Kanematsu et al. [37], who, in Japan, showed that they were responsible for shoot cankers.

Regarding Diaporthe species, our results from pathogenicity tests are similar to those reported by Sessa et al. [64], who investigated the diversity and the virulence of the Diaporthe species associated with wood disease symptoms in deciduous fruit trees in Uruguay. The same authors recognized them to be the causal agents of twig and branch cankers, showing that D. ursos and D. foeniculina produced necrosis. Furthermore, another study by Abramczyk et al. [62] characterized isolates of D. ursos based on morphological and pathological characteristics, which were isolated from fruit plants and genetically identified as D. ursos species complex [78]. Additionally, they demonstrated that in pathogenicity tests D. ursos produced small necrosis of about 12–17 mm in diameter, occurring at the site of inoculation. D. ursos colonies were obtained from the artificially inoculated tissue, again confirming the results obtained in our study on this fungus, showing its pathogenic abilities towards apple trees.

Pathogenicity tests results showing that N. parvum was the most virulent among all fungal species isolated and identified on apple trees from the Val d’Agri region match those by Cloete et al. [6]. The authors analyzed fungi associated with die-back symptoms of apple and pear trees cultivated in proximity of grapevine in Western Cape, South Africa, and found that a species of Neofusicoccum (N. australe) was among the most virulent species towards apple, with mean necrotic lesions of about 40.2 mm length. In the same study, Phomopsis sp. was observed to be less virulent (necrotic lesions of about 11.8 mm in length), and this was similar to the results obtained in the present study (necrotic lesions of about 8 mm in length). Another study by Espinoza et al. [80] found that Neofusicoccum spp. was associated with the stem canker and dieback of blueberry in Chile and reported, for the first time, N. parvum as a canker-causing agent on blueberry. In their study, the same authors performed pathogenicity tests on kiwi, blueberry and apple and found that N. parvum was the most aggressive fungus, in all hosts, and this is also in accordance with our results.

4. Materials and Methods

4.1. Biologic Material

Pieces of symptomatic trunks from the apple cv. “Golden Delicious”, showing die-back symptoms, were collected in autumn/winter 2019. During this period, apple orchards located in the Val d’Agri area were surveyed for the presence of apple die-back symptoms. A total number of 50 samples, made of pieces of living material (bark and cankered trunks) showing die-back symptoms, more specifically, “scaly bark” and extensive cankers, mainly located in the lower part of the trunk, and wood decay were obtained from trees 3–12 years old (Figure 6). They were brought to the Plant Pathology Laboratory at the University of Basilicata and stored in fridges, at 4 °C, until used.
Pieces of symptomatic trunks from the apple cv. “Golden Delicious”, showing die-back symptoms, were collected in autumn/winter 2019. During this period, apple orchards located in the Val d’Agri area were surveyed for the presence of apple die-back symptoms. A total number of 50 samples, made of pieces of living material (bark and cankered trunks) showing die-back symptoms, more specifically, “scaly bark” and extensive cankers, mainly located in the lower part of the trunk, and wood decay were obtained from trees 3–12 years old (Figure 6). They were brought to the Plant Pathology Laboratory at the University of Basilicata and stored in fridges, at 4 °C, until used.

Figure 6. Apple trees located in the Val d’Agri area showing die-back symptoms (a–c) and death of tree caused by die-back (d). Figure a—courtesy of Dr. Camilla Nigro, ALSIA, Basilicata Region.

4.2. Pathogen Isolation

Symptomatic wood pieces were cut under laminar flow sterile conditions into small parts, surface-sterilized by soaking in a 70% ethanol solution for 1 min, in a 1% NaOCl solution for 1 min, in 70% ethanol solution for another 30 sec and finally rinsed in sterile water for 2 min. After sterilization, the trunk pieces were dried on a sterile paper and cut into small parts. Small parts of about 2 × 2 mm taken from the margins between necrotic and healthy tissue were placed on petri plates containing potato dextrose agar (PDA, Oxoid Ltd., Hants, UK), amended with streptomycin sulphate (40 mg L⁻¹, Merck KGaA, Darmstadt, Germany) and were incubated at 25 °C in the dark until growth could be detected. Subcultures were performed from the growing hyphae onto PDA and incubated under the same conditions. Pure cultures were created for all obtained PDA plates.

To isolate and identify bacterial pathogens probably linked to the die-back syndrome symptomatic wood trunk, samples were first surface sterilized and prepared, as reported by Schaad et al. [81].

4.3. Morphological Identification

All fungal isolates obtained in this study were stored, as pure cultures (PFC), in the culture collection of the Plant Pathology Laboratory of the School of Agriculture, Forestry, Food and Environmental Sciences (SAFE) at the University of Basilicata on PDA slants and maintained at 4 °C in fridge.

Fungal isolates were examined using a Axioscope microscope (Zeiss, Jena, Germany) and preliminary identified by morphological characteristics.

