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Botryosphaeria Dothidea and Neofusicoccum Yunnanense Causing Canker and Die-Back of Sequoiadendron Giganteum in Croatia

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Abstract: *Sequoiadendron giganteum* Lindl. [Buchholz] is a long-lived tree species endemic to the Sierra Nevada Mountains in California. Due to its massive size and beauty, *S. giganteum* is a popular ornamental tree planted in many parts of the world, including Europe. Since 2017, scattered branch die-back has been observed on *S. giganteum* trees in Zagreb, Croatia. Other symptoms included resinous branch cankers, reddish-brown discoloration of the sapwood and, in severe cases, crown die-back. Branches showing symptoms of die-back and cankers were collected from six *S. giganteum* trees in Zagreb and the aim of this study was to identify the causal agent of the disease. The constantly isolated fungi were identified using morphology and phylogenetic analyses based on the internal transcribed spacer (ITS) of the ribosomal DNA (rDNA), and partial sequencing of two housekeeping genes, i.e., translation elongation factor 1-α (TEF 1-α), and β tubulin 2 (TUB2). The fungi were identified as *Botryosphaeria dothidea* (Moug.) Ces. and De Not. and *Neofusicoccum yunnanense* G.Q. Li & S.F. Chen. The pathogenicity test was conducted in a plant growth chamber on *S. giganteum* seedlings and revealed that *N. yunnanense* was more aggressive compared to *B. dothidea. N. yunnanense* was able to reproduce symptoms of canker and die-back and kill plants seven weeks after inoculation whereas *B. dothidea* produced cankers. To the best of our knowledge, this is the first report of *B. dothidea* and *N. yunnanense* causing canker and die-back disease of *S. giganteum* in Croatia. It is also the first record on the identity and pathogenicity of any fungal species associated with *S. giganteum* in this country. The study expended the known host range of *N. yunnanense* to include *S. giganteum*, which is a valuable ornamental tree in Croatian landscapes. Disease management strategies should be developed to mitigate or reduce the impact of the disease.

Keywords: *Botryosphaeriaceae*; phylogeny; giant sequoia; die-back disease; canker disease; urban tree pathogens

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1. Introduction

Giant sequoia (*Sequoiadendron giganteum* Lindl. [Buchholz]) is a massive, long-lived, coniferous tree species endemic to the western slopes of the Sierra Nevada Mountains in California, in western North America. Its natural populations are confined to distinct groves with a narrow mid-elevation range of 1400–2150 m and occupy about 14,600 ha. These groves are in areas with moderate winter temperatures, and relatively abundant summer soil water supply, such as valley bottoms or plateaus receiving snowmelt [1]. *S. giganteum* is threatened by climate changes due to expected decrease in the amount of soil moisture throughout Sierra Nevada [2]. Moreover, the species is endangered due to competition in the absence of periodic fires, and loss of genetic diversity and has been listed on the IUCN red list of threatened plants [3,4]. Outside its natural range, *S. giganteum*

is highly regarded as ornamental and it is frequently planted as a large tree for shade or specimen in city parks and private gardens. It is also often planted in the living collections of woody plants such as botanical gardens and arboreta [1].

Sequoiadendron giganteum has been known for its remarkable fire, pest, and pathogen resistance. This is mainly due to its thick resin-free bark and hardwood that is resistant to fungal decay, enabling trees to live for >3000 years in the species' native range [1]. However, recently, it was found that severe drought stress in combination with cambial damage from fire and bark beetle attack (*Phloeosinus* spp.) can increase mortality of *S. giganteum* [5]. Moreover, in the last decade, a die-back and canker disease of *S. giganteum* was observed outside of its native range, including urban landscapes of California, France, Germany, Austria, Switzerland, Hungary, Greece, Bulgaria, and Serbia [6–16]. Tests of pathogenicity have shown that the disease has been caused by fungi in the *Botryosphaericeae* (Ascomycota: Botryosphaeriales), namely *Neofusicoccum parvum* (Pennycook and Samuels) Crous, Slippers and A.J.L. Phillips, *Neofusicoccum nonquaesitum* Inderb., Trouillas, Bostock and Michailides, *Botryosphaeria dothidea* (Moug.) Ces. and De Not. and *Dothiorella omnivora* Linaldeddu, Deidda and Scanu [6,14].

Fungi in the *Botryosphaeriaceae* are important tree pathogens causing disease symptoms such as canker, die-back, leaf blight, fruit rot in a wide variety of hosts, including ornamental trees [14–18]. They are also saprophytes, endophytes and latent pathogens behaving pathogenic when plants are exposed to stress conditions such as drought or high temperatures [19]. *Botryosphaeriaceae* are distributed worldwide and most of these fungi have broad host affinities [14,19]. In the last decade, incidence of diseases caused by *Botryosphaeriaceae* have been increasing and climate change was speculated to be the main cause for this increase [13,19].

For many years, delimitation of species boundaries within *Botryosphaeriaceae* has been challenging. This is due to overlapping morphology between species and the existence of cryptic species complexes that cannot be distinguished based on morphology alone or combination of morphology and single gene sequencing, including *Neofusicoccum parvum* species complex. In recent years, DNA sequence-based phylogenetics and the introduction of a "one fungus–one name" concept have strongly shaped the taxonomy of members of this fungal family. Therefore, the analyses of combined multilocus DNA sequences and genealogical concordance phylogenetic species recognition (GCPSR) principle have become an important tool to infer phylogenetic relationships within *Botryosphaeriaceae*. The most used loci include the internal transcribed spacer (ITS) of the ribosomal DNA (rDNA), partial translation elongation factor 1-α (TEF 1-α), partial β-tubulin 2 gene (TUB2) and DNA-directed RNA polymerase II subunit (rpb2) [20,21].

Since 2017, mature *S. giganteum* trees (50–140 years old) planted as ornamentals in Zagreb, Croatia have been showing disease symptoms such as scattered branch die-back, resinous branch cankers, reddish-brown internal discoloration of sapwood and in severe cases, crown die-back (Figure 1). About fifty *S. giganteum* trees had been planted in Zagreb, of which up to 70% have been showing disease symptoms described above. The symptoms resemble those of a *Botryosphaeriaceae*-related die-back and canker disease of *S. giganteum* in California and Europe [6–16]. The aim of this study was to determine the causal agent of the disease observed on *S. giganteum* trees in Zagreb. This was done using morphological characteristics of the asexual morph, phylogenetic analyses of the ITS rDNA, partial TEF 1-α and partial TUB2 gene regions, and a pathogenicity test.

Figure 1. Disease symptoms observed on *Sequoiadendron giganteum* Lindl. [Buchholz] trees in Zagreb, Croatia. (**a**). Scattered branch die-back. (**b**). Top die-back and scattered branch die-back. (**c**,**e**). Resinous branch canker (**d**). Needle die-back.

