New and unexpected host associations for *Diplodia sapinea* in the Western Balkans

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Summary

Diplodia sapinea is an important pathogen of pine trees in plantations and urban areas in many parts of the world. This pathogen has recently also been isolated from diseased *Cedrus atlantica*, *C. deodara* and *Picea omorika* planted as ornamentals across the Western Balkans. The aim of this study was to consider the host range of *D. sapinea* in Serbia and Montenegro. *Diplodia sapinea* was identified from a broader collection of Botryosphaeriaceae from the Western Balkans region, based on the DNA sequence data for the internal transcribed spacer (ITS) rDNA and the translation elongation factor 1α (TEF 1- α). The *D. sapinea* isolates were obtained from sixteen tree species in the genera *Abies*, *Cedrus*, *Chamaecyparis*, *Juniperus*, *Picea*, *Pinus*, *Pseudotsuga* and *Fagus*. Four species represented new hosts in the Balkans and this is the first report of *D. sapinea* from *F. sylvatica* anywhere in the world. Pathogenicity tests were conducted on the tree hosts from which *D. sapinea* was isolated, as well as on *P. abies*, *Thuja occidentalis*, *Prunus laurocerasus*, *Eucalyptus grandis* and *P. patula*. Inoculations were made on seedlings in the field, in the greenhouse or on freshly detached branches. Inoculations on *P. pungens*, *P. omorika, P. abies, P. menziesii, A. concolor*, *P. nigra* and *P. sylvestris* resulted in death of the seedlings 5-16 weeks after inoculation. *Diplodia sapinea* produced lesions on *J. horizontalis* and *P. patula* seedlings and *F. sylvatica* cut branches. Reciprocal inoculations showed that *D. sapinea* is not a pine-specific pathogen, causing disease on tree species, including those from which it had not been isolated. Not surprisingly, the pathogen was most aggressive on some species of Pinaceae.

1 Introduction

Diplodia sapinea (Fr.) Fuckel 1870 (syn. *Diplodia pinea* (Desm.) Kickx., *Sphaeropsis sapinea* (Fr.: Fr.) Dyko & Sutton) is an important pathogen of pine trees (*Pinus* spp.) in various countries of the world (Punithalingam and Waterston 1970; Phillips et al. 2013). It is the causative agent of *Diplodia* blight (*Sphaeropsis* blight) and causes needle and shoot blight, branch and stem cankers, die-back, collar rot, blue stain of the sapwood and, where severe damage occurs, also tree death (Sinclair et al. 1987; Phillips et al. 2013). *Diplodia sapinea* readily forms pycnidia on

dead shoots and mature seed cones, which can play an important role in the survival of the fungus and serves as an inoculum source (Palmer et al. 1988). Conidia are wind and rain-dispersed during the summer growing season (Swart et al. 1987; Palmer et al. 1988). *Diplodia sapinea* is well known as an endophyte and latent pathogen residing in infected pine host tissue in the absence of symptoms (Swart and Wingfield 1991; Stanosz et al. 2007; Bihon et al. 2011). Disease outbreaks generally emerge after host trees have been subjected to environmental stress such as high temperatures, drought, hail damage or unfavourable site conditions (Swart et al. 1987; Stanosz et al. 2001; Zwolinski et al. 1990; Swart and Wingfield 1991). Damage mostly occurs in nurseries, plantations (particularly of non-native trees) and to ornamental plantings, on pine trees of all ages (Gibson 1979; Swart and Wingfield 1991).

Diplodia sapinea has been isolated from 33 pine species (Punithalingam and Waterston 1970; Gibson 1979; Phillips et al. 2013). Severe infections have been reported in exotic pine plantations in South Africa, Australia, New Zealand and USA (Chou 1976; Swart and Wingfield 1991). In Europe, *D. sapinea* was reported for the first time as a serious pathogen of *P. nigra* and *P. sylvestris* in the Netherlands in 1982 (Dam and de Kam 1984). The pathogen has been found to cause severe die-back of native *P. sylvestris* and *P. nigra* in countries of the Southern and South Eastern Europe (Capretti et al. 1987; Rossnev and Petkov 1994; Karadžić and Milijašević 2008), Central Europe (Piou et al. 1991; Cech and Carpetti 1995; Jankovský and Palovćiková 2003; Blaschke and Cech 2007) and has been reported recently from Northern Europe (Drenkhan and Hanso 2009; Adamson et al. 2015). In the Balkans, *D. sapinea* was first reported from *P. nigra* nursery seedlings in Croatia in 1957 (Böhm 1959) and later from *P. nigra* seedlings in Serbia (Karadžić 1987). Subsequently, *Diplodia* blight has been recorded on pine trees in a number of localities in Bulgaria, Croatia, Montenegro, Slovenia and Serbia (Rossnev and Petkov 1994; Diminić and Jurc 1999; Karadžić and Milijašević 2008). In Serbia and Montenegro, *D. sapinea* has become the most common cause of damage to plantation-grown pines and pine trees in urban environments (Karadžić and Vujanović 1992; Karadžić and Milijašević 2008).

Diplodia sapinea has only occasionally been found associated with other conifers. These include species in the genera *Abies*, *Picea*, *Pseudotsuga*, *Cedrus*, *Juniperus*, *Chamaecyparis*, *Araucaria*, *Thuja*, *Cupressus* and *Larix* (Punithalingam and Waterston 1970; Gibson 1979; Phillips et al. 2013). In Serbia, the fungus has occasionally been isolated from *Abies concolor*, *Cedrus atlantica*, *Chamaecyparis lawsoniana*, *Thuja occidentalis, Cupressus sempervirens, Juniperus virginiana* and *Sequoiadendron giganteum* (Karadžić and Stojadinović 1988; Milijašević 2003, 2009). Apart from diseased pine trees, various other ornamental trees and shrubs in the Western Balkans

region have recently been reported to suffer extensive die-back and mortality. Isolations from cankers and other symptoms by Zlatković et al. (2016) yielded isolates of ten Botryosphaeriaceae species, including *D. sapinea,* which was found associated with diseased trees of *C. atlantica*, *C. deodara* and *Picea omorika*. The species in Serbia and Montenegro has been characterized using phylogenetic inference based on multiple gene regions and morphology; a *Dichomera*-like syn-asexual morph, some unique morphological characteristics of the species were described and the pathogen was reported on *P. omorika* for the first time (Zlatković et al. 2016).

