



Virulence of *Dothistroma septosporum* and *D. pini* on *Pinus nigra* and *P. mugo* under conditions of natural infection

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Abstract *Dothistroma* needle blight (DNB) is a severe needle disease of pines worldwide, caused by two closely related species, *Dothistroma septosporum* and *D. pini*. The two fungal species are similar not only in their morphological characteristics, but also cause very similar symptoms in their hosts, and have a similar ecology. The aim of this study was to compare the virulence of the two *Dothistroma* species in natural infection experiments on 2-year-old seedlings of two DNB susceptible pine species, *Pinus nigra* and *P. mugo*, in two seedling stands for each pathogen species. The virulence of the pathogens and presence of symptoms (symptomatic needles, red bands and acervuli) were assessed after 2 years of exposure to inoculum. The incidence of seedlings with DNB symptoms was 65% and 76% for *P. nigra* and *P. mugo*, respectively. No difference was found between *D. septosporum* and *D. pini* in any of the three DNB symptoms evaluated on seedlings of *P. mugo*. However, symptoms of disease differed between the two *Dothistroma* species on *P. nigra*. Variables that reflect the intensity of disease development, the number of red bands and acervuli per needle, showed a difference in virulence between *D. septosporum* and *D. pini*, but only in the case of the host species *P. nigra*.

The results suggest that the virulence of the two *Dothistroma* species could be affected by host pine species and that there are differences in the susceptibility of individual pine species to *D. septosporum* and *D. pini*. Further factors could affect the virulence of these pathogens, including isolate origin, climatic or environmental factors.

Keywords Needle blight · *Pinus nigra* · *Pinus mugo* · Symptomatic needles · Red bands · Acervuli

Introduction

Dothistroma needle blight (DNB) is a serious needle disease of conifers that primarily affects pine species. Two ascomycete fungi are known to cause DNB: *Dothistroma septosporum* (Dorog.) Morelet and *D. pini* Hulbary (Barnes et al., 2004). The morphology, taxonomy, genetics and phylogenetics, host and geographical range of the two *Dothistroma* species have been intensively studied. The two species of *Dothistroma* are similar in their ecology and cause indistinguishable symptoms (Barnes et al., 2008). Additionally, the microscopic morphological characteristics of the two DNB fungi are very similar: the only credible difference is the width of the conidia (Barnes et al., 2004, 2008). Furthermore, differences in culture morphology and growth rate were investigated between the two *Dothistroma* species. Substantial variability has been observed in their culture morphology

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(Barnes et al., 2004). Based on statistical analysis, highly significant interactions and differences were noted between the two species in the pigmentation of fungal cultures (Adamčíková et al., 2021a).

Dothistroma pini has a more limited geographic distribution and host range but does overlap with *D. septosporum* (except in the USA). *D. septosporum* has a worldwide geographical distribution and a broad host range (Drenkhan et al., 2016). However, more recent records of *D. pini* in many areas of Europe suggest a wider distribution than initially thought (Drenkhan et al., 2016; Jánošíková-Hečková et al., 2018; Matsiakh et al., 2018; Mullett et al., 2018; Ondrušková et al., 2017; Ortiz de Urbina et al., 2017).

DNA-based molecular approaches are reliable for distinguishing *D. septosporum* and *D. pini* (Barnes et al., 2004). The two species can be differentiated by conventional PCR and qPCR, use species-specific primers (Ioos et al., 2010), and by sequencing the nuclear ribosomal internal transcribed spacer region (ITS), elongation factor and β tubulin gene regions or ITS RFLPs (Barnes et al., 2004). Using these diagnostic methods, both *Dothistroma* species have been detected in Slovakia. *Dothistroma septosporum* has a wider geographical range and is detected six times more frequently than *D. pini*, while the host species range was comparable (Jánošíková-Hečková et al., 2018). Both mating types of *D. septosporum* were found at approximately a 1:1 ratio in Slovakia. The results of genetic diversity and population structure analyses of Slovak populations of *D. septosporum* show that the fungus has a high genetic diversity, numerous haplotypes, low clonality, and is a long-established pathogen in Slovakia, with an asexual and sexual mode of reproduction, and which spreads both naturally and by human activity (Jánošíková et al., 2021). In contrast, the populations of *D. pini* are characterised by high clonality, low gene and genetic diversity, with a single mating type predominating, and are maintained by asexual reproduction. *D. pini* is considered a recently introduced pathogen in Slovakia (Adamčíková et al., 2021b).