2. Materials and Methods

2.1. Sample Collection, Fungal Isolation, and Morphological Characterization

In March 2017, samples were collected from six randomly selected *S. giganteum* trees experiencing branch and crown die-back in Zagreb, Croatia (Table S1, Figure 1). Five branch parts approximately 20 cm long with needles showing die-back symptoms and cankered sapwood, which was resin-soaked and discolored at the cross section, were collected from each sampled tree, placed in paper bags, and transferred to the laboratory. Small tissue pieces (3–4 mm diameter) from apparently healthy-to-diseased margins of needles and discolored, resinous sapwood were cut, and surface sterilized as described in Zlatković et al. [13]. Two hundred samples (100 samples of needles and 100 samples of sapwood, five samples per Petri dish) were plated onto 2% malt extract agar (LabM, Lancaster, UK) plates (MEA) amended with lactic acid (1.6 mL/L, NRK, Belgrade, Serbia, AMEA) to prevent bacterial growth. Petri dishes were sealed using parafilm (Brand, Wertheim, Germany) and incubated at 25 °C in the dark for two weeks.

The consistently recovered colonies were *Botryosphaeriaceae*-like (mycelium fast-growing, grey to black, fluffy to appressed) [13]. These colonies were selected, and hyphal tips were

transferred to fresh MEA using a sterile hypodermic needle. Based on colony morphology, the isolates were then divided into two groups. Depending on the number of isolates available, up to ten isolates from each group were further morphologically characterized. To induce the formation of fruit bodies, isolates were grown on 2% water agar (WA, LabM, Lancaster, UK) overlaid with triple sterilized *Pinus nigra* J. F. Arnold needles and kept in the laboratory at room temperature (22–24 °C) under natural day and night cycle for six weeks. The fruiting bodies were sectioned by hand and mounted on microscope slides in distilled water. Morphological characteristics of the fruit bodies and spores were observed using an Olympus SZX10 stereo microscope (Olympus Co., Tokyo, Japan), and an Olympus BX53F light microscope with differential interference contrast. Photographs were taken with an Olympus SC50 digital camera and accompanying software. The lengths and widths of 20 conidia per isolate were measured and the ratio of average length to average width (l/w) for each species was calculated.

Two representative isolates of each morphologically different group were selected, and further used in this study to identify the fungi. Isolates were stored as mycelium plugs in sterile distilled water at 4 °C and in 40% glycerol at −80 °C. The isolates were deposited in the collection of microorganisms of the Institute of Lowland Forestry and Environment (ILFE).

2.2. DNA Extraction, PCR Amplification and Phylogenetic Analyses

Genomic DNA was extracted from one-week-old fungal cultures by gently scraping the mycelium using a sterile scalpel and by following the manufacturer's protocol for the PrepMan Ultra Sample Preparation Reagent (Applied Biosystems, Warrington, UK). The DNA concentrations and quality were measured using a BioSpec-nano spectrophotometer (Shimadzu, Biotech, Japan), at 260 and 280 nm. Afterwards, the DNA was diluted to the concentration of 20 ng/µL. The ITS region of the rDNA was amplified using primers ITS1F [22] and ITS 4 [23]. Part of the TEF 1-α gene and part of the TUB2 gene were amplified using primers described in Zlatković et al. [13]. The 25 µL PCR reaction mixtures contained 2.5 µL of 10 × PCR buffer (100 mM Tris-HCl, 500 mM KCl (pH 8.3), Roche Diagnostics GmbH, Mannheim, Germany), 2–4 µL of 25 mM $MgCl_2$ (Roche), 1 µL of 100 µM of each dNTPs (Thermo Scientific, Vilnius, Lithuania), 0.5 µL of 10 µM of each primer (Invitrogen, Paisley, UK), 2 µL (40 ng) of genomic DNA, 0.5 µL (2.5 U) of Taq polymerase (Roche) and 14–16 µL of autoclave-sterilized ultra-pure water treated with a carbon filtration-reverse osmosis-deionizing system (Ecosoft Water Systems GmbH, Nettetal, Germany) and filtered with an Acrodisc Syringe Filter with 0.2 µm HT Tuffryn membrane (Pall Corporation, Ann Arbor, MI, USA). Control samples contained sterile ultra-pure water instead of the DNA. The PCR was performed in an Eppendorf Mastercycler epgradient S thermal cycler (Eppendorf AG, Hamburg, Germany) under the following conditions: initial denaturation of 5 min at 95 °C, followed by 35 cycles of 30 s at 95 °C, 30 s at 55 °C, and 1 min at 72 °C, and a final extension step of 8 min at 72 °C. However, the ITS region and TUB2 failed to amplify for some isolates with amplification conditions described above. In these situations, PCRs were performed using a touchdown protocol [24] with annealing temperatures ranging from 61 °C to 55 °C or 65 °C to 55 °C (Table S2).

The amplified products were analysed by electrophoresis on 2% (*w*/*v*) agarose gels (Serva Electrophoresis GmbH, Heidelberg, Germany) in 0.5 × TBE buffer, stained with Roti-GelStain (Carl Roth GmbH + Co. KG, Karlsruhe, Germany) and visualized with UV illumination. The DNA molecular weight marker (O'GeneRuler 100 bp DNA ladder, Thermo Scientific, Vilnius, Lithuania) was used to estimate the size of the products. The PCR products were purified with the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions, and sequenced by Macrogen Europe (Amsterdam, The Netherlands). Sequencing proceeded in both directions, using the same primers as used for the PCR reactions.

Sequences were examined, edited, assembled, and multiple sequence alignments were done using BioEdit v. 7.2.5, MEGA X and MAFFT v. 7 as described in Zlatković

et al. [25]. Sequence data of the three loci (ITS, tef 1-α, TUB2) were analysed individually and in combination, to assure that the evolutionary lineage is consistent in most of the analysed phylogenies [26]. The phylogenetic analyses of the individual genes were done using Maximum Likelihood (ML) and Bayesian Interference (BI) analyses, whereas the analyses of the combined dataset (ITS + tef 1-α + TUB2) were performed using ML, Maximum parsimony (MP) and BI. The ML and MP analyses were conducted using PhyML v. 3.0 (http://www.atgc-montpellier.fr/phyml/, accessed on 13 April 2021) and PAUP v. 40b10 following the methods described by Zlatković et al. [25] and Zlatković et al. [13], respectively. The partition homogeneity test (PHT) was done in PAUP v. 40B10 [13]. The BI analyses were done in MrBayes v. 3.0b4 as described previously [27] but using the best nucleotide substitution model previously generated by the smart model selection option based on the Akaike information criterion during the ML analyses [28]. *Pseudofusicoccum stromaticum* Mohali, Slippers and M.J. Wingfield (CBS 117448, CBS 117449) served as outgroup. The DNA sequences generated in this study were deposited in the GenBank database (Table 1).