4.4. Molecular Characterization

For molecular characterization, genomic DNA was extracted from fresh PFC mycelia of each isolate, 7–10 days old, through an extraction protocol described by Mang et al. [43]. Genomic DNA quality and quantity were checked using a Nanodrop ND-1000 spectrophotometer (Thermo Scientific Inc., Willmington, DE, USA) and the material was stored at −20 °C in 1.5 mL Eppendorf tubes until further use. In order to determine the fungal species, three different genes/regions were amplified. Namely, the internal transcribed spacers (ITS1 and ITS2) of the ribosomal RNA (ITS); β-tubulin (tub-2) and actin (ACT). The oligonucleotides used for PCR amplifications were: ITS5/ITS4 [82], Bt2a/Bt2b [83] and ACT512F/ACT783R [84] (Table 2).
Table 2. Details of primers pairs used in this study for the amplification and sequencing of fungal DNA.

| Locus * | Primer | Sequences 5′→3′ | Reference |
|---------|--------|-----------------|-----------|
| ITS     | ITS5   | 5′-GGA AGT AAA AGT CGT AAC AAG G-3′ | White et al., 1990 |
|         | ITS4   | 5′-TCC TCC GCT TAT TGA TAT GC-3′ |           |
| TUB-2   | Bt2a   | 5′-GGT AAC CAA ATC GGT GCT GCT TTC-3′ | Glass and Donaldson, 1995 |
|         | Bt2b   | 5′-ACC CTC AGT GTA GTG ACC CTT GGC-3′ |           |
| ACT     | ACT-512F | 5′-ATG TGC AAG GCC GGT TTC GC-3′ | Carbone and Kohn, 1999 |
|         | ACT-783R | 5′-TAC GAG TCC TTC TGG CCC AT-3′ |           |

* ITS: internal transcribed spacer regions and intervening 5.8S rRNA gene; TUB-2: partial beta tubulin gene; ACT: actin gene.

PCR amplifications were performed under the conditions explained in Mang et al. [43,85] for ITS only. For the other two genes the Phire Direct PCR Master mix (Thermo Scientific Inc., USA) was used, following manufacturer’s instructions with some modifications. PCR mixtures were composed of 10 µL of 2X Phire Plant PCR Buffer (including 1.5 mM MgCl₂ and 20 µM of dNTPs), Primers 0.5 µM each; 0.4 µL of Phire Hot Start II DNA polymerase enzyme, 5 µL of template DNA (20 ng/µL) and double distilled water up to 20 µL. The PCR cycling protocol consisted of: an initial denaturation at 98 °C for 5 min for 1 cycle; then 40 cycles of denaturation at 98 °C for 5 s; annealing at 60 °C for ITS and at 62 °C for tub-2 and ACT genes for 5 s; extension at 72 °C for 20 s, followed by a final extension at 72 °C for 1 min for 1 cycle. All PCR products were separated in 1.5% agarose gels in Tris-Acetic acid-EDTA (TAE) buffer and visualized under the UV after staining with SYBR Safe DNA Gel Stain (ThermoFisher Scientific™ , Carlsbad, CA, USA). A 100-bp GeneRuler Express DNA Ladder (ThermoFisher Scientific™ Baltics UAB, Vilnius, Lithuania) was used as a molecular weight marker. Direct sequencing of all PCR products was performed by BMR Genomics [Padua, Italy], using a 3130xl automatic sequencer in both directions and using the same primers as for the PCR. Subsequently, the sequence information was analyzed by the local alignment search tool using BLASTn [86,87] in the National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov/BLAST, accessed on 12 January 2022). Annotations were based on BLAST searches with a minimum of 99–100% identity over at least 80% of the length of the nucleotide sequence, which are the commonly used thresholds for reliable sequence annotation [88]. Nucleotide sequences primary identification was carried out through the BLASTn search tool program [86,87] of the NCBI by comparing all sequences obtained in this study with those already present in the database.

4.5. Sequences Alignments and Phylogenetic Analysis

All nucleotide sequences produced by this study and identified based on high sequence identity (>99–100%) to similar species already present in nucleotide databases, along with few additional reference sequences downloaded from GenBank (http://www.ncbi.nlm.nih.gov/GenBank, accessed on 12 January, 2022), were used for the phylogenetic analysis. Subsequently, they were manually edited and aligned with the ClustalX version 2.0 [88] program, using the MEGA X (Molecular Evolutionary Genetic Analysis) [89] phylogeny package to build representative alignments (Table 3). As reported in previous studies by Slippers et al. [66] and by Crous et al. [24], N. parvum and N. ribis are closely related cryptic species within the recently described Genus Neofusicoccum (Botryosphaeriaceae, Ascomycetes). Therefore, in case of the ACT gene, when no other reference species were available in the GenBank nucleotide database, this fungal species was also used, allowing us to perform the phylogenetic investigation (Table 3).
Table 3. List of taxa, fungal isolates and GenBank accession numbers of the genes analyzed in this study and used for phylogenetic analysis.