Diplodia sapinea has also been recorded having undergone host jumps to angiosperms, including *Malus domestica, Olea europaea*, *Eucalyptus* spp., *Blepharocalyx salicifolius* and *Prunus persica* (Bettucci et al. 1999, 2004; Damm et al. 2007; Lazzizera et al. 2008; Inderbitzin et al. 2010). However, *D. sapinea* is not considered an important pathogen of non-pine hosts (Swart and Wingfield 1991).

The aim of this study was to investigate the host range of *D. sapinea* in the Western Balkans. This was done by identifying isolates of *D. sapinea* from a broader collection of Botryosphaeriaceae isolates collected in the region, using the DNA sequence data for the internal transcribed spacer (ITS) rDNA and the translation elongation factor 1α (TEF 1- α). A subset of these isolates was then used to conduct reciprocal inoculations on the hosts from which *D. sapinea* had been isolated, as well as on *P. abies*, *T. occidentalis*, *Prunus laurocerasus*, *Eucalyptus grandis* and *P. patula* seedlings.

2 Materials and methods

2.1 Sampling and isolation

Samples were collected from diseased trees and shrubs between 2009 and 2014 across Serbia and Montenegro as described in Zlatković et al. (2016, Fig. S1). Two symptomatic *P. radiata* trees from a plantation in the Mount Athos region in northern Greece were sampled, and those *D. sapinea* isolates were also included in the study. A list of all isolates obtained can be found in Table S1. *Diplodia sapinea* was isolated from symptomatic tissues (Fig. S1) and directly from pycnidia on 2 % malt extract agar (MEA) plates acidified with lactic acid (AMEA) as described in Zlatković et al. (2016). Cultures were purified using hyphal tip transfers and isolates with the *D. sapinea*-like culture morphology (Zlatković et al. 2016) were transferred to new Petri dishes. Representative isolates from each host were

deposited in the Culture Collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa.

2.2 Phylogenetic analyses

DNA was extracted from one-week-old cultures using PrepMan Ultra reagent (Applied Biosystems, Foster City, California) or using a CTAB-based protocol of Möller et al. (1992). The ITS region of rDNA operon was amplified using primers ITS-1 or ITS1F and ITS-4 (White et al. 1990; Gardes and Bruns 1993) and part of the TEF-1-α gene using primers EF1-728F and EF1-986R (Carbone and Kohn 1999) or EF1-F and EF2-R (Jacobs et al. 2004). PCR amplification, sequencing and sequence alignments followed the methods described by Zlatković et al. (2016).

Phylogenetic analyses were performed using Maximum parsimony (MP), Maximum likelihood (ML) and Bayesian analyses (BI). MP analyses were conducted using PAUP version 4.0b10 (Swofford 2003) and ML analyses were performed using an online version of PhyML 3.0 (Guindon et al. 2010). The reliability of each node was assessed using 1000 bootstrap replications (Felsenstein 1985). BI analyses were made using MrBayes v. 3.0b4 (Ronquist and Huelsenback 2003). For ML and BI analyses, the best nucleotide substitution model was determined with Jmodeltest v.0.1 (Posada 2008). Markov Chain Monte Carlo (MCMC) analyses of 4 chains started from random tree topology and lasted three million generations. Trees were sampled every 100th generation resulting in 30,000 trees. The first 3000 trees were discarded as the “burn-in” phase and posterior probabilities (PP) were determined from the remaining trees. Phylogenetic trees were visualized in MEGA v.6. Sequences obtained in this study were deposited in GenBank (Table S1).

2.3 Seedling inoculations in the field

Diplodia sapinea isolates used for seedling inoculations were obtained from symptomatic *C. atlantica* (CMW 39329, CMW 39337, CMW 39338), *P. nigra* (CMW 39332) and *P. sylvestris* (CMW 39343) trees planted in Serbia and the Mediterranean part of Montenegro (CMW 39337). Among these, isolate CMW 39338 from *C. atlantica* was chosen because it had an unusual colony morphology compared to other isolates characterized in the study by Zlatković et al. (2016).

The inoculation tests were carried out during the growing seasons of 2014 and 2015 (April to October and April to June, respectively) in a nursery of the Faculty of Forestry in Belgrade, Serbia. Inoculations were made on some of the natural hosts of *D. sapinea*, i.e. *C. atlantica*, *C. deodara*, *P. pungens*, *P. omorika*, *A. concolor*, *Pseudotsuga menziesii*, *P. nigra*, *P. sylvestris*, *J. horizontalis* and *C. lawsoniana*. In addition, some of the common ornamental species i.e. *P. abies*, *T. occidentalis* and *P. laurocerasus* were also inoculated. Combinations of tree species and isolates used in the pathogenicity trial in the field are shown in Table 1, while details of the inoculated species are presented in Table S2. All seedlings were two or three-year-old potted plants purchased from the commercial ornamental nursery located in Novi Sad, Serbia or obtained from the nursery of the Faculty of Forestry, University of Belgrade. Seedlings were arranged in a completely randomized design.

Table 1. Tree species and *Diplodia sapinea* isolates used for seedling inoculations in the field.

Inoculated species	Isolates				
	CMW 39329	CMW 39337	CMW 39338	CMW 39332	CMW 39343
Cedrus atlantica	+	+	−	−	−
C. deodara	+	+	−	−	−
Picea pungens	+	+	−	−	−
P. omorika	+	+	−	+	+
P. abies	+	+	−	−	−
Abies concolor	+	+	−	−	−
Pseudotsuga menziesii	+	+	−	−	−
Pinus nigra	+	+	+	−	+
P. sylvestris	+	+	+	−	+
Juniperus horizontalis	+	+	−	−	−
Chamaecyparis lawsoniana	+	+	−	−	−
Thuja occidentalis	+	+	−	−	−
Prunus laurocerasus	+	+	−	−	−

'+' indicates that the isolate was used for inoculation. '−' indicates that the isolate was not used for inoculation.