Table 1. Sequences used in the phylogenetic analyses.

Isolate No. [1,2]	Identity	Host	Location	Collector	GenBank Accession No. [3,4]		
					ITS	tef 1-α	TUB2
ILFE 2	Botryosphaeria dothidea	Sequoiadendron giganteum	Zagreb, Croatia	M. Kovač	**MW033525**	**MW051688**	**MW051690**
ILFE 3	B. dothidea	S. giganteum	Zagreb, Croatia	M. Kovač	**MW033526**	**MW051689**	**MW051691**
CMW 39308	B. dothidea	S. giganteum	Valjevo, Serbia	N. Keča	KF575008	KF575040	KF575104
CBS 110302	B. dothidea	Vitis vinifera	Portugal	A.J.L. Phillips	AY259092	AY573218	EU673106
CMW 8000	B. dothidea	Prunus sp.	Crocifisso, Switzerland	B. Slippers	AY236949	AY236898	AY236927
CERC 2947	B. qingyuanensis	Eucalyptus hybrid	China	S.F.Chen & G.Q.Li	KX278001	KX278106	KX278210
CERC 2946	B. qingyuanensis	Eucalyptus hybrid	China	S.F.Chen & G.Q.Li	KX278000	KX278105	KX278209
CBS 135219	B. kuwatsukai	Malus domestica	China	C.S. Wang	KJ433388	KJ433410	-
LSP 5	B. kuwatsukai	Pyrus sp.	China	C.S. Wang	KJ433395	KJ433417	-
CBS 127194	B. fabicerciana	Eucalyptus sp.	China	M.J. Wingfield	HQ332198	HQ332214	KF779069
CBS 127193	B. fabicerciana	Eucalyptus sp.	China	M.J. Wingfield	HQ332197	HQ332213	KF779068
ATCC 22927	B. corticis	Vaccinium sp.	North Carolina, USA	R.D. Millholland	DQ299247	EU673291	EU673108
CBS 119047	B. corticis	V. corymbosum	USA	P.V. Oudemans	DQ299245	EU017539	EU673107
CBS 124702	B. scharifii	Magnifera indica	Iran	J. Abdol-lahzadeh & A. Javadi	JQ772019	JQ772056	-
CBS 124703	B. scharifii	M. indica	Iran	J. Abdol-lahzadeh	JQ772020	JQ772057	-
CGMCC 3.18004	B. ramosa	Acacia sp.	Hainan, China	Z.P. Dou & W. He	KX197073	KX197093	KX197100
CBS 122069	B. ramosa	E. camaldulensis	Australia	T.I. Burgess	EU144055	EU144070	KF766132
CBS 141505	B. agaves	Agave sp.	France	P.W. Crous	KX306750	MT592030	MT592463
CBS 133992	B. agaves	Agave sp.	Thailand	R. Phookamsak	JX646791	JX646856	JX646841

Table 1. *Cont.*

Isolate No. [1,2]	Identity	Host	Location	Collector	GenBank Accession No. [3,4]		
					ITS	**tef 1-α**	**TUB2**
CBS 116131	Neofusicoccum arbuti	Arbutus menziesii	USA	M. Elliott	AY819720	KF531792	KF531793
CBS 117090	N. arbuti	A. menziesii	USA	M. Elliott	AY819724	KF531791	KF531794
CBS 126655	N. nonquaesitum	Umbellularia californica	USA	F.P. Trouillas	GU251163	GU251295	GU251823
CBS 133501	N. nonquaesitum	Persea americana	USA	A. Eskalen	MT587498	MT592213	MT592705
CGMCC 3.18313	N. illicii	Illicium verum	Guangxi, China	L. Wang	KY350152	KY817758	KY350158
CGMCC 3.18311	N. illicii	I. verum	Guangxi, China	L. Wang	KY350150	KY817756	KY350156
CBS 123634	N. cordaticola	Syzygium cordatum	South Africa	D. Pavlić	EU821898	EU821868	EU821838
CBS 123635	N. cordaticola	S. cordatum	South Africa	D. Pavlić	EU821903	EU821873	EU821843
CMM 1285	N. brasilliense	Mangifera indica	Brazil	M.W. Marques	JX513628	JX513608	KC794030
CMM 1338	N. brasilliense	M. indica	Brazil	M.W. Marques	JX513630	JX513610	KC794031
CBS 123639	N. kwambonambiense	S. cordatum	South Africa	D. Pavlić	EU821900	EU821870	EU821840
CBS 123641	N. kwambonambiense	S. cordatum	South Africa	D. Pavlić	EU821919	EU821889	EU821859
CBS 121.26	N. ribis	Ribes rubrum	New York, USA	N.E. Stevens	AF241177	AY236879	AY236908
CBS 115475	N. ribis	Ribes sp.	USA	B. Slippers & G. Hudler	AY236935	AY236877	AY236906
CERC 3416	N. sinoeucalypti	Eucalyptus hybrid	Guangxi, China	S.F. Chen & G.Q. Li	KX278064	KX278169	KX278273
CERC 2005	N. sinoeucalypti	E. urophylla × E. grandis	Guangdong, China	S.F. Chen & G.Q. Li	KX278061	KX278166	KX278270
CBS 128008	N. occulatum	E. grandis hybrid	Australia	T.I. Burgess	EU301030	EU339509	EU339472
MUCC 286	N. occulatum	E. pellita	Australia	T.I. Burgess	EU736947	EU339511	EU339474
CSF 5721	N. dianense	E. globulus	YunNan, China	S.F. Chen & G.Q. Li	MT028608	MT028774	MT028940
CSF 6075	N. dianense	E. urophylla × E. grandis	YunNan, China	S.F. Chen & G.Q. Li	MT028605	MT028771	MT028937
CSF 6142	*N. yunnanense*	*E. globulus*	YunNan, China	S.F. Chen & G.Q. Li	MT028667	MT028833	MT028999
CSF 6034	*N. yunnanense*	*E. urophylla* × *E. grandis*	YunNan, China	S.F. Chen & G.Q. Li	MT028672	MT028838	MT029004
ILFE 4	*N. yunnanense*	*S. giganteum*	Zagreb, Croatia	M. Kovač	**MW085805**	**MW071142**	**MW071144**
ILFE 5	*N. yunnanense*	*S. giganteum*	Zagreb, Croatia	M. Kovač	**MW085806**	**MW071143**	**MW071145**
CMW 39327 *	*N. yunnanense*	*S. giganteum*	Belgrade, Serbia	M. Zlatković	KF729050	KF729380	KF729340
CMW 9080	*N. parvum*	*Populus nigra*	New Zealand	G. J. Samuels	AY236943	AY236888	AY236917
CMW 9081	*N. parvum*	*P. nigra*	New Zealand	G.J. Samuels	AY236943	AY236888	AY236917
CGMCC 3.18748	*N. hongkongense*	*Araucaria cunninghamii*	Hong Kong, China	S.F. Chen	KX278051	KX278156	KX278260
CGMCC 3.18747	*N. hongkongense*	*A. cunninghamii*	Hong Kong, China	S.F. Chen	KX278050	KX278155	KX278259
CBS 117448	*Pseudofusicoccum stromaticum*	*Eucalyptus* hybrid	Venezuela	S. Mohali	AY693974	AY693975	EU673094
CBS 117449	*P. stromaticum*	*Eucalyptus* hybrid	Venezuela	S. Mohali	DQ436935	DQ436936	EU673093