Despite earlier reports of successful pathogenicity tests (Gadgil, 1974, 1977; Gadgil & Holden, 1976; Parker, 1972), artificial inoculation and infection of pine seedlings with *Dothistroma* spp. in containment is notoriously unreliable, particularly with cultured inoculum (Bradshaw et al., 2006; Devey et al.,

2004; Schwelm et al., 2009). Continual leaf wetness is crucial for controlled inoculation and symptom development (Gadgil, 1977; Muir & Cobb, 2005). Temperature and light intensity are also important factors (Gadgil, 1974; Gadgil & Holden, 1976). To maintain optimal conditions for artificial inoculation, appropriate laboratory equipment and cultures with excellent sporulation potential are required. In addition to the low and variable infection rates, the use of cultured inoculum might cause morphological instability and attenuation of metabolite production in the fungi (Kale et al., 1996; Schwelm et al., 2009). Furthermore, penetration behaviour by *Dothistroma* spp. on pine seedlings in growth chambers can differ from that under natural (forest) conditions (Muir & Cobb, 2005). Based on the reports described above, we chose to use natural sources of infection in the experiments in this study.

To the best of our knowledge there is no information on differences in virulence between *D. septosporum* and *D. pini*. We aimed to study and compare the virulence of *D. septosporum* and *D. pini* on two pine species, *P. nigra* and *P. mugo*, which are both known to be highly susceptible to DNB (Drenkhan et al., 2016). Only landscape shrubs of *P. mugo* in urban stands have been reported to have symptoms of DNB in Slovakia—no DNB symptoms have been reported in natural and naturally regenerated stands of *P. mugo* in the country (Ondrušková et al., 2017). So, in addition, we tested the local, Slovak *P. mugo* provenances for susceptibility to the two DNB pathogens.

Materials and methods

Experimental sites

Pines in plantations or arboretums in Jahodná (in the Dunajská Streda District in the Trnava Region), Kálnica (in the Nové Mesto nad Váhom District in the Trenčín Region) and Mlyňany (in Zlaté Moravce District of the Nitra Region) in west Slovakia that were naturally infected with DNB were selected for the experiment (Fig. 1, Table 1).

The Jahodná site was a Christmas tree (*P. nigra*) plantation, approximately 33 years old. *Dothistroma pini* was identified here for the first time in 2014, although symptoms of DNB have been present since 2000 (Adamčíková et al., 2021b; Ondrušková et al.,



Fig. 1 Locations of the three experiment sites (Jahodná, Kálnica and Mlyňany) where pines were used to study *Dothistroma* needle blight in west Slovakia

Table 1 Some characteristics of the experimental sites and arboretum with pine trees naturally infected with *Dothistroma* needle blight

Characteristic of stand	Jahodná	Kálnica	Mlyňany 1	Mlyňany 2
Type of planting	Christmas tree plantation	Christmas tree plantation	Arboretum	Arboretum
<i>Pinus</i> sp.	<i>P. nigra</i>	<i>P. nigra</i>	<i>P. ponderosa</i>	<i>P. mugo</i>
Host age (years)	33	23	45–50	n.a
Tree stand area or number of infected trees	2.8 ha	1.0 ha	21 trees	8 m ²
DNB pathogen present	<i>D. pini</i>	<i>D. septosporum</i>	<i>D. pini</i>	<i>D. septosporum</i>
Coordinates	N48.042883, E17.727464	N48.762536, E17.974826	N48.322212, E18.371260	N48.317548, E18.370643
Altitude (m a.s.l.)	103	404–428	188	216

2018). The mean disease incidence of trees in the stand in 2018 was 68.81% (Ondrušková et al., 2020).