Ten seedlings per species per isolate were inoculated on the stems, 3-9 cm above the soil level. The same number of seedlings of each species served as mock-inoculated controls. The *D. sapinea* inoculum consisted of 3-mm or 6-mm-diameter plugs taken from the margins of 7-day-old cultures growing on MEA that had been maintained in the dark at a room temperature (20-23°C). A colonized agar plug was placed mycelium-side down into a wound made on the surface disinfested stem with a flame-sterilized cork borer (3 or 6 mm diameter, Table S2) to expose the cambium. The inoculated wounds were covered with the bark that had been removed to make the wounds and a piece of cotton wool soaked in distilled water and wrapped with Parafilm to limit desiccation and contamination. Control seedlings were treated in the same manner as the fungus-treated plants, but were inoculated with plugs of sterile-water agar (WA).

The inoculated seedlings were maintained in an open-air nursery under natural urban forest conditions and irrigated daily to attain field water capacity. Seedlings were inspected for disease symptoms and mortality (flagging of the terminal shoot, needle drying or needle fall) at weekly intervals. Disease assessment for seedlings of *A. concolor*, *P. pungens*, *P. abies*, *P. omorika* and *P. menziesii* was based on the percentage of dead seedlings recoded over time. The area under disease progress curve (AUDPC) was used to quantify disease intensity over time (Madden et al. 2007). The AUDPC was calculated by the trapezoidal integration method using the equation:

$$\mathrm{AUDPC} = \sum_{i=1}^{n-1} [(y_i + y_{i+1})/2]*(t_{i+1} - t_i)$$

Where 'n' is the total number of observations, 'y_i' the severity of disease at the 'i^{th}' observation and 't_i' time (week) at the 'i^{th}' observation.

For *P. sylvestris* and *P. nigra* seedlings, disease severity was determined as the percentages of dead seedlings six weeks after inoculation. Since no mortality was observed in *J. horizontalis*, *T. occidentalis*, *C. atlantica*, *C. deodara*, *C. lawsoniana* and *P. laurocerasus* seedlings six weeks (*C. deodara*) or six months (all other species) after inoculation, seedlings were sectioned longitudinally and the extent of vascular discoloration/resin accumulation was measured above and below the points of inoculation.

When the experiment was terminated, pycnidia were collected from the surface of the dead tissue and examined as described by Zlatković et al. (2016). Tissue segments (3 x 3mm) from the margin between necrotic and apparently healthy tissue were surface disinfested (1 min in 70 % ethanol), rinsed in sterile distilled water, dried on sterile paper towels, flamed briefly and placed onto 2 % malt extract agar (MEA) plates. Plates were incubated on a laboratory bench at room temperature (20 ± 3°C) for 2 weeks. *Diplodia sapinea* identity was verified based on the morphology of cultures and conidia in order to confirm Koch's postulates. All inoculation experiments were repeated once.

2.4 Cut branch inoculations

Branch pieces, 30-40 cm in length and 1.3-1.5 cm thick were cut from healthy, mature *Fagus sylvatica* trees growing in experimental forest of the Faculty of Forestry located in Belgrade, Serbia. Branches were collected in May 2015, at the start of the growing season and inoculated with five isolates of *D. sapinea* from *C. atlantica* (CMW 39329 and CMW 39337), *P. nigra* (CMW 39332), *P. sylvestris* (CMW 39343) and *F. sylvatica* (CMW 39336). Ten branches per isolate were inoculated by placing 6 mm diameter mycelial plugs into wounds made with

a cork borer of equal size, at the centres of the branches. Branches with one end cut diagonally to increase water uptake, were maintained in jars of distilled water for 3 weeks at room temperature (20 ± 3°C) on a laboratory bench. The water was replaced every second day and branch ends were re-cut at the same time. In order to maintain a moist environment, branches were covered with a plastic bag during the first week and sprayed with water daily until the end of the experiment. Ten control branches for each tree species were inoculated with sterile WA plugs as controls. After three weeks, the branches were sectioned longitudinally and the extent of vascular discoloration was measured. Re-isolations were made from the discoloured tissue and the resulting isolates were identified as previously described. The entire experiment was repeated once.

2.5 Inoculations in the greenhouse

Three-year-old potted *E. grandis* seedlings and 5-year-old potted *P. patula* seedlings (Table S2) were inoculated with two isolates of *D. sapinea* obtained from *C. atlantica* (CMW 39329, CMW 39337) trees from Serbia and the Mediterranean part of Montenegro. For each isolate, ten replicate seedlings were used for inoculations and ten seedlings of each species served as controls. To inoculate seedlings, wounds were made in the lower parts (15-20 cm above soil level) of the stems using a 9 mm diameter cork borer and 9 mm diameter mycelial plugs were placed in the wounds as described previously. The same numbers of seedlings served as mock-inoculated controls. Seedlings were maintained at 25°C under natural day/night cycles for six weeks and watered daily. At the end of the experiment, lesion lengths were measured and *D. sapinea* was re-isolated and identified as described previously.

2.6 Statistical analyses

Data from the pathogenicity tests were analysed using Statistica 8.0 (StatSoft Inc., Tulsa, OK, USA) and IBM SPSS Statistics 20.0 (New York, U.S.A). The normality of the data was checked using the Kolmogorov-Smirnov test and homogeneity of variances using Levene's test. The results of the two subsequent pathogenicity trials were compared using Student's t-test at $\alpha = 0.05$ (data not shown). No significant differences were found between the two repeats of the trials, so the data for the different tree species were pooled in a single dataset for further analyses. AUDPC values for *A. concolor*, *P. pungens*, *P. abies*, *P. omorika* and *P. menziesii* were analysed using z-test (Pocock 2006) at the 5% significance level ($z>1.96$, $p<0.05$). Differences in the lengths of resinous lesions on *T. occidentalis, C. lawsoniana, C. atlantica*, *C. deodara*, vascular discoloration of *P. laurocerasus*, *P. patula*, *E. grandis* and percent of

dead *P. sylvestris* and *P. nigra* seedlings were determined using one way analyses of variance (ANOVA). The lg10 transformation was used to improve homogeneity of variances in order to analyse the effect of two *D. sapinea* isolates on vascular discoloration of *P. patula* and *E. grandis* seedlings. Treatment means were compared using post-hoc least significant difference (LSD) test at α= 0.05. The extent of vascular discoloration associated with *F. sylvatica* twigs, *J. horizontalis* seedlings and *E. grandis* seedlings (isolate CMW 39329) was assessed using non-parametric Kruskal-Wallis H test (α= 0.05).