[1] Ex-type or ex-epitype strains are shown in italic. [2] Strains sequenced in this study are shown in bold. [3] ITS: Internal Transcribed Spacer; tef 1-α: partial translation elongation factor 1-alpha gene; TUB2: partial beta-tubulin 2 gene. [4] Dashes indicate that the corresponding sequence was not available in GenBank. * Previously identified as *N. parvum* in [14]. ATCC: American Type Culture Collection, Virginia, USA; CBS: Culture Collection of the Westerdijk Fungal Biodiversity Institute, Utrecht, the Netherlands; CERC: Culture Collection of the China Eucalypt Research Centre, Chinese Academy of Forestry, ZhanJiang, GuangDong Province, China; CGMCC: China General Microbiological Culture Collection, Beijing, China; CMM: Culture Collection of Phytopathogenic Fungi "Prof. Maria Menezes", University of Pernambuco, Recife, Brazil; CMW: Culture Collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa; ILFE: Collection of Microorganisms of the Institute of Lowland Forestry and Environment, University of Novi Sad, Serbia; MUCC: Culture Collection of Murdoch University, Perth, Australia.

2.3. Path ogenicity Test and Statistical Analysis

One isolate of each species identified, i.e., ILFE 2 (*B. dothidea*) and ILFE 4 (*N. yunnanense* G.Q. Li & S.F. Chen) was randomly selected for pathogenicity test. The test was performed on potted three-years-old *S. giganteum* seedlings purchased from an ornamental plant nursery Iris MBM d.o.o. After purchase, the seedlings (40–45 cm high with a 0.7 to 0.8 cm trunk diameter at the soil line) were kept in a growth chamber (23 °C, 70% humidity, 12/12 h day/night cycle) for two weeks and watered every other day to attain field water capacity. Ten plants per isolate were used to inoculate a total of 20 plants and ten plants were mock inoculated using uncolonized MEA plugs to serve as controls. The bark was surface sterilized using 70% ethanol (*v/v*) and a 3 mm wound was made 3–4 cm above the soil line using a sterilized cork borer. Mycelial plugs (3 mm diam.) were taken from the margins of one week old colonies and placed in the wounds, with the mycelium facing the vascular tissue. Inoculation points were covered with sterile moist cotton wool and wrapped with parafilm to prevent desiccation and contamination. The inoculated plants were arranged in a completely randomized design and maintained in a growth chamber under the conditions described above. The test lasted for seven weeks, i.e., until the first inoculated plant was killed. When the experiment was terminated, the outer bark was removed and the lengths of cankers upwards and downwards from the point of inoculation were measured. Pieces of necrotic tissue from the edge of each canker were surface disinfected as described in Zlatković et al. [13] and plated onto AMEA to isolate the inoculated fungi and complete Koch's postulates. The identity of the recovered fungi was verified using morphology of fungal colonies and spores as described previously.

Statistical analyses were done with Statistica 12.0 (StatSoft Inc., Tulsa, OK, USA). Normality and homogeneity of variances were checked using One sample Kolmogorov–Smirnov test and Leven's test. The differences in canker lengths between the two fungal isolates were assessed using nonparametric Mann–Whitney U test at $\alpha = 0.05$.

3. Results

3.1. Fungal Isolation, and Morphological Characterization

In total, isolations yielded 91 *Botryosphaeriaceae*-like isolates (63.6% were isolated from cankered sapwood, and 36.4% from needles experiencing die-back). The isolates were divided into two groups based on colony morphology, i.e., group 1: colonies grey and fluffy (91.2% of all isolates), group 2: colonies black, with appressed mycelial mat (8.8%, Figure 2).

Isolates produced mature fruit bodies (pycnidia) after seven weeks of growing on WA overlaid with pine needles. Pycnidia of the group 1 were black, sometimes with sparse grey mycelial hairs, globose, up to 500 µm wide, solitary, semi-immersed to superficial, unilocular with a central ostiole. Conidiogenous cells were with annellations. Conidia were hyaline, narrowly fusiform, or fusiform, smooth with granular content, aseptate, measuring $21 - 31.9 \times 7.1 - 8.7$ (av. 25.3×7.8, l/w 3.2) µm. Pycnidia of the group 2 were black, covered with long, light grey mycelial hairs, globose, up to 700 µm wide, solitary, semi-immersed to superficial, unilocular with a central ostiole. Conidiogenous cells were proliferating percurrently with indistinct annellations. Conidia were hyaline, fusiform to ellipsoidal, smooth with granular content, aseptate, measuring $15.4 - 19.4 \times 6 - 8.1$ (av. 17.8×6.7, l/w 2.7) µm (Figure 2). Based on morphological characteristics the isolates were preliminary identified as *Botryosphaeria* spp. (group 1) and *Neofusicoccum* spp. (group 2) in the *Botryosphaeriaceae* (Ascomycota).