The Kálnica site was an abandoned Christmas tree plantation, where the symptoms of DNB have been assessed since 2002. DNB caused by *D. septosporum* had an incidence of 80.39% in 2018 (Jánošíková-Hečková et al., 2018; Ondrušková et al., 2020).

Two experimental plots were chosen at the Mlyňany site, which was an arboretum. The first was a stand of *P. ponderosa* with approximately 21 trees heavily infected with *D. pini* (Adamčíková et al., 2021b), and the second was a stand of *P. mugo* shrubs, severely diseased with DNB caused by *D. septosporum* (Ondrušková et al., 2017). The two stands are approximately 900 m apart, but isolated by

a tall, dense continuous screen of vegetation comprising trees and shrubs.

Plant material used in the natural infection susceptibility tests

To assess the virulence of *D. septosporum* and *D. pini* and the relative susceptibility of host species, two-year-old seedlings of two susceptible pine species were obtained. Seedlings of *P. nigra* of regional origin were obtained from the tree nursery Forests of the Slovak Republic (Lovce, Hladomer) and the *P. mugo* seedlings were from seeds collected in natural or natural regenerated stands (State Forests of TANAP), representing populations of trees in Rakúske Lúky

(Rakúsy, in the Kežmarok District in the Prešov Region of north-eastern Slovakia). The seedlings were healthy, without any damage and had received no application of fungicides.

Fifty seedlings of each pine species were planted at each experimental site in May 2019. The seedlings were planted manually using a spade, directly under a DNB-diseased tree, thus exposed to natural inoculum of *Dothistroma*. The seedlings were assessed over two growing seasons by visual inspection for seedling survival and symptoms of DNB once each year at the end of the growing season (in November 2019 and 2020). For PCR confirmation of *Dothistroma* species and evaluation of their virulence, all seedlings were harvested in March and April 2021, by cutting the stem near ground level, placing in separate plastic bags and storing in a freezer at -20 °C until processing.

For negative controls, thirty seedlings of both pine species were grown outdoors in the experimental garden of the Slovak Academy of Sciences in Nitra (Fig. 1). There was no known local source of *Dothistroma* inoculum.

Evaluation of virulence of DNB pathogens

To assess the virulence of *D. septosporum* and *D. pini*, the presence of symptoms (needles with typical symptoms of reddish-brown spots scattered on green needles, that develop into red bands with acervuli)

were scored (Fig. 2). Needles were categorized as healthy (green), chlorotic (with no evidence of DNB infection) or symptomatic (infected). Disease incidence was calculated as the percentage of symptomatic needles (=infected needles) per seedling. The number of red bands was counted on 30 randomly selected needles per seedling, and the number of acervuli per needle was counted on a subsample of 10 randomly selected needles from those 30.

Statistical analyses

All analyses and associated graphics were conducted using R 4.2.2 (R Development Core Team, 2022). Two separate mixed models were used with a generalised least squares framework (GLS; Pinheiro & Bates, 2000). The models aimed to explain: i) the prevalence of symptomatic needles, and ii) the number of red bands and acervuli per symptomatic needle based on "pathogen species" and "*Pinus* host species" (the two fixed effects and their interaction), while "site" and "measured object" (i.e. tree or needle) were treated as random effects. The significance of the model terms was assessed via the F- and P-values. *Pseudo-R*², which determines the proportion of explained variance, was obtained from a correlation between the dependent variable and squared predicted values. Subsequently, results were visualised as predicted mean values with corresponding 95% confidence intervals with the package "AICcmodavg"