3 Results

3.1 *Diplodia sapinea* isolation

A total of 83 isolates were collected in this study. These included 42 isolates from *C. atlantica*, ten isolates from *P. nigra*, nine isolates from *P. sylvestris*, four isolates from *P. pungens*, three isolates from *P. menziesii*, two isolates each from *P. halepensis*, *P. radiata*, *P. omorika*, *J. horizontalis*, *A. concolor*, *C. lawsoniana* trees and one isolate each from *P. pinea*, *P. pinaster, F. sylvatica* and *C. libani* (Table S1). Two isolates of *D. sapinea* from *P. omorika* and one isolate each from *C. atlantica* and *C. deodara* were from a previous study (Zlatković et al. 2016). These isolates were identified based on sequence comparisons of the ITS, TEF-1-*α,* β-tub and LSU gene regions as well as morphological characteristics of conidia and cultures and included in the study.

3.2 Phylogenetic analyses

Preliminary identification of *D. sapinea*-like isolates was conducted based on the sequence comparisons of the ITS or combined ITS and TEF-1 *α* gene regions and MP analyses (data not shown). Representative isolates from each host (including those from the previous study) were further analysed using MP, ML and BI analyses. The combined data set of four loci (ITS, TEF-1-*α,* β-tub and LSU) contained 26 sequences and included 13 sequences obtained in this study, 3 sequences obtained in a previous study (Zlatković et al. 2016) and 10 sequences retrieved from GenBank, with *Sphaeropsis visci* as an outgroup. The sequence dataset contained 1816 characters of which 163 were parsimony informative, 1653 were parsimony uninformative, with CI=0.8, RI=0.9 and TL=188. The PHT test gave a P-value of P=0.8. The model TrN+G was chosen for the ML and BI analyses (G=0.1390). The topologies of the trees emerging from MP, ML and BI analyses were similar with regards to the clade representing *D. sapinea* and

therefore, only the MP tree (Fig.1) is presented. Based on these phylogenetic analyses, all isolates considered in this study were identified as *D. sapinea* (Table S1, Fig.1).

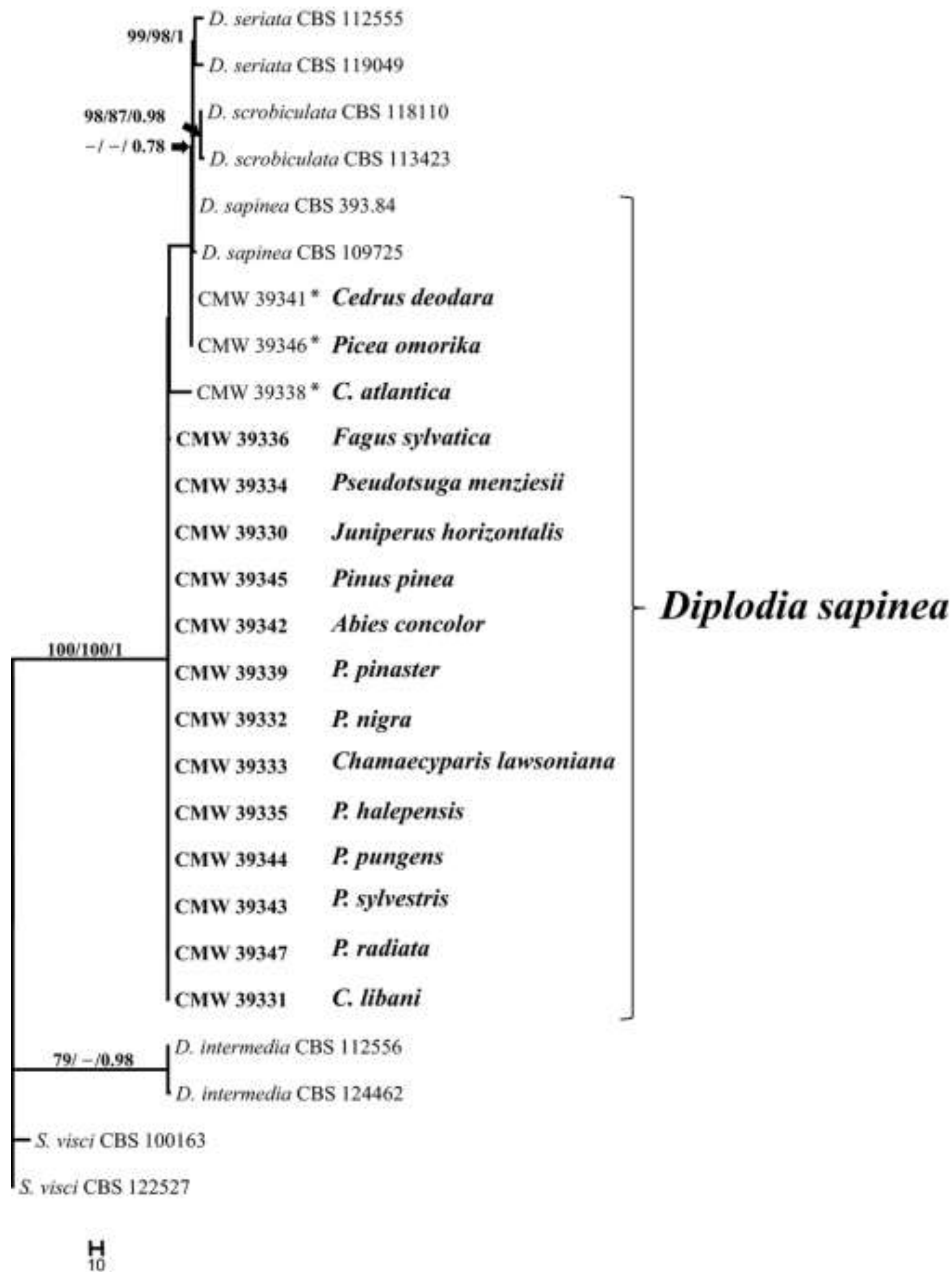

Figure 1. The first of 1000 equally most parsimonious trees resulting from parsimony analyses of the combined ITS, TEF-1-α, β-tub and LSU alignment. The bootstrap support values (MP/ML ≥ 70%) and posterior probabilities are indicated at the nodes, and the scale bar represents the number of changes. The tree was rooted to *S. visci* CBS 100163 and CBS 122527. Hosts from this study are given in bold. ___ Hosts from the previous study (Zlatković et al., 2016). Sequences which are not given in bold or marked with an asterisk were obtained from GenBank