| Taxa                        | Isolate Name | Culture No. | Gene       | GenBank Accession Number | Reference                  |
|-----------------------------|--------------|-------------|------------|--------------------------|----------------------------|
| Diaporthe eres             | CBS 186.37   | CPC 30116   | ITS        | MH855881                 | Wu et al., 2019            |
|                             | SS48         | NEFF 3-23-4 | TUB-2      | MK281261                 | GenBank                    |
|                             | STEU 8322    | DB414AG027  | ACT        | MIFCC 316                 | GenBank                    |
|                             | STEU 8323    | Nc1         | ITS        | KY312645                 | GenBank                    |
|                             | F2333        | MJL13       | TUB-2      | KR023623                 | GenBank                    |
| Diaporthe foeniculina      | ISPaVe 2156  | CoPat-560   | N/A        | LN651172                 | GenBank                    |
|                             | A1907B       | Av-1        | ISPaVe 2156| MT230444                 | Udayanga et al., 2014      |
|                             | PI01b        | CAA133      | N/A        | MT173564                 | GenBank                    |
| ** Diaporthea corylina     | CBS121124    | CBS121124   | N/A        | KC343004                 | Mathioudakis et al., 2020  |
| ** Diaporthe helianthi     | N/A          | N/A         | AR4131     | KC343972                 | Hunter et al., 2006        |
| Neofusicoccum parvum       | B32          | ACBA15      | YELO-21a   | KJ499738                 | Zhang et al., 2004         |
| N. parvum                  | B65          | AKKA308.2   | CMW 7773   | KJ499740                 | Slippers et al., 2004      |
| N. ribis                   | -            | -           | CMW 7773   | -                        | N/A                        |
| N. parvum                  | B135         | 11215_3     | N/A        | JX989444                 | GenBank                    |
| N. parvum                  | B146         | MFLUCC_12-0380 | N/A | KJ499743                 | GenBank                    |
| N. parvum                  | F1236        | GDTCMF23    | N/A        | MT424811                 | GenBank                    |
| N. parvum                  | JRad16       | CBS:130994  | N/A        | KY680281                 | GenBank                    |
| N. parvum                  | CMW9081; ICMP 8003 | CMW994 | N/A        | N/A                      | GenBank                    |
| ** Dipodina seriata        | ASJ297       | HL1         | N/A        | MH221123                 | GenBank                    |
| Botryosphaeria dothidea    | N/A          | N/A         | HPLW1      | N/A                      | N/A                        |
| Pestalotiopsis funerea     | ML4DY        | ML4DY       | N/A        | EF055197                 | Tang et al., 2012          |
| Pestalotiopsis sp.         | SKS0416      | TAP18N030   | N/A        | EU715650                 | Liu et al., 2010           |
| Pestalotiopsis sp.         | ZC-W-1-1     | BRIP 66615  | N/A        | KR822153                 | GenBank                    |
| Pestalotiopsis sp.         | NW-FA2867    | ZX18A       | N/A        | MG088325                 | GenBank                    |
| ** Sordaria alcina         | CBS 109460   | CBS 109460  | N/A        | AY681198                 | Lai et al., 2010           |

* Reference numbers for GenBank accessions.
Table 3. Cont.

| Taxa                        | Isolate Name | Culture No. | Gene | GenBank Accession Number * | Reference          |
|-----------------------------|--------------|-------------|------|-----------------------------|--------------------|
| **Valsa japonica**          | CBS375.29    | N/A         | N/A  | AF191185                    | Adams et al., 2002 |
| **Grifola frondosa**        | WC835        | N/A         | N/A  | N/A                         | Shen et al., 2002  |

Notes: * ITS-Nuclear ribosomal internal transcribed spacer regions; TUB- (tub-2) gene; ACT-actin gene. ** This fungal species has been used as outgroup for the phylogenetic analysis. The sign “-” in the column table indicates either that the nucleotide sequences do not exist in the GenBank or were not obtained in this study for that particular fungal isolate. * This study was considered only for the tub-2 nucleotide gene sequence. b This fungal species was used only for the phylogenetic analysis involving the tub-2 gene nucleotide sequences. c This GenBank origin of the accession regards only ACT gene nucleotide sequences used for the phylogenetic analysis. d This fungal species was used only for the phylogenetic analysis involving the ACT gene. N/A: not available. e Data not considered.
Phylogeny reconstructions were performed with MEGAX [89] for each gene using the neighbor-joining (NJ) statistical method [90] with an interior branch test and 1000 bootstrap replications [91,92], nucleotide substitution type and the Tajima–Nei substitution model [93] with uniform rates among sites. A deletion was used as treatment for gaps and missing data and the codon positions included were 1st, 2nd, 3rd and noncoding sites. The evolutionary distances computed using the Tajima–Nei method [93] are in the units of the number of base substitutions/site. The same procedure described previously was used for the tub-2 and ACT genes. An unequal number of nucleotide sequences were involved in the phylogenetic analyses for each gene investigated, which was caused by the lack of positive PCR and sequencing results for some of the genes and also the nonexistence of nucleotide sequences in the GenBank database. Therefore, only single gene phylogenies could be performed, each one containing all nucleotide sequences obtained in this study for the examined gene plus reference species downloaded from the GenBank.