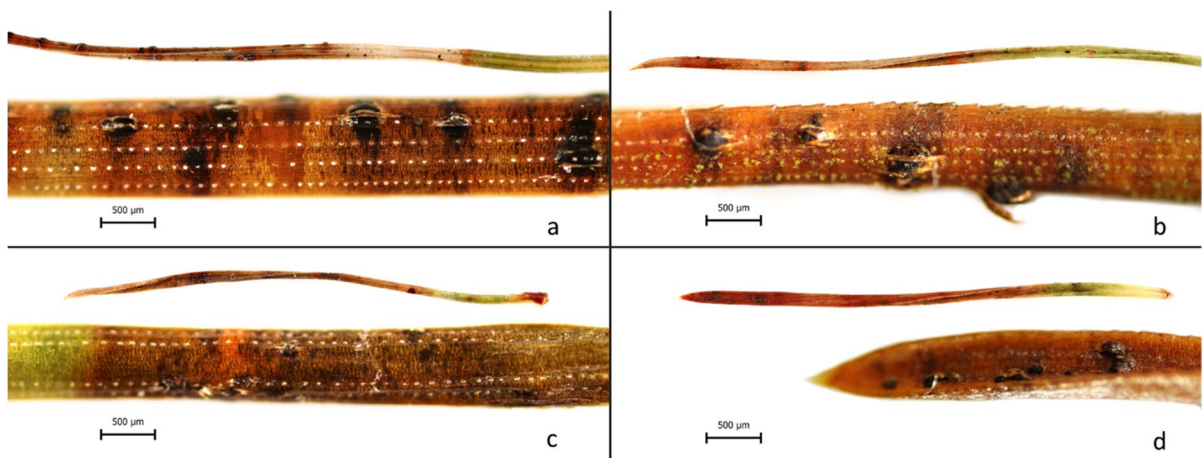


Fig. 2 Pine needles diseased with *Dothistroma* needle blight. Whole needles with typical symptoms and details showing acervuli **a** *Pinus nigra* infected with *D. pini*, **b** *P. nigra* infected

with *D. septosporum*, **c** *P. mugo* infected with *D. pini*, **d** *P. mugo* infected with *D. septosporum*

(Mazerolle, 2020). Generalized least squares were analyzed with the "glS" command of the "nlme" package (Pinheiro et al., 2014).

Detection of *Dothistroma* species

Conventional PCR was used for the identification of *Dothistroma* species on the harvested seedlings at each site that had been stored -20 °C. DNA was extracted directly from randomly selected symptomatic needles from both pine species (Table 1). A symptomatic section with acervuli was cut from one needle per seedling after surface sterilization with 96% ethyl alcohol. A DNA sample consisted of needle pieces with acervuli from 3–7 different needles from different seedlings from the same pine species

and experimental site. Seven to 20 needles were screened per site, pine species and pathogen species (Table 2). A single sample of DNA per host tree was analyzed for the presence of *Dothistroma* from negative control seedlings. An E.Z.N.A.® Fungal DNA Mini Kit (Omega Bio-Tek, Inc., Norcross, GA, USA) was used for DNA extraction following the manufacturer's instructions.

PCR was conducted with each DNA sample using two diagnostic methods for *Dothistroma* species and mating type. First, mating type idiomorph (*MAT1-1* and *MAT1-2*) species-specific primers were used according to Groenewald et al. (2007), and second, general, species-specific primers were used according to Ioos et al. (2010). In cases where

Table 2 The presence of *Dothistroma septosporum* and *D. pini* as detected by PCR in pine needle samples collected from 2-year-old pine seedlings (*Pinus nigra* and *P. mugo*) planted

at different sites with trees having natural *Dothistroma* needle blight infection in west Slovakia