3.3 Seedling inoculations in the field

Isolates from *C. atlantica* were shown to infect seedlings of *P. abies*, *P. pungens*, *P. omorika*, *A. concolor* and *P. menziesii* and they eventually killed the plants (Fig. S2). Initial symptoms included wilting of shoots and yellowing and browning of the needles. Initial symptoms were first observed at the following intervals after inoculation: two weeks for *A. concolor* and *P. menziesii*; three weeks for *P. abies*; four weeks for *P. pungens*, four weeks (3-years old seedlings) and one week (2-years old seedlings) for *P. omorika* seedlings.

The first mortality was seen at the following intervals after inoculation: four weeks for *P. omorika* (2-years old), *P. abies* and *P. menziesii*; five weeks for *A. concolor* and *P. pungens* and six weeks for *P. omorika* (3-years old) seedlings (Fig. 2). There was no mortality in the controls and no visible symptoms were observed on these seedlings. The AUDPC value (740) was significantly higher ($z = 17.2$, $p < 0.0001$) for seedlings of *P. pungens* inoculated with *D. sapinea* isolate from *C. atlantica* CMW 39337 compared to inoculations with isolate CMW 39329 (AUDPC = 210). There were no significant differences in the AUDPC values between *D. sapinea* isolates inoculated into other species ($p < 0.05$). 100 % seedling mortality occurred between the 5th and 16th week of the experiment, depending on the *D. sapinea* isolate and tree species used (Fig. 2).

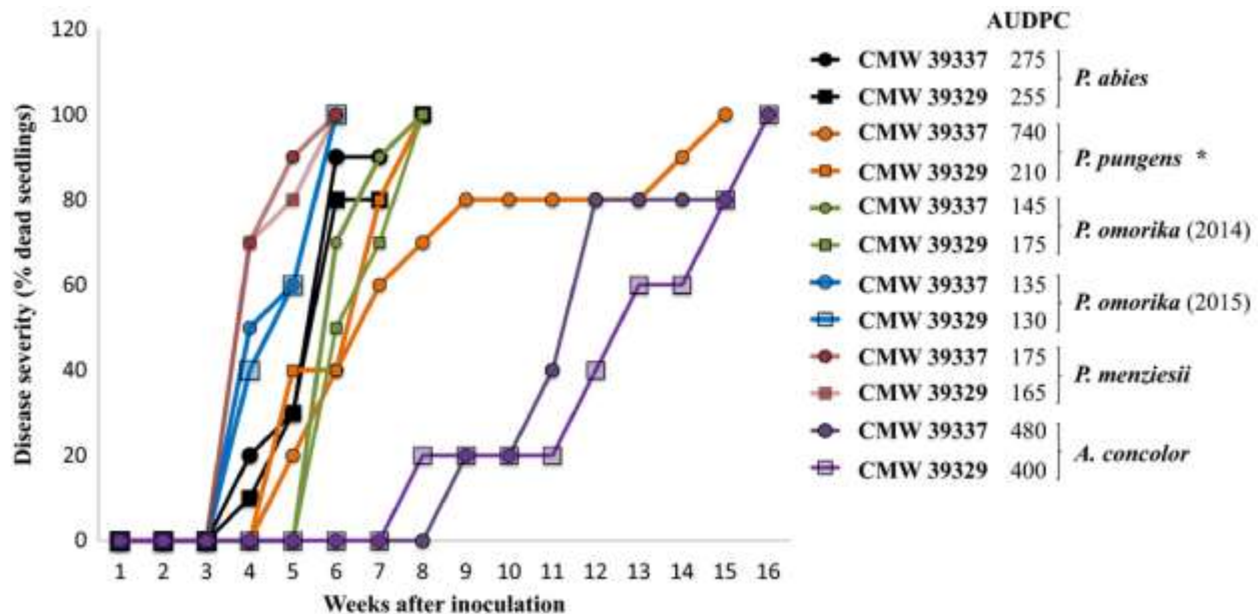

Figure 2. Disease progress curves and area under the disease progress curve (AUDPC) of seedlings of 3-year-old *Picea abies*, 3-year-old *Picea pungens*, 3-year-old *Picea omorika* (inoculated in 2014), 2-year-old *P. omorika* (inoculated in 2015), 2-year-old *Pseudotsuga menziesii* and 2-year-old *Abies concolor* inoculated with *D. sapinea* isolates CMW 39337 and CMW 39329. AUDPC values were significantly different (*z*-test, $z = 17.2$, $p < .00001$)

Table 2. Mean lesion lengths of a variety of tree species inoculated with *Diplodia sapinea* isolates.

Seedling species	Lesion length (cm) [1]						Control (cm)	Inocultions
	CMW 39329	**CMW 39337**	**CMW 39338**	**CMW 39332**	**CMW 39343**	**CMW 39336**		
Juniperus horizontalis	1.6 (1-2.3)	1.2 (1.1-1.3)	–	–	–	–	–	field
Thuja occidentalis	1.2 (1.1-1.4)	1.2 (1.1-1.3)	–	–	–	–	1.1 (0.9-1.2)	field
Cedrus atlantica	0.6 (0.5-0.7)	0.7 (0.5-0.6)	–	–	–	–	0.6 (0.5-0.6)	field
C. deodara	0.6 (0.5-0.7)	0.6 (0.5-0.7)	–	–	–	–	0.6 (0.5-0.6)	field
Chamaecyparis lawsoniana	1 (0.9-1.3)	1 (0.9-1.1)	–	–	–	–	1 (0.9-1.1)	field
Prunus laurocerasus	1.2 (0.8-1.8)	1.1 (0.8-1.2)	–	–	–	–	1 (0.8-1.2)	field
Fagus sylvatica	1.1 (0.9-1.3)	1.08 (0.9-1.3)	1.09 (0.9-1.3)	1.11 (1-1.3)	1.1 (1-1.3)	1.1 (0.9-1.1)		branch
Pinus patula	78.8 (26-160)	44.6 (23-72)	–	–	–	–	6 (6-7)	greenhouse
Eucalyptus grandis	12.5 (10-22)	11.8 (9-15)	–	–	–	–	6 (6-7)	greenhouse

[1] Mean length of the discoloured or resinous area at the site of inoculation. Range is given in brackets. '–' indicates that the isolate was not used for inoculations. '/' indicates that lesions were not observed in the controls.