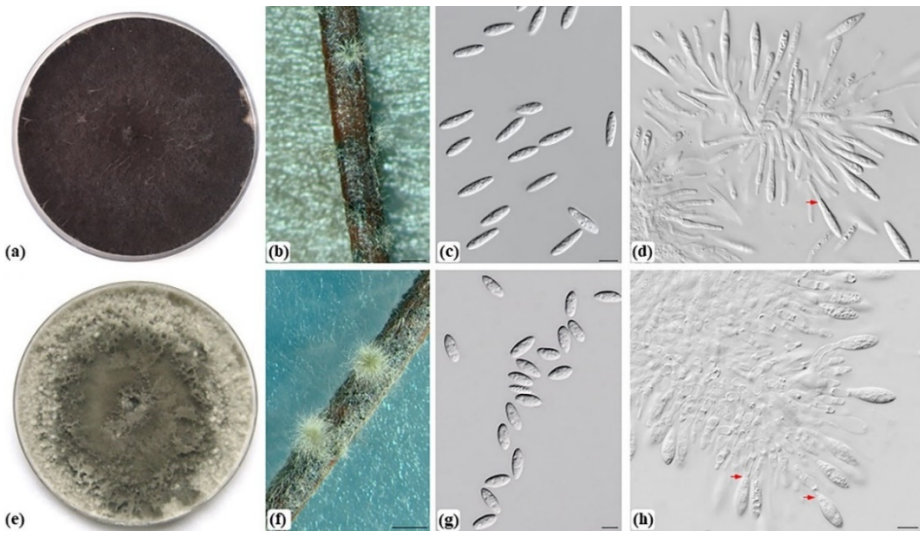

Figure 2. Morphological characteristics of *Botryosphaeria dothidea* (ILFE 2) and *Neofusicoccum yunnanense* (ILFE 4) isolated and identified in this study. (**a**,**e**). 14-day old culture of *B. dothidea* (**a**) and *N. yunnanense* (**e**) growing on MEA at 25 °C. (**b**,**f**). Superficial globose pycnidia of *B. dothidea* (**b**) and *N. yunnanense* (**f**) covered with mycelium. (**c**). Hyaline, aseptate fusiform conidia of *B. dothidea*. (**d**,**h**). Conidia of *B. dothidea* (**d**) and *N. yunnanense* (**h**) developing on conidiogenous cells. Annellations are marked with arrows (**d**,**h**). (**g**). Hyaline, aseptate, fusiform to ellipsoidal conidia of *N. yunnanense*. Scale bars: 1 mm (**b**,**f**), 10 µm (**c**,**d**,**g**,**h**).

3.2. Phylogenetic Analyses

The best fit models of nucleotide substitution used in the analyses of the individual genes were as follows: GTR + G (ITS; G = 0.499), GTR + G + I (tef 1-α; G = 0.499, I = 0.105), and HKY 85 + G (TUB2; G = 0.279). ML and BI analyses of the individual genes resulted in trees with congruent topologies (Figure S1). The concatenated dataset with the tree loci (ITS + tef 1-α + TUB2) of 50 sequences including *P. stromaticum* as an outgroup (12 sequences generated in this study and 38 retrieved from GenBank) resulted in 1292 characters of which 453 characters were parsimony informative and 839 characters were parsimony uninformative (Table 1). For ML and BI analyses, the model GTR + G + I (G = 0.791, I = 0.401) was selected as the best fit model of nucleotide substitution. For MP analyses, PHT test indicated that the loci are suitable to be combined (p = 0.03). Tree topologies resulting from ML, BI and MP analyses (37 equally most parsimonious trees, CI = 0.8, RI = 0.9, TL = 643) of the concatenated dataset were similar, with minor differences in the positions of subclades and the ML tree is shown in Figure 3. Phylogenetic analyses of the combined ITS/tef 1-α/TUB2 dataset revealed two major and strongly supported clades, each representing a separate genus, including *Botryosphaeria* and *Neofusicoccum* (Figure 3).

In the ITS, tef 1-α and combined ITS/tef 1-α/TUB2 trees isolates ILFE 2 and ILFE 3 clustered within the clade corresponding to *B. dothidea* (Figure S1, Figure 3). These isolates had only one single nucleotide polymorphism (SNP) that differentiated them from the type strain of *B. dothidea* CMW 8000 (Table 2).

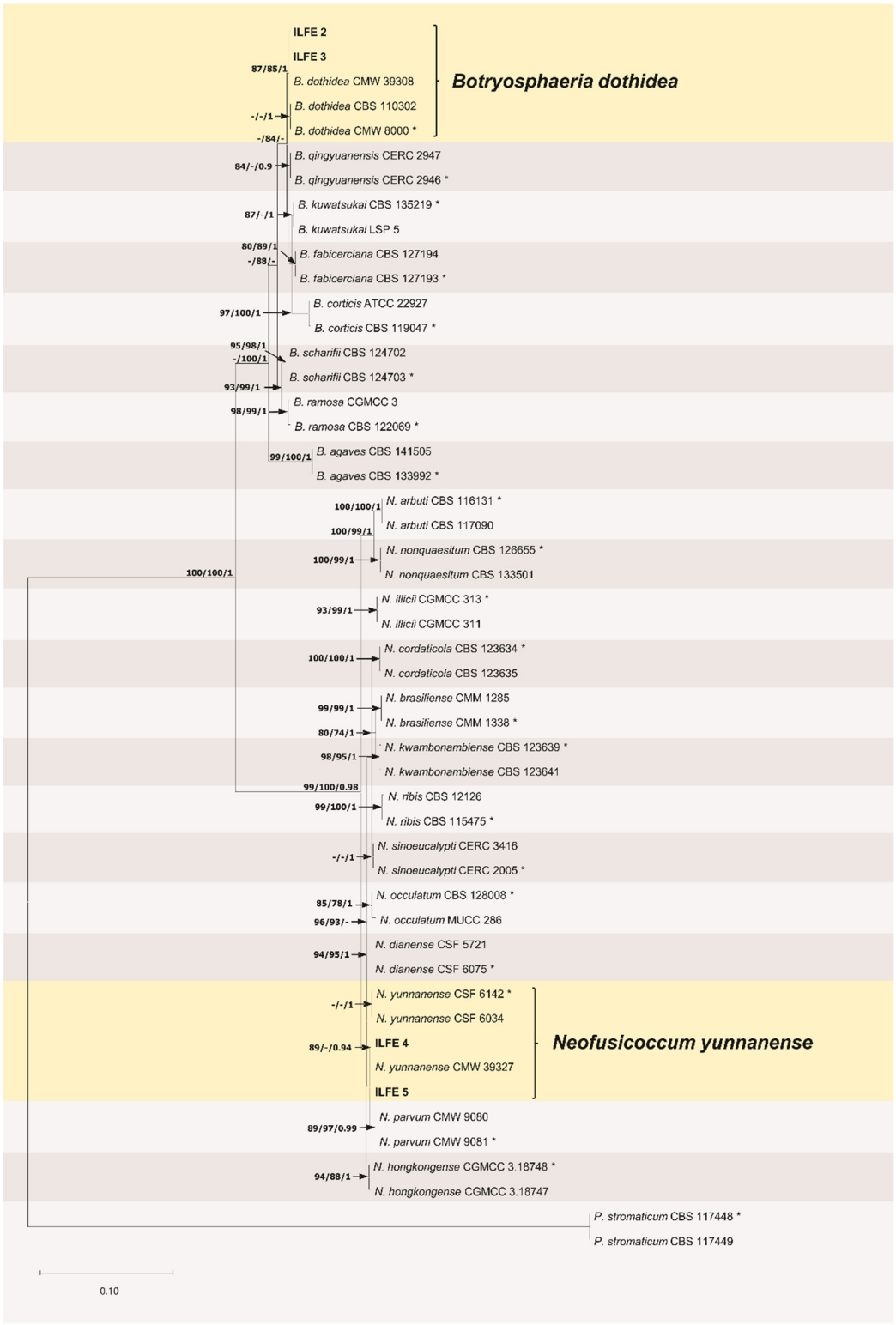

Figure 3. Phylogenetic tree generated from a maximum likelyhood analyses (ML) based on a concatenated alignment of ITS, tef 1-α and TUB2 sequences data showing the position of *Botryosphaeria dothidea* and *Neofusicoccum yunannense* in relation to their closely related species. ML and MP bootstrap support values greater than 70% and Bayesian posterior probability values (PP) greater than 0.90 are indicated at the tree nodes (ML/MP/PP). Clades corresponding to *N. yunnanense* and *B. dothidea* are highlighted. The type strains are marked with an asterisk and isolates obtained in this study are shown in bold. *Pseudofisicoccum stromaticum* (CBS 117448 and CMW 117449) was included as an outgroup. Scale bar indicates expected number of substitutions per site.