In particular, the reference sequences representing the relevant species used to build alignments for species identification were: for D. eres and D. foeniculina (D. corylina strain CBS121124 and only for ACT gene D. helianthi strain AR4131), N. parvum (Diplodia seriata strain ASJ297; or Botryosphaeria dothidea strains HL1 and HPLW1 for the tub-2 and ACT genes, respectively), Pestalotiopsis sp. (Sordaria alcina strain CBS 109460), Phomopsis sp. (Valsa japonica isolate CBS375.29) and Trametes versicolor (Grifola frondosa isolate WC835) (Table 3).

A different number of nucleotide sequences were obtained for each gene and fungal species in this study; therefore, a multilocus phylogeny with three genes (ITS + tub-2 + ACT) was possible only for Diaporthe and Neofusicoccum spp., while for Pestalotiopsis spp. a two-gene phylogeny (ITS + tub-2) was performed using the Seaview5 program, as presented in Table S1 and in Figures S1–S3. (Supplementary material).

4.6. Pathogenicity Trials

A trial was conducted under field conditions to examine the formation of lesions on twigs of 2-year-old apple trees (cv. “Golden Delicious”), using a common protocol. In particular, the pathogenicity tests were performed using 4 mm diameter mycelial plugs taken from the margins of 7-day-old cultures on PDA amended with antibiotic streptomycin sulphate (40 mg L\(^{-1}\), MerckKGaA, Darmstadt, Germany). An equal number of young apple shoots were equally treated but using only sterile agar plugs, which were left as controls. One fungal isolate was used for each apple tree, according to the fungal species identified and characterized in this study, and each treatment was replicated four times. A wound of the about the size of the agar plug was made on each woody shoot, in the phloem and cortex tissue, with a sterile scalpel. Immediately after wounding, the plug was positioned in the center of the wound and covered by a sterile water wetted cotton piece. In order to avoid a rapid dehydration, lesion sites were wrapped with parafilm (Pechiney Plastic Packaging, Menasha, WI, USA). For each fungal pathogen the trial layout was a randomized block design with four repetitions using twigs as experimental units. The whole pathogenicity trial consisted of six fungal pathogens, isolated in this study from apple trees in Val d’Agri, and an agar plug only. Following inoculation, all young apple trees were placed in a greenhouse, where they were kept under natural light conditions at 22 °C and at about 70% relative humidity. After 30 days of inoculation, apple twigs were inspected for lesion development and after 45 days post-inoculation, when their necrosis was evident, the twigs were removed and brought to the laboratory for immediate analysis. The number of twigs with necrosis was recorded and, after the removal of the bark, the length of the developed canker lesions was measured. In order to reisolate the causal agent, small pieces (approx. 5 mm length) of diseased wooden tissue were cut from the edge of the necrotic lesions from the inoculated twigs and, after surface disinfection, were placed in petri dishes containing PDA and antibiotic streptomycin sulphate. Plates were incubated for 7 days at 20 °C in an incubator, under dark conditions, until growth was detected. Subsequently, the identification of the reisolated fungi was carried out by both morphological features and
molecular analysis, using the protocols for morphological identification, DNA extraction and PCR conditions described above.

4.7. Statistical Analysis

Since the data obtained from the lesion measurements were normally distributed (Shapiro–Wilk tests [94], followed by a Holm–Bonferroni [95] correction), a one-way ANOVA was used to test for mean differences among the investigated fungal isolates. Tukey post hoc tests for multiple comparisons of means were also performed to detect significant differences among the treatments. The statistical analyses performed in this study were performed using the R version 3.6.2 software (R Core Team, Vienna, Austria) [96].

5. Conclusions

Fungal species investigated in this study are well known to be involved in the fruit tree trunk diseases. The present study demonstrated that among all fungi investigated, *N. parvum* was the most aggressive and may be involved in the heavy decline of apple trees in the Val D’Agri area. In addition, other fungi, such as *D. eres, D. foeniculina, P. funerea, T. versicolor* and *Phomopsis* spp., could have contributed to the aggravation of the existing symptoms. Our field observations allowed us to assume that fungi, and in particular *N. parvum*, could penetrate the trees through wounds created by cuttings. Therefore, to avoid this, it is necessary to protect the wounds, in particular after cuttings.

Given the economic importance of apples worldwide, more investigations related to the role played by the phytopathogens discovered in this study, which are involved in die-back disease on apple trees, seem necessary. Future outcomes will be expected to add beneficial knowledge to better understand this complex disease in order to establish appropriate strategies to protect this regionally relevant and worldwide nutritionally important crop.