Isolates from *C. atlantica* were able to infect seedlings of *J. horizontalis* and lesion lengths ranged from 1 to 2.3 cm (Table 2). There were no significant differences in lesion lengths produced by the two isolates from *C. atlantica* (Table 3). Lesions were not observed on the control plants.

Table 3. Analyses of variance (ANOVA) and Kruskal-Wallis H test results for the effect of *D. sapinea* isolates on the percent of dead *P. sylvestris* and *P. nigra* seedlings, and on lengths of resionus lesions/vascular discoloration associated with *Juniperus horizontalis*, *Thuja occidentalis*, *Cedrus atlantica*, *C. deodara*, *Chamaecyparis lawsoniana*, *Prunus laurocerasus, Pinus patula*, *Eucalyptus grandis* seedlings and *Fagus sylvatica* shoots.

Seedling species	CMW 39329 × C			CMW 39337 × C			CMW 39329 × CMW 39337			CMW 39337 × CMW 39329 × CMW 39338 × CMW 39343			CMW 39329 × CMW 39337 × CMW 39338 × CMW 39332 × CMW 39343 × CMW 39336		
	F	df	P	F	df	P	F	df	P	F	df	P			
P. sylvestris										6.98	1	0.12			
P. nigra										140.67	2	**0.05**			
T. occidentalis	3.1	1	0.2	1.8	1	0.2									
C. atlantica	1.33	1	0.26	1.33	1	0.26									
C. deodara	2.77	1	0.11	1	1	0.33									
C. lawsoniana	1.17	1	0.29	0.11	1	0.75									
P. laurocerasus	1.9	1	0.18	0.36	1	0.55									
P. patula	27.14	1	**0.00006**	49.41	1	**0.000001**	4.43	1	**0.05**						
E. grandis				75.48	1	**0**	0.29	1	0.60						
	H	df	P				H	df	P				H	df	P
J. horizontalis							2.87	1	0.09						
F. sylvatica													0.85	5	0.97
E. grandis	15.3	1	**0.0001**												

'C' indicates control seedlings; 'F' indicates one-way ANOVA F-test statistic; 'H' indicates Kruskal-Wallis Test statistic; 'df' indicates degrees of freedom; 'P' indicates probability value. The P values are bold where they are statistically significant at α= 0.05.

Isolates from *C. atlantica*, *P. nigra* and *P. sylvestris* were able to infect seedlings of *P. nigra* and *P. sylvestris*. Initial disease symptoms, including wilting of shoots were observed three weeks post inoculation on *P. nigra* and four weeks post inoculation on *P. sylvestris*. The first mortality for both tree species occurred five weeks after inoculation. Statistical analyses showed that there were no significant differences in the disease severity among

P. sylvestris seedlings (Table 3). In contrast, significant differences were observed among seedlings of *P. nigra* (Table 3). Isolate CMW 39343 from *P. sylvestris* was the most aggressive and isolate CMW 39337 from *C. atlantica* was the least aggressive (Fig. 3). Inoculated plants exhibited resin bleeding, death of the needles from the base of the needles towards their tips, needle chlorosis and desiccation and resin-soaked girdling lesions on the stems. *Diplodia sapinea* was re-isolated and identified from inoculated seedlings.