Table 2. Polymorphisms in the nucleotide sequences of ITS, tef 1-α and TUB2 loci between isolates ILFE 4, ILFE 5, CMW 39327, *N. yunnanense*, and *N. parvum* and between isolates ILFE 2, ILFE 3, and *B. dothidea*. The ex-type strains are marked with an asterisk and shared polymorphisms are highlighted in grey.

Isolates	Locus						
	ITS			tef 1-α		TUB2	
	48	147	290	294	322	386	404
N. parvum CMW 9081 *	T	C	A	A	T	T	T
N. parvum CMW 9080	T	C	A	A	T	T	T
N. yunnanense CSF 6142 *	A	T	G	C	C	C	C
N. yunnanense CSF 6034	A	T	G	C	C	C	C
CMW 39327	A	C	G	C	C	C	C
ILFE 4	A	C	G	C	C	C	C
ILFE 5	A	C	G	C	C	C	C
B. dothidea 8000 *					T		
B. dothidea CBS 110302					T		
B. dothidea CMW 39308					C		
ILFE 2					C		
ILFE 3					C		

In both analyses of individual genes and combined ITS/tef 1-α/TUB2 tree isolates ILFE 4 and ILFE 5 clustered within the clade corresponding to *N. yunnanense*. There was only one SNP that differentiated these isolates from the type strain of *N. yunnanense* CSF 6142 and six SNPs differentiated them from the type strain CMW 9081 of the phylogenetically close species *N. parvum* (Table 2). In the combined ITS/tef 1-α/TUB2 tree the clade corresponding to *N. parvum* was strongly supported in all three analyses (89/97% ML, MP bootstrap support; Posterior probability: 1) and the clade corresponding to *N. yunnanense* was strongly supported in ML, weakly supported in MP analyses, and moderately supported in BI analyses (89/66% ML, MP bootstrap support; Posterior probability: 0.94). Based on phylogenetic analyses, isolates from this study were identified as *B. dothidea* and *N. yunnanense*.

3.3. Pathogenicity Test

Three weeks after inoculation, 30% of the plants inoculated with *N. yunnanense* showed disease symptoms such as wilting and die-back. Moreover, sunken, resinous cankers (<2 cm) were evident on the stems after the parafilm was removed. The disease progressed; the cankers enlarged, leading further to wilting and consequent death of the plants. Needles were dry and necrotic and fruit bodies (pycnidia) containing fusiform to ellipsoidal spores typical for *Neofusicoccum* spp. developed within cankers. The remaining plants developed resinous cankers with cracked bark in some of the plants measuring 2.1–2.6 cm (av. 2.3 cm). In addition, 40–60% of foliage of these plants was wilted, dry and necrotic. Plants inoculated with *B. dothidea* exhibited only resinous cankers measuring 1.3–1.8 cm (av. 1.4 cm). There was a statistically significant difference between the canker lengths produced by *N. yunnanense* and *B. dothidea* ($U = 0.00$, $p < 0.001$ two-tailed). Control seedlings showed no disease symptoms (Figure 4). Both fungi were re-isolated from the canker margins on inoculated plants, but not from the control plants, completing Koch's postulates.