**Supplementary Materials:** The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/plants11101374/s1, Figure S1: PhyML tree (ITS + *tub-2* + *ACT*) for *Diaporthe* spp.; Figure S2: PhyML tree (ITS + *tub-2* + *ACT*) for *Neofusicoccum* spp.; Figure S3: PhyML tree (ITS + *tub-2*) for *Pestalotiopsis* spp.; Table S1: Multilocus phylogeny parameters used in the study.

**Author Contributions:** Conceptualization, S.M.M. and I.C.; methodology, S.M.M.; software, S.M.M. and A.M.; validation, S.M.M., C.M., A.M. and I.C.; formal analysis, S.M.M.; investigation, S.M.M.; resources, I.C.; data curation, S.M.M., I.C., C.M. and A.M.; writing—original draft preparation, S.M.M.; writing—review and editing, S.M.M., I.C., C.M. and A.M.; visualization, S.M.M., I.C., C.M. and A.M.; supervision, I.C. and S.M.M.; project administration, I.C. and S.M.M.; funding acquisition, I.C. All authors have read and agreed to the published version of the manuscript.

**Funding:** This research was funded by the Basilicata Region-Phytosanitary Office (Matera, Italy), under the project entitled: “Epidemiological studies regarding the presence and spread in Basilicata of pathogens of agricultural and forestry plants with particular concern on those of quarantine. Molecular characterization of the pathogens and possibilities of fighting against them”.

**Institutional Review Board Statement:** Not applicable.

**Informed Consent Statement:** Not applicable.

**Data Availability Statement:** The authors declare that the data supporting the findings of this study are available within the article and in the supplementary materials (Figures S1–S3 and Table S1).

**Acknowledgments:** The authors thank Giuseppe Malvasi and Vincenzo Pucciariello (Phytosanitary Office of Matera, Department of Agricultural and Forestry Politics, Basilicata Region, Italy) for furnishing the information regarding the presence of the disease investigated in this study and for their valuable help with the field investigations.