Site/ <i>Pinus</i> sp.	Sample number	No. of needles in sample	Expected <i>Dothistroma</i> sp. ^c	<i>Dothistroma</i> spp. detected	
				Species specific mating type primers (Groenewald et al., 2007)	Species specific primers (Ioos et al., 2010)
Kálnica					
<i>P. nigra</i>	D980	6	<i>D. septosporum</i>	<i>D. septosporum</i>	<i>D. septosporum</i>
<i>P. nigra</i>	D981	7	<i>D. septosporum</i>	<i>D. septosporum</i>	<i>D. septosporum</i>
<i>P. nigra</i>	D982	7	<i>D. septosporum</i>	Neg ^a	<i>D. septosporum</i>
<i>P. mugo</i>	D983	6	<i>D. septosporum</i>	<i>D. septosporum</i>	<i>D. septosporum</i>
<i>P. mugo</i>	D984	7	<i>D. septosporum</i>	<i>D. septosporum</i>	<i>D. septosporum</i>
<i>P. mugo</i>	D985	7	<i>D. septosporum</i>	<i>D. septosporum</i>	<i>D. septosporum</i>
Jahodná					
<i>P. nigra</i>	D986	7	<i>D. pini</i>	<i>D. pini</i> + <i>D. septosporum</i> ^b	<i>D. pini</i>
<i>P. nigra</i>	D987	7	<i>D. pini</i>	<i>D. pini</i>	<i>D. pini</i>
<i>P. mugo</i>	D988	7	<i>D. pini</i>	<i>D. pini</i>	<i>D. pini</i>
<i>P. mugo</i>	D989	4	<i>D. pini</i>	<i>D. pini</i>	<i>D. pini</i>
Mlyňany 1					
<i>P. nigra</i>	D990	3	<i>D. pini</i>	<i>D. pini</i>	<i>D. pini</i>
<i>P. nigra</i>	D991	4	<i>D. pini</i>	<i>D. pini</i>	<i>D. pini</i>
<i>P. mugo</i>	D993	7	<i>D. pini</i>	<i>D. pini</i> + <i>D. septosporum</i> ^b	<i>D. pini</i>
<i>P. mugo</i>	D996	6	<i>D. pini</i>	<i>D. pini</i>	<i>D. pini</i>
Mlyňany 2					
<i>P. mugo</i>	D994	7	<i>D. septosporum</i>	<i>D. septosporum</i>	<i>D. septosporum</i>
<i>P. mugo</i>	D995	5	<i>D. septosporum</i>	Neg ^a	<i>D. septosporum</i>

^a Neg = negative result

^b Poor signal for *D. septosporum*

^c the expected presence of *Dothistroma* species based on earlier species detection (Jánošíková-Hečková et al., 2018; Ondrušková et al., 2018)

discordant results were obtained with the two diagnostic methods, the PCR was repeated.

PCRs were performed in a total volume of 20 µl per sample containing 4 µl of 5×HOT FIREPol® Blend Master Mix (Solis BioDyne, Tartu, Estonia), 1–2 µl of template DNA, 1 µl of each specific forward and reverse primers (10 mol/µl), and purified water to make 20 µl.

Primers DpiniMat1f2/DotMat1r and DpiniMat2f/DotMat2r were used in single PCR to identify mating types of *D. pini*, and primers DseptoMat1f/DotMat1r and DseptoMat2f/DotMat2r for mating types of *D. septosporum* (Groenewald et al., 2007). The cycle conditions for PCR consisted of an initial denaturation step for 15 min at 95 °C, followed by 40 cycles each comprising a denaturation step at 94 °C for 20 s, an annealing step (65 °C for *D. pini* and 63 °C for *D. septosporum*) for 30 s, and an extension step at 72 °C for 40 s followed by a final extension step for 5 min at 72 °C. The primers amplify regions of approximately 820 bp of the *MAT1-1* and 480 bp of the *MAT1-2* mating type idiomorph of *D. septosporum*, and 823 bp of the *MAT1-1* and 480 bp of the *MAT1-2* mating type idiomorph of *D. pini*.

For the species-specific primers, DStub2F/DStub2R were used to identify *D. septosporum*, and DPtefF/DPtefR to identify *D. pini* (Ioos et al., 2010). A 231 bp amplicon is diagnostic for *D. septosporum* and a 193 bp amplicon for *D. pini*. The cycling conditions were the same for both *Dothistroma* species, which included an initial denaturation step at 95 °C for 15 min, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s, and elongation at 72 °C for 60 s, and a final extension at 72 °C for 10 min.