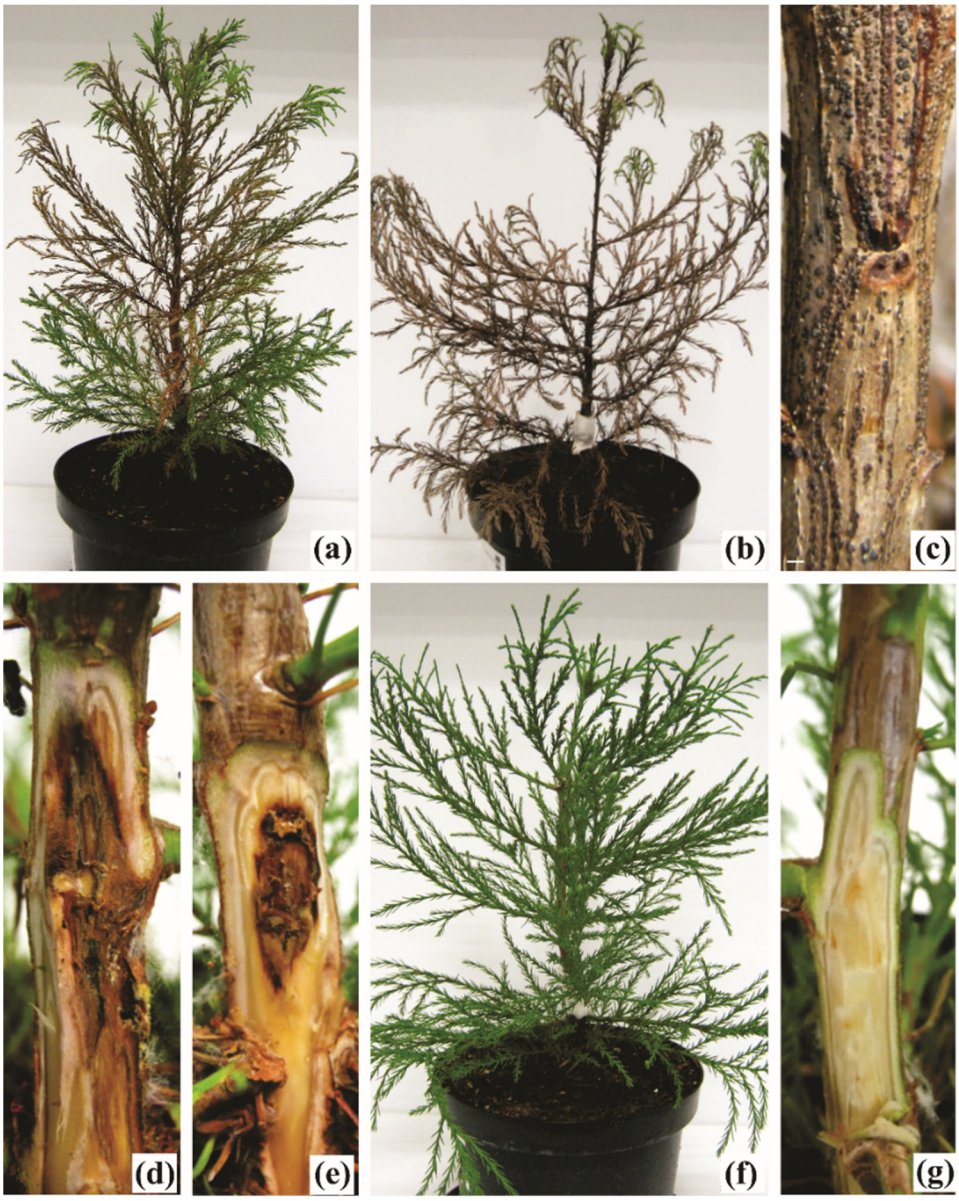

Figure 4. Disease symptoms and signs associated with *Neofusicoccum yunnanense* and *Botryosphaeria dothidea* on inoculated *Sequoiadendron giganteum* seedlings. (**a**,**b**). Browning and drying of the needles from inoculation point upwards after inoculation with *N. yunnanense* ILFE 5 (**a**) and ILFE 4 (**b**). (**c**). Pycnidia of *N. yunnanense* formed in the cankered tissue after inoculation with *N. yunnanense* ILFE 4. (**d**). Girdling, resin-soaked canker formed after inoculation with *N. yunnanense* ILFE 5. (**e**). Resinous canker formed after inoculation with *B. dothidea* ILFE 2. (**f**,**g**). Control seedling inoculated with sterile MEA plug showing no disease symptoms. Scale bar: 5 mm (**c**).

4. Discussion

The current study presents the first record of *B. dothidea* and *N. yunnanense* on *S. gigateum* in Croatia. The known geographic and host range of *N. yunnanense* was expanded and the host association of *B. dothidea* with *S. giganteum* was confirmed. The fungi were identified using morphology, phylogenetic analyses of the ITS rDNA and two housekeeping genes (tef 1-α and TUB2). The pathogenicity test showed that *B. dothidea* and *N. yunnanense* are the causal agents of the canker and die-back disease of *S. giganteum* in Croatia.

The present study is also the first report of any disease of *S. giganteum* in Croatia. Even though these trees have been planted as ornamentals for more than 150 years in this

country (the first *S. giganteum* tree was planted in 1862 in Zagreb) [29], prior to this study no research has been conducted on diseases of *S. giganteum* in Croatia. Moreover, little research has been conducted on diseases of *S. giganteum* in Southeastern Europe and the only studies are related to the die-back caused by *Botryosphaeriaceae* fungi, including *B. dothidea*, *N. parvum* and *D. omnivora* in Serbia and Greece [11,13,14]. Die-back symptoms have also recently been observed in Bulgaria, and *Botryosphaeriaceae* have been suspected to be the cause of the disease [12].

The isolation and pathogenicity of *B. dothidea* towards *S. giganteum* in Croatia is not unexpected given that it is a plurivorous and widespread species, and a well-known pathogen of forest and ornamental trees [14,17]. *B. dothidea* produced cankers on stems when inoculated, although it was less aggressive compared to *N. yunnanense* and did not cause symptoms of wilting and die-back. In contrast, in a study of Zlatković et al. [14] *B. dothidea* was able to produce girdling cankers and cause death of *S. giganteum* seedlings 13 weeks after inoculation. However, the test was carried out under field conditions, the seedlings were younger (2-year-old compared to 3-year-old seedlings used in this study) and the *B. dothidea* isolates used for inoculation originated from *Chamaecyparis lawsoniana* (A. Murray) Parl. Moreover, Worral et al. [30] isolated *B. dothidea* from *S. giganteum* in California. The later study showed that *B. dothidea* can produce symptoms of die-back and cause death of 2-year-old *S. giganteum* seedlings five weeks after inoculation in the greenhouse. However, the isolates were identified using morphological data alone and hence their identity remained unclear. In addition, Haenzi et al. [16] reported *B. dothidea* from symptomatic *S. giganteum* trees in Switzerland, but the pathogenicity of the fungus towards *S. giganteum* was not tested. Furtermore, Morelet et al. [7], Kehr et al. [8], Cech et al. [9], Vajna et al. [10] and Georgieva [12] isolated *B. dothidea* from *S. giganteum* showing die-back symptoms in France, Germany, Austria, Hungary, and Bulgaria, respectively. However, in these studies the species was identified using morphology only and the pathogenicity test was not conducted. Although *B. dothidea* has a worldwide distribution, in Croatia the species has been previously isolated only from *Vitis vinifera* L, but it remained unknown if *B. dothidea* is a pathogen of grapevine in this country [31].

Neofusicoccum yunnanense was isolated from *S. giganteum* in this study. *Neofusicoccum* species have previously been reported associated with *S. giganteum* trees and are known to have a broad host range [14,17,19]. For example, *Neofusicoccum mediterraneum* Crous, M.J. Wingf. and A.J.L. Phillips and *N. nonquaesitum* have been isolated from *S. giganteum* in California (USA) [6,32]; *Neofusicoccum australe* (Slippers, Crous and M.J. Wingf.) Crous, Slippers and A.J.L. Phillips has been found associated with *S. giganteum* in Australia [33]; *N. parvum* has been isolated from *S. giganteum* in Greece, Switzerland, and Serbia, respectively [11,14,16]. *N. yunnanese* has recently been described from China, where it was isolated from *Eucalyptus globulus* Labill., *E. urophylla* × *E. grandis* hybrid, and *Eucalyptus* sp. [34]. In this study however, *N. yunnanense* was isolated from *S. giganteum* and it seems that this species is not host-specific, as with most of the members of the *Botryosphaeriaceae* [14,19]. Conidia of isolates of *N. yunnanense* from this study (17.8 × 6.7 µm, l/w 2.7) were on average bigger and less narrow compared to those of the type strain of *N. yunnanense* (15.6 × 4.4 µm, l/w 3.5) [34]. However, this is consistent with the view that the use of morphological data for species identification in the *Botryosphaeriaceae* is unreliable. This was highlighted in previous studies [13,17,21], including in that by Slippers et al. [20] who also provided the argument that *Botryosphaeriaceae* morphological characters have evolved more than once.

Interestingly, in the phylogenetic analyses, isolates of *N. yunnanense* from China (including the type strain) clustered with isolates from this study and with an isolate CMW 39327 isolated from *S. giganteum* in Serbia. Previous study of Zlatković et al. [14] has identified the isolate CMW 39327 as *N. parvum*, but this was before the description of *N. yunnanense*. Recently, Zhang et al. [21] reassessed the identity of the 499 isolates in the culture collection (CBS) of the Wersterdijk Institute in the Netherlands, including species of the *Botryosphaeriaceae*. However, the work did not consider all strains of *N. parvum*

isolated from *S. giganteum* in the previous studies and did not include *N. yunnanense*. Further studies are necessary to clarify the identity of the global collection of isolates of the *N. parvum* species complex from *S. giganteum*. Moreover, given the small number of isolates sequenced in this study along with restricted sampling area, we cannot exclude the possibility of presence of *N. parvum* along with *N. yunnanense* on *S. giganteum* in Croatia and further investigations are required to better understand the diversity and impact of *Botryosphaeriaceae* on this host.