**Conflicts of Interest:** The authors declare no conflict of interest.
31. Justo, A.; Hibbett, D.S. Phylogenetic classification of Trametes (Basidiomycota, Polyporales) based on a five-marker dataset. *Taxon* 2011, 60, 1567–1583. [CrossRef]
32. Welti, S.; Moreau, P.-A.; Favel, A.; Courtecuisse, R.; Haon, M.; Navarro, D.; Taussac, S.; Lesage-Meessen, L. Molecular phylogeny of Trametes and related genera, and description of a new genus Leotrametes. *Fungal Divers.* 2012, 55, 47–64. [CrossRef]
33. Carlson, A.; Justo, A.; Hibbett, D.S. Species delimitation in Trametes: A comparison of ITS, RPB1, RPB2 and TEF1 Gene Phylogenies. *Mycologia* 2014, 106, 735–745. [CrossRef]
34. Crous, P.W.; Summerell, B.A.; Swart, L.; Denman, S.; Taylor, J.E.; Beziudenhout, C.M.; Palm, M.E.; Mariconowitz, S.; Groenewald, J.Z. Fungal pathogens of Proteaceae. *Persoonia* 2011, 27, 20–45. [CrossRef]
35. Maharachchikumbura, S.S.N.; Guo, L.-D.; Chukeatirote, E.; Bahkali, A.H.; Hyde, K.D. Pestalotiopsis—Morphology, phylogeny, biochemistry and diversity. *Fungal Divers.* 2011, 50, 167–187. [CrossRef]
36. Maharachchikumbura, S.S.N.; Hyde, K.D.; Groenewald, J.Z.; Xu, J.; Crous, P.W. Pestalotiopsis revisited. *Stud. Mycol.* 2014, 79, 121–186. [CrossRef]
37. Kanematsu, S.; Kobayashi, T.; Kudo, A.; Ohtsu, Y. Conidial morphology, pathogenicity and culture characteristics of *Phomopsis* isolates from peach, Japanese pear and apple in Japan. *Ipom. J. Phytopathol.* 1999, 65, 264–273. [CrossRef]
38. Santos, J.M.; Phillips, A.J.L. Resolving the complex of *Diaporthe* (Phomopsis) species occurring on *Foeniculum vulgare* in Portugal. *Fungal Divers.* 2009, 34, 111–125.
39. Van Niekerk, J.M.; Crous, P.W.; Groenewald, J.Z.; Fourie, P.H.; Halleen, F. DNA phylogeny, morphology and pathogenicity of Botryosphaeria species on grapevines. *Mycologia* 2004, 96, 781–798. [CrossRef] [PubMed]
40. Van Niekerk, J.M.; Groenewald, J.Z.; Farr, D.F.; Fourie, P.H.; Halleen, F.; Crous, P.W. Reassessment of *Phomopsis* species on grapevine. *Australas. Plant Pathol.* 2005, 34, 27–39. [CrossRef]
41. Koenraadt, S.; Somerville, S.C.; Jones, A.L. Characterization of mutations in the beta-tubulin gene of benomyl-resistant field strains of *Venturia inaequalis* and other plant pathogenic fungi. *Phytopathology* 1992, 82, 1348–1354. [CrossRef]
42. Begerow, D.; Beate, J.; Oberwinkler, F. Evolutionary relationships among β-tubulin gene sequences of basidiomycetous fungi. *Mycol. Res.* 2004, 108, 1257–1263. [CrossRef]
43. Mang, S.M.; Scrano, L.; Camele, I. Preliminary studies on fungal contamination of two rupetianche from Matera (Southern Italy). *Sustainability* 2020, 12, 6988. [CrossRef]
44. Hyde, K.D.; Dong, Y.; Phookamsak, R.; Jeewon, R.; Bhat, D.J.; Jones, E.B.; Liu, N.G.; Abeywickrama, P.D.; Mapook, A.; Wei, D.; et al. *Fungal diversity notes* 1151–1276: Taxonomic and phylogenetic contributions on genera and species of fungal taxa. *Fungal Diver.* 2020, 100, 5–277. [CrossRef]
45. Mang, S.M.; Raciopi, R.; Camele, I.; Rana, G.L.; D’Auria, M. Use of metabolic profiles to distinguish three *Monilinia* species. *Plant Pathol.* 2015, 97, 55–59. [CrossRef]
46. Sofò, A.; Elshafie, H.S.; Scopa, A.; Mang, S.M.; Camele, I. Impact of airborne zinc pollution on the antimicrobial activity of olive oil and teh microbial metabolic profiles of Z-contaminated soils in an Italian olive orchard. *J. Trace Elem. Med. Biol.* 2020, 60, 1099–1109. [CrossRef]
47. Hofstetter, V.; Buyck, B.; Eyssartier, G.; Schnee, S.; Gindro, K. The unabearable lightness of sequence-based identification. *Fungal Diver.* 2019, 96, 243–284. [CrossRef]
48. Senanayake, I.C.; Rathnayaka, A.R.; Marasinghe, D.S.; Calabon, M.S.; Gentekaki, E.; Lee, H.B.; Hurdeal, V.G.; Pem, D.; Disnar, S.; Cormier, X.C. Biochemical diversity of *Phomopsis* species. *Mycologia* 2012, 104, 111–125. [CrossRef] [PubMed]
49. Senanayake, I.C.; Jeewon, R.; Chomnunti, P.; Wanasinghe, D.N.; Norphanphoun, C.; Karunarathna, A.; Pem, D.; Perera, R.H.; et al. Large-scale generation and analysis of filamentous fungal DNA barcodes boosts coverage for kingdom fungi taxa. *Fungal Divers.* 2011, 96, 95–100. [CrossRef]
50. Schoch, C.L.; Seifert, K.A.; Huhndorf, S.; Robert, V.; Spouge, J.L.; Levesque, C.A.; Chen, W.; Fungal Barcoding Consortium Author List; Bolchacova, E. Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for Fungi. *Proc. Natl. Acad. Sci. USA* 2012, 109, 6241–6246. [CrossRef]
51. Kiss, L. Limits of nuclear ribosomal DNA internal transcribed spacer (ITS) sequences as species barcode for fungi. *Proc. Natl. Acad. Sci. USA* 2012, 109, E1811. [CrossRef]
52. Xiao, C.L.; Boal, R.J. A New Canker and Twig Dieback Disease of Apple and Crabapple Trees Caused by *Sphaeropsis pyriputrescens* in Washington State. *Plant Dis.* 2005, 89, 10. [CrossRef]
53. Jabiri, S.; Lahlali, R.; Bahra, C.; Amraoui, M.B.; Tahiri, A.; Amiri, S. First report of *Phytophthora vexans* associated with dieback disease of apple trees in Morocco. *J. Plant Pathol.* 2020, 102, 1319. [CrossRef]
56. Bai, Q.; Zhai, L.F.; Chen, X.R.; Hong, N.X.; Xu, W.X.; Wang, G.P. Biological and molecular characterization of five Phomopsis species associated with pear shoot canker in China. Plant Dis. 2015, 99, 1704–1712. Available online: http://apsjournals.apsnet.org/loi/pdis (accessed on 18 January 2021). [CrossRef]

57. Bertetti, D.; Guarnaccia, V.; Spadaro, D.; Gullino, M. First report of fruit rot caused by Diaporthe eris Nitschke on European pear cultivated in Italy. (Diaporthe eris Nitschke, agente di marciu me frutti di pero in Italia). Prot. Delle Colt. 2018, 5, 10–13. [CrossRef]

58. Dissanayake, A.J.; Camporesi, E.; Hyde, K.D.; Zhang, W.; Yan, J.Y.; Li, X.H. Molecular phylogenetic analysis reveals seven new Diaportha species from Italy. Mycosphere 2017, 8, 853–877. [CrossRef]