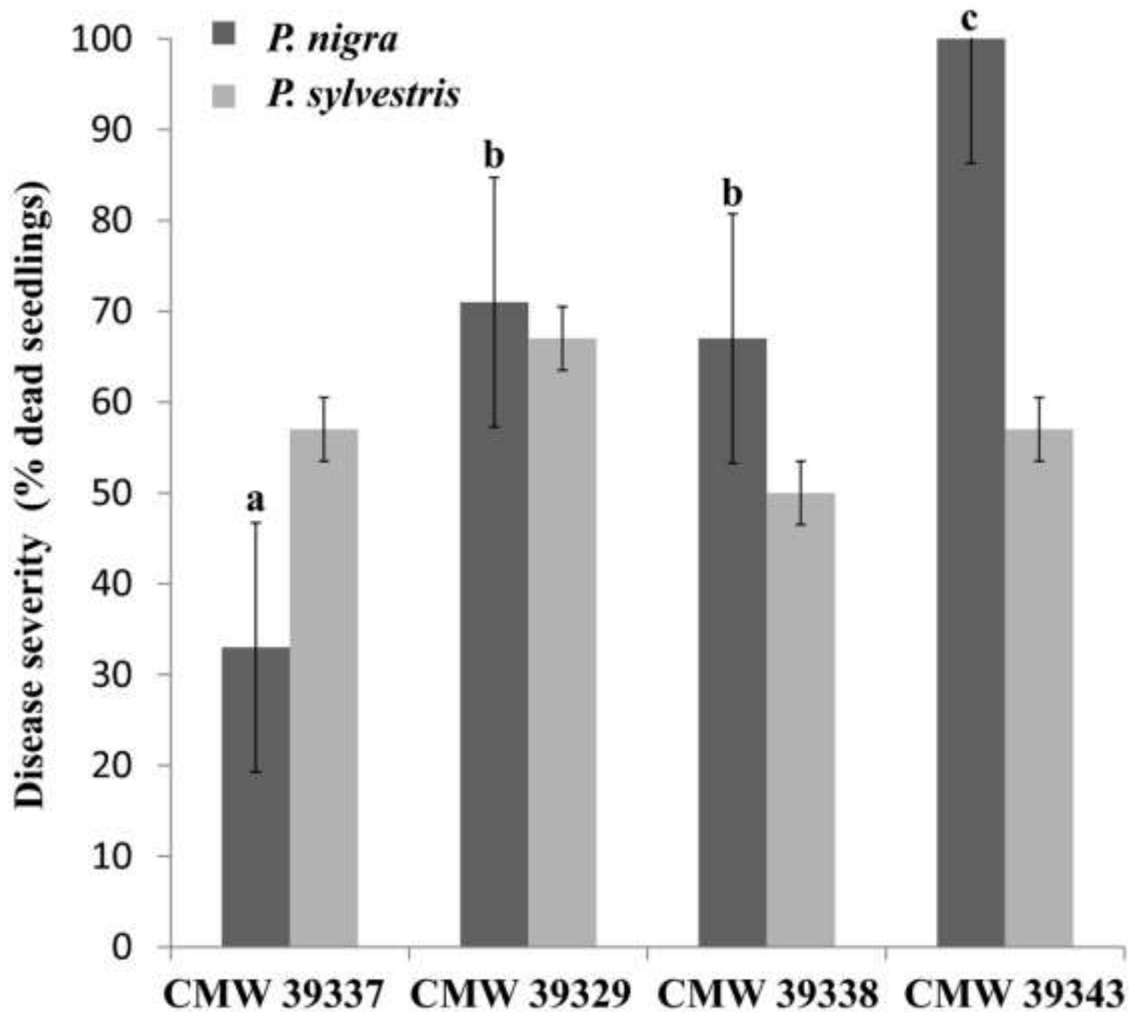

Figure 3. Disease severity (percentage of dead seedlings) of *P. sylvestris* and *P. nigra* seedlings inoculated with four *D. sapinea* isolates 6 weeks after inoculation. For each vertical bar, error bars are ±*SEM*. Isolates of *P. sylvestris* did not differ significantly. Isolates of *P. nigra* with the same letter above the bar did not differ significantly, as determined by the LSD test at the $\alpha = 0.05$ level

Isolates from *C. atlantica* did not cause lesions or disease on seedlings of *T. occidentalis*, *C. lawsoniana*, *C. atlantica*, *C. deodara* and *P. laurocerasus*. Inoculations on *P. laurocerasus* seedlings produced dark brown to black wood discoloration, while other species exhibited resinous lesions (Table 2). Lesion lengths were not significantly different to that observed in the controls (Table 3) and *D. sapinea* was not re-isolated from these tissues.

3.4 Cut branch inoculations

Isolates from *C. atlantica*, *P. nigra*, *P. sylvestris* and *F. sylvatica* produced dark brown lesions ranging from 0.9-1.3 cm on cut branches of *F. sylvatica* (Table 2). There were no significant differences in lesion lengths produced by *D. sapinea* isolates (Table 3). Branches inoculated as controls produced no lesions. *Diplodia sapinea* was re-isolated from lesions on all inoculated branches, but not from the controls.

3.5 Inoculations in the greenhouse

Isolates from *C. atlantica* produced lesions on both *P. patula* and *E. grandis* seedlings (Table 2). There were significant differences in lesion lengths resulting from inoculation with the two *D. sapinea* isolates on *P. patula* seedlings (Table 3). Lesions produced by isolate CMW 39337 were significantly longer than those produced by isolate CMW 39329, and both were significantly longer than those of the controls (Table 3). Lesions on *E. grandis* were small, but significantly different compared to those of the controls (Tables 2, 3). *Diplodia sapinea* was successfully re-isolated from all inoculated seedlings, maintained either in the field or in the greenhouse, thus fulfilling the conditions of Koch’s postulates. The pathogen was never isolated from the controls.

4 Discussion

In this study, the known host range of *D. sapinea* in the Western Balkans has been substantially expanded, including the discovery of unexpected host trees. The fungus was associated with 16 tree species, including 15 conifers and one angiosperm (*F. sylvatica*). The majority of the *D. sapinea* isolates were from *C. atlantica,* followed by *P. nigra* and *P. sylvestris,* whereas it was isolated from other tree species only occasionally. *Diplodia sapinea* was shown to be able to cause lesions on seven tree species and also *P. abies* and *P. patula* from which the pathogen was not isolated in this study.

It was not surprising to commonly isolate *D. sapinea* from pine trees in this study, as the fungus has previously been found on ten pine species in the Western Balkans region by Milijašević (2009). The latter study showed that Balkan isolates of *D. sapinea* could infect unwounded shoots of *P. nigra*. In the present study, *D. sapinea* showed high levels of aggressiveness on *P. nigra* and *P. sylvestris.* This is consistent with observations of high levels of damage caused by *D. sapinea* on *P. nigra* and *P. sylvestris* in European countries where these pines are native (Dam and de Kam 1984; Piou et al. 1991; Diminić and Jurc 1999; Jankovský and Palovćiková 2003; Blaschke and Cech 2007; Karadžić and Milijašević 2008; Fabre et al. 2011). However, it is in contrast to a study by Iturritxa et al. (2013) where *P. nigra* and *P. sylvestris* seedlings were moderately susceptible to Spanish isolates of *D. sapinea* compared to highly susceptible *P. radiata* when inoculated in a greenhouse.

Diplodia sapinea is confirmed in this study as a pathogen of *Picea* and *Abies* species through sequence data and inoculation experiments. Prior reports of *D. sapinea* in Serbia were based on morphology (Karadžić and Stojadinović 1988; Milijašević 2009), which is not conclusive for this species given how easily it can be confused with the cryptic sister species *D. scrobiculata* (Phillips et al. 2013). The pathogenicity trials showed that Balkan isolates of *D. sapinea* were able to cause disease on the tested species of *Picea* and *Abies* with symptoms similar to those seen on mature trees under natural conditions. The aggressiveness of *D. sapinea* on *P. omorika* seedlings is especially disturbing because this is a species that is in danger of extinction (Alberto et al. 2013). *Diplodia sapinea* has also been identified from *Picea* and *Abies* species (*P. pungens*, *P. abies, P. menziesii* and *A. concolor*) in the USA (Hepting 1971; Luley and Gleason 1988), although identification was based on morphology and prior to the description of *D. scrobiculata*. The distribution of the association between *D. sapinea* and hosts such as *Picea* and *Abies* deserves wider study, especially in the light of *D. sapinea* potential pathogenicity on these hosts, as illustrated in this study.