In this study, *N. yunnanense* was pathogenic towards *S. giganteum* and it was more aggressive compared to *B. dothidea.* Apart from producing cankers on the inoculated seedlings, *N. yunnanense* was able to produce symptoms of wilting and die-back such as those seen on mature trees under natural conditions and kill the seedlings seven weeks after inoculation. This is consistent with the results of the previous studies where *Neofusicoccum* species were among the most aggressive members of the *Botryosphaeriaceae*. For example, pathogenicity tests on *Sequoia sempervirens* (D. Don) Endl., the closest relative of *S. giganteum* have shown that *Neofusicoccum* species were more aggressive compared to *B. dothidea* and produced the largest lesions [35]. Moreover, in a study of Lazzizera et al. [36] *Neofusicoccum vitifusiforme* (Van Niekerk and Crous) Crous, Slippers and A.J.L. Phillips was more aggressive compared to *B. dothidea* when inoculated onto *Olea europaea* L. Furthermore, a study of Rooney-Latham et al. [6] had shown that *N. nonquaesitum* can produce black, sunken cankers and cause wilting of *S. giganteum* seedlings 14 days after inoculation in the greenhouse. In addition, in a study by Tsopelas et al. [11] *N. parvum* caused cankers and die-back of branches of mature *S. giganteum* trees eight weeks after inoculation. Additionally, pathogenicity results of Zlatković et al. [14] and Haenzi et al. [16] suggested that *N. parvum* is an important pathogen of *S. giganteum*, able to produce cankers and die-back when inoculated onto seedlings of *S. giganteum*.

The die-back of *S. giganteum* observed in this study could be related to various forms of stress to which trees growing in unique ecological conditions of urban sites are exposed (i.e., soil compaction, air pollution, "Heat island effect"). During the last decade, Croatia has experienced several warmest and driest years since measurements begun, accompanied by several "Heat waves" [37]. Similarly, Morelet et al. [7], Kehr et al. [8], Cech et al. [9] and Zlatković et al. [15] speculated that Botryosphaeriaceae related die-back of *S. giganteum* could be linked to an increase in extreme weather events, and other stresses that trees planted in urban areas experience. In addition, *S. giganteum* is a tree species with high water demand that naturally occurs in an area with relatively abundant water supply which is much higher than that in Zagreb where symptoms of die-back have been observed [2,37]. Additionally, in its native range, *S. giganteum* populations are confined to a mid-elevation range (1400–2150 m) [2], whereas, in Zagreb, these trees had been planted on low elevation sites (115–254 m) [29]. These conditions could have suppressed *S. giganteum* health and triggered the *Botryosphaeriaceae* related disease. This is consistent with the opportunistic nature of these fungi that are typically associated with plant stress [19].

In this study, *N. yunnanense* and *B. dothidea* have been isolated from *S. giganteum* trees planted in urban green spaces, i.e., a city park, a private garden, a botanical garden, and an arboretum. Botanical gardens and arboreta with diverse international plant collections represent dense assemblages of various tree species, including conifers and broadleaves, native and introduced trees, and are standing sentinels for the potentially invasive pathogens [38]. *Botryosphaeriaceae* are known to infect a wide range of hosts and can move between tree species [14,19] and it might be possible that other nearby trees have also been infected. Moreover, in this study, die-back symptoms have been observed on mature trees, and it is not known if the disease is also present in Croatian nurseries. Similarly, die-back of mature *S. giganteum* trees has been found in Switzerland, USA, and Greece [6,11,16], whereas Kehr et al. [8] and Georgieva et al. [12] reported the presence of disease symptoms on trees of all ages in Germany and Bulgaria, respectively. In addition, crown die-back has been associated with an advance stage of the disease development in this study but standing dead *S. giganteum* trees have not been found. In contrast, *Botryosphaeriaceae* related

S. giganteum tree mortality has been reported from Switzerland and Bulgaria [12,16], and it has also recently been observed in Serbia [39]. Therefore, a detailed tree health survey of the rest of the tree species planted in Zagreb's green places, as well as *S. giganteum* seedlings and trees in nurseries and landscapes across Croatia is urgently needed to examine the possibility or the magnitude of the spread of the disease and develop measures for disease prevention and control to minimize economic and environmental impacts.

Botryosphaeriaceae invade vascular tissues of trees and thus the safe and effective control of diseases caused by these fungi represents a challenge [19,40]. Moreover, management options for trees in urban environments are limited due to the potentially harmful impacts of residues of chemical fungicides on human health and the environment [41]. Horticultural practices such as mulching, watering, pruning of the infected branches, pruning during the dormant season or at least during dry periods to prevent infection by water splashed spores, can be used to reduce stress to trees and prevent die-back [36]. Additionally, various biological control strategies for management of the *Botryosphaeriaceae* diseases are being developed and the preliminary results are promising [42–44].

5. Conclusions

To the best of our knowledge, this work represents the first report of *B. dothidea* and *N. yunnanense* as pathogens of *S. giganteum* in Croatia. It is also the first report on the identity and pathogenicity of any fungal species associated with *S. giganteum* in this country. The host range of *N. yunnanense* has been expanded and the host association of *B. dothidea* with *S. giganteum* has been confirmed. Considering high social, and landscape value that *S. giganteum* trees have in Croatian urban areas, and the fact that most trees have been protected by the Law as horticultural monuments [29], the magnitude of this problem should not be neglected, and special attention should be paid to those trees. An integrated disease management approach, focusing on horticultural practices and biological control is needed to mitigate or reduce the impact of the disease.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/f12060695/s1, Table S1: *Sequoiadendron giganteum* trees sampled in this study, Table S2: PCR conditions used in this study, Figure S1: Phylogenetic trees generated from Bayesian interference analyses based on a single gene alignment of ITS, tef 1-α and TUB2 sequences data showing the relationships of *Botryosphaeria dothidea* and *Neofusicoccum yunannense* with closely related species. ML bootstrap support values greater than 70% and Bayesian posterior probability values (PP) greater than 0.90 are indicated at the tree nodes (ML/PP). Clades corresponding to *N. yunnanense* and *B. dothidea* are highlighted. The type strains are marked with an asterisk and isolates sequenced in this study are marked with degree sign. *Pseudofisicoccum stromaticum* (CBS 117448 and CMW 117449) was included as an outgroup. Scale bar indicates expected nuber of substitutions per site.

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