59. Ali, S.; Renderos, W.; Bevis, E.; Hebb, J.; Abassi, P.A. Diaportha eris causes stem cankers and death of young apple rootstocks in Canada. Can. J. Plant Pathol. 2020, 42, 217–227. [CrossRef]

60. Floriani, V.C.; Puia, C.; Groza, R.; Saciu, L.A.; Floriani, T. Study of the major pathogens that lead to apple fruit decay during storage. Not. Bot. Horti Agrobot. Cluj-Napoca 2018, 46, 538–545. [CrossRef]

61. Sun, W.; Huang, S.; Xia, J.; Zhang, X.; Li, Z. Morphological and molecular identification of Diaportha species in south-western China, with description of eight new species. MycoKeys 2021, 77, 65–95. [CrossRef]

62. Abramczyk, B.; Król, E.D.; Zalewska, E.D.; Zimowska, B. Morphological characteristics and pathogenicity of Diaportha eris isolates to the fruit tree shoots. Acta Sci. Pol. - Horitum Cultus 2018, 17, 125–133. [CrossRef]

63. Chaisiri, C.; Liu, X.; Lin, Y.; Fu, Y.; Zhu, F.; Luo, C. Phylogenetic and Haplotype Network Analyses of Diaportha species in China Based on Sequences of Multiple Loci. Biology 2021, 10, 179. [CrossRef]

64. Sessa, L.; Abreò, E.; Bettucci, L.; Lupò, S. Diversity and virulence of Diaportha species associated with wood diseases symptoms in deciduous fruit trees in Uruguay. Phytopathol. Mediterr. 2017, 56, 431–444. [CrossRef] [PubMed]

65. Thompson, S.M.; Tan, Y.P.; Young, A.J.; Neate, S.M.; Atikin, E.A.B.; Shivash, R.G. Stem cankers on sunflower (Helianthus annuus) in Australia reveal a complex of pathogenic Diaportha (Phomopsis) species. Pers. Molec. Phylogen. Evol. Fungi 2011, 27, 80–89. [CrossRef]

66. Slippers, B.; Smit, W.A.; Crous, P.W.; Countinho, T.A.; Wingfield, M.J. Taxonomy, phylogeny and identification of Botryosphaeriaceae associated with pome and stone fruit trees in South Africa and other regions of the world. Plant Pathol. 2007, 56, 128–139. [CrossRef]

67. Yu, Z.D.; Tang, G.H.; Peng, S.B.; Chen, H.; Zhai, M.Z. Neofusicoccum parvum causing canker of seedlings of Juglans regia in China. J. For. Res. 2015, 26, 1019–1024. [CrossRef]

68. Shen, Y.-M.; Chao, C.-H.; Liu, H.L. First Report of Neofusicoccum parvum Associated with Stem Canker and Dieback of Asian Pear Trees in Taiwan. Plant Dis. 2010, 94, 1062. [CrossRef] [PubMed]

69. Choi, S.; Paul, N.C.; Lee, K.-H.; Kim, H.-J.; Sang, H. Morphology, Molecular Phylogeny, and Pathogenicity of Neofusicoccum parvum, Associated with Leaf Spot Disease of a New Host, the Japanese Bay Tree (Machilus thunbergii). Forests 2021, 12, 440. [CrossRef]

70. Zmitrovich, I.V.; Ezhov, O.N.; Wassser, S.P. A survey of species of genus Trametes Fr. (higher basidiomycetes) with estimation of their medicinal source potential. Int. J. Med. Mushrooms 2012, 14, 307–319. [CrossRef]

71. Habtemariam, S. Trametes versicolor (Synn. Coriolus versicolor) Polysaccharides in Cancer Therapy: Targets and Efficiency. Biomedicines 2020, 8, 135. [CrossRef]

72. Pop, R.M.; Puia, I.C.; Puia, A.; Chedea, V.S.; Leopold, N.; Bocsan, I.C.; Buzoianu, A.D. Characterization of Trametes versicolor: Medicinal Mushroom with Important Health Benefits. Not. Bot. Horti Agrobot. Cluj-Napoca 2018, 46, 343–349. [CrossRef]

73. Kile, G. The Host-Pathogen Relationship between Apple and Trametes versicolor and Factors Affecting Host Susceptibility. Ph.D. Thesis, University of Tasmania, Hobart, Australia, 1971.

74. Darbyshire, B. Factors Influencing the Susceptibility of Apple Trees to Trametes (Polystictus) versicolor. Ph.D. Thesis, University of Tasmania, Hobart, Australia, 1968.