Diplodia sapinea was only occasionally isolated from *C. libani*, *C. lawsoniana* and *J. horizontalis* in this study and from *C. deodara* in a previous study by Zlatković et al. (2016). The pathogen has been reported to cause symptoms such as cankers, branch die-back and collar rot on *C. deodara* in USA and France (Hepting 1971; Morelet and Chandelier 1993). The results of the present study, however, showed that *D. sapinea* was not pathogenic to *C. deodara* or *C. lawsoniana*. In contrast, inoculation trials showed that the fungus can cause disease on *J. horizontalis*, suggesting that it is most likely contributing to the observed die-back of *J. horizontalis* shrubs. Turkish isolates of *D. sapinea* have also been shown to be highly aggressive on *C. libani* seedlings (Doğmuş-Lehtijärvi et al. 2009).

Diplodia sapinea is predominantly known as an endophyte and pathogen of conifers, especially *Pinus* spp., but it has also been isolated from several angiosperms (Bettucci et al. 1999, 2004; Damm et al. 2007; Lazzizera et al. 2008; Inderbitzin et al. 2010). In this study, *D. sapinea* was found associated with the angiosperm *F. sylvatica* for the first time Isolations yielded only a single isolate from *F. sylvatica* and inoculation tests showed that it is likely not a pathogen of this tree. Lazzizera et al. (2008) isolated *D. sapinea* from olive trees in orchards surrounded by pine trees and authors suggested that isolation of this fungus from olives was most likely due to the high inoculum pressure. We believe a similar situation is true for *D. sapinea* on *F. sylvatica* in this study.

Given the evidence that *D. sapinea* could infect both conifers and angiosperms, we conducted a separate experiment on economically important *P. patula* and *E. grandis*, in a setting where both species are grown in a close proximity. Inoculations of *D. sapinea* on *P. patula* resulted in lesions significantly larger than those on the control plants. *Pinus patula* is known to be susceptible to *D. sapinea* infections under controled conditions (Swart et al. 1988). Lesions produced on *E. grandis* seedlings were not significantly larger compared to those produced on the control plants. It is thus not likely that *D. sapinea* represents a potential pathogen of this tree.

Reciprocal inoculations presented in this study indicated that *D. sapinea* is not host specific in the Pinaceae (including *Pinus*, *Abies*, *Pseudotsuga* and *Picea*), but pathogenic only on one member of the Cupressaceae (*J. horizontalis*). Selective host preference in *D. sapinea* has also been suggested by de Wet et. al. (2008) and Fabre et al. (2011), and has recently been noted for some other Botryosphaeriaceae (Jami et al. 2014). This is consistent with the fact that Botryosphaeriaceae host specifity is influenced by the host itself and by specific environment in which a given host occurs (Swart and Wingfield 1991; Slippers and Wingfield 2007). Moreover, this study confirmed the movement of Botryosphaeriaceae between native hosts to introduced tree species and *vice versa* (Pavlic et al. 2007; Slippers and Wingfield 2007). For example, *D. sapinea* was shared between native *P. sylvestris*, *P. nigra*, *P. omorika*, *P. abies* and introduced *P. pungens*, *A. concolor*, *P. menziesii* and *J. horizontalis*.

The results of this study showed that *D. sapinea* can be a significant pathogen of *P. nigra*, *P. sylvestris*, *P. patula*, *P. abies*, *P. pungens*, *P. omorika*, *A. conclor*, *P. menziesii* and can cause a disease in *J. horizontalis*. In contrast, the fungus was not particularly pathogenic to *E. grandis* and resides as an endophyte in *T. occidentalis, C. lawsoniana, C. atlantica* and *C. deodara.* However, reciprocal inoculations from this study were carried out under field conditions. Although plants were watered daily to field capacity, the level of plant stress was not measured and

can not be completely excluded. *Diplodia sapinea* is an opportunistic pathogen, able to infect physiologically stressed tissues (Swart and Wingfield 1991). Thus, the aggressiveness of this pathogen might have been higher than it would have been under controlled conditions. In addition, seedlings were wounded and a large amount of inoculum was placed directly in contact with the living tissues. Although this method guarantees pathogen entry, plant external resistance mechanisms are circumvented (Capretti and Dorworth 1989).

Sudden and severe die-back and mortality of conifers sampled in this study might be associated with inapropriate forest management practices. These trees could be ecophysiologically maladapted to their current habitat and consequently susceptible to *D. sapinea* infection. Conifers from this study were planted on low elevation sites that would, naturally have been covered by broadleaves, mainly oak and beech. Site-related influences on *D. sapinea*-associated disease have been reported by Munck et al. (2009). These authors found more conidia on cones in plantations in northern Wisconcin (USA) planted on sites historically dominated by *P. banksiana* compared to areas of less sandy soils and presettlement red pine dominance.

Other factors that may have predisposed conifers sampled in this study to *D. sapinea* infection include climate extremes (e.g. high temperatures, drought) and additional stresses that trees growing in urban environments are likely to encounter (Zlatković et al. 2016), leading to lowered defence potential against pathogens (Allen et al. 2010). Moreover, *Pinus* spp. sampled in this study were frequently planted in a close proximity to other ornamental conifers. This could have lead to an increase in pathogen pressure (Ennos 2015) which, along with the above mentioned stress factors, allowed *D. sapinea* to cross-infect near-by ornamentals. High propagule pressure and geographical proximity have also been hypothesized to be the cause of the outbreaks of *D. sapinea* in a *P. menziesii* plantation in Turkey (Kaya et al. 2014) and in nurseries of pine species in the USA and South Africa (Palmer and Nicholls 1985; Stanosz et al. 2007; Bihon et al. 2011).

Acknowledgements

We thank members of the Tree Protection Co-operative Programme (TPCP), the University of Pretoria, South Africa and the Ministry of Education and Science of the Republic of Serbia (TR37008) for the financial support that made this study possible. The first author also wishes to acknowledge partial financial support from European Cooperation in Science and Technology (COST) Actions Pathway Evaluation in Pest Risk Management In Transport (PERMIT

FP1002), ALIEN Challenge (TD1209) and A global network of nurseries as early warning system against alien tree pests (Global Warning FP1401). Nursery of the Faculty of Forestry (University of Belgrade) is acknowledged for providing some of the seedlings used in the pathogenicity trials. Dr. Jelena Lazarević is thanked for help with sampling in Montenegro and prof. Dr. Dragan Karadžić and Dr. Ivan Milenković for providing some of the samples used in this study.

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