75. Dilley, M.A.; Covey, R.P., Jr. Association of Coriolus versicolor with a dieback disease of apples in Washington State. Plant Dis. 1981, 65, 75–77. [CrossRef]

76. Udayanga, D.; Liu, X.Z.; McKenzie, E.H.C.; Chukeatirote, E.; Bahkali, A.H.A.; Hyde, L.D. The genus Phomopsis: Biology, applications, species concepts and names of common phytopathogens. Fungal Divers. 2011, 50, 189–225. [CrossRef]

77. Karaoglanidi, G.; Bardas, G. First Report of Phomopsis Fruit Decay on Apple Caused by Phomopsis mali in Greece. Plant Dis. 2006, 90, 375. [CrossRef]

78. Abramczyk, B.; Król, E.D. Use of RAPD-PCR and its markers for identification of Diaporthe/Phomopsis from fruit trees in south-eastern Poland. Acta Sci. Pol. - Horitum Cultus 2016, 15, 161–175. Available online: http://www.acta.media.pl/pl/action/getfullphp?id=5213 (accessed on 8 January 2020).

79. Pertot, I.; Vindimian, M.E. Diffusione di Phomopsis mali, agente di moria in giovani impianti di melo in Trentino. Inform. Fitopatol. 1998, 48, 45–49. Available online: http://hdl.handle.net/10449/17658 (accessed on 8 January 2020).

80. Espinoza, J.G.; Briceño, E.X.; Chávez, E.R.; Urbez-Torrez, J.R.; Latorre, B.A. Neofusicoccum spp. Associated with Stem Canker and Dieback of Blueberry in Chile. Plant Dis. 2009, 93, 1187–1194. [CrossRef] [PubMed]

81. Schaad, N.W.; Jones, J.B.; Chun, W. Laboratory Guide for the Identification of Plant Pathogenic Bacteria, 3rd ed.; The American Phytopathological Society: St. Paul, MN, USA, 2001; 373p.
82. White, T.J.; Bruns, T.; Lee, S.; Taylor, J. Amplified and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In PCR Protocols: A Guide to Methods and Applications; Innis, M.A., Gelfand, D.H., Sninsky, J.J., White, T.J., Eds.; Academic: San Diego, CA, USA, 1990; pp. 315–322.

83. Glass, N.L.; Donaldson, G.C. Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous ascomycetes. Appl. Environ. Microbiol. 1995, 61, 1323–1330. [CrossRef] [PubMed]

84. Cabone, I.; Kohn, L.M. A method for designing primer sets for speciation studies in filamentous ascomycetes. Mycologia 1999, 91, 553–556. [CrossRef] [PubMed]

85. Mang, S.M.; Bonomo, M.G.; Camele, I.; Saturnino, C.; Capasso, A.; Salzano, G. Preliminary Investigation of Allergenic Fungi in an Indoor Environment. Pharmacologyonline 2020, 2, 314–320.

86. Altschul, S.F.; Gish, W.; Miller, W.; Myers, E.W.; Lipman, J.D. Basic Local Alignment Search Tool. J. Mol. Biol. 1990, 215, 403–410. [CrossRef]

87. Johnson, M.; Zaretskaya, I.; Raytselis, Y.; Merezhuk, Y.; McGinnis, S.; Madden, T.L. NCBI BLAST: A better web interface. Nucl. Acids Res. 2008, 36 (Suppl. 2), W5–W9. [CrossRef] [PubMed]

88. Larkin, M.A.; Blackshields, G.; Brown, N.P.; Chenna, R.; McGettigan, P.A.; McWilliam, H.; Valentin, F.; Wallace, L.M.; Wilm, A.; Lopez, R.; et al. Clustal W and Clustal X version 2.0. Bioinformatics 2007, 23, 2947–2948. [CrossRef]

89. Kumar, S.; Stecher, G.; Li, M.; Knyaz, C.; Tamura, K. MEGA X: Molecular Evolutionary Genetic Analysis across computing platforms. Mol. Biol. Evol. 2018, 35, 1547–1549. [CrossRef]

90. Saitou, N.; Nei, M. The neighbor-joining method: A new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 1987, 4, 406–425. [CrossRef]

91. Felsenstein, J. Confidence intervals on phylogenetics: An approach using bootstrap. Evolution 1985, 39, 783–791. [CrossRef]

92. Dopazo, J. Estimating errors and confidence intervals for branch lengths in phylogenetic trees by a bootstrap approach. J. Mol. Evol. 1994, 38, 300–304. [CrossRef] [PubMed]

93. Tajima, F.; Nei, M. Estimation of evolutionary distance between nucleotide sequences. Mol. Biol. Evol. 1984, 1, 269–285. [PubMed]

94. Shapiro, S.S.; Wilk, M.B. An analysis of variance test for normality (complete samples). Biometrika 1965, 52, 591–611. [CrossRef]

95. Holm, S. A simple sequentially rejective multiple test procedure. Scand. J. Stat. 1979, 6, 65–70.

96. R Core Team. R: A Language and Environment for Statistical Computing, 2019; R Foundation for Statistical Computing: Vienna, Austria; Available online: https://www.R-project.org/ (accessed on 15 January 2022).