All PCR products were visualized by horizontal electrophoresis on 1% (w/v) TBE agarose gels stained with Simply Safe (EURx Ltd. Gdansk, Poland). The length of PCR products was determined using 100-bp size markers (Solis BioDyne, Tartu, Estonia). The absence of bands of expected size were scored as a negative result. Molecular-grade water was used as a negative amplification control and *D. septosporum* and *D. pini* samples confirmed from previous studies (Jánošíková – Hečková et al., 2018) were used as positive controls.

Results

Confirmation of *Dothistroma* species in needle tissue of pine seedlings

All 16 DNA samples gave positive results for the presence of *D. pini* and/or *D. septosporum* in at least one of the two PCR detection assays (Table 2).

PCR failed to detect *D. septosporum* in two samples (D982 and D995). Weak positive results were obtained for *D. septosporum* in two DNA samples (D986 from the Jahodná site and D993 from the Mlyňany site) which were also confirmed positive for *D. pini*. All other samples were diagnosed as either *D. septosporum* or *D. pini* (Table 2). Only *D. septosporum* was detected from the Kálnica and Mlyňany 2 sites, and *D. pini* from the Jahodná and Mlyňany 1 sites (with the two weak positives for *D. septosporum*). The results agree with the expected presence of *Dothistroma* species based on earlier species detection according to Jánošíková-Hečková et al. (2018) and Ondrušková et al. (2018). DNA samples collected from needles from control seedlings were negative for both *Dothistroma* spp.

Susceptibility of pine species to *Dothistroma septosporum* and *D. pini*

Of the 200 *P. mugo* seedlings planted, 145 survived at the end of the experiment, from which 110 (almost 76%) had symptoms of DNB. With *P. nigra*, seedling survival rate was slightly higher, with 159 of 200 seedlings surviving until the end of the experiment, with a final incidence of DNB of 65% (Table 3).

There was no significant difference (GLS; $F=0.11$; $p=0.802$) in disease incidence, expressed as the proportion of DNB symptomatic pine needles caused by either *Dothistroma* species, on either of the two hosts, *P. mugo* or *P. nigra* (Fig. 3a). The incidence (~20%) was similar regardless of *Dothistroma* species or host. However, our data indicate differences in some of the symptoms of DNB, specifically the red bands and acervuli. The mean number of red bands is higher (GLS: $F=7.68$; $p=0.006$) on needles of *P. nigra* when compared to *P. mugo* for both *D. septosporum* and *D. pini* (Fig. 3b). On *P. mugo* needles, the maximum number of red bands was 11 and 12 with *D. pini* and *D. septosporum*, respectively, while on *P. nigra* the maximum number of red bands

was almost double, with 21 and 20 for *D. pini* and *D. septosporum*, respectively (Table 4).

Greater numbers of acervuli (GLS: $F=3.97$; $p=0.046$) were observed on needles of *P. nigra* infected with *D. septosporum* (Fig. 3c). The maximum number of acervuli counted per needle was 41

(Table 5) and differed significantly from the numbers of acervuli on *P. mugo*. There was no significant difference (GLS: $F=0.37$; $p=0.718$) in the number of acervuli of *D. pini* on *P. mugo* or *P. nigra* (Fig. 3c).

Table 3 The total numbers of seedlings of each pine species evaluated for Dothistroma needle blight (DNB), and the number and percentage with symptoms of DNB and the species causing the disease

Site / <i>Dothistroma</i> species	Number of seedlings					
	<i>Pinus mugo</i>			<i>Pinus nigra</i>		
	Evaluated	Diseased	Diseased (%)	Evaluated	Diseased	Diseased (%)
Jahodná/ <i>D. pini</i>	41	18	44%	49	35	71%
Kálnica/ <i>D. septosporum</i>	47	47	100%	46	43	93%
Mlyňany 1 / <i>D. pini</i>	17	16	94%	18	16	89%
Mlyňany 2 / <i>D. septosporum</i>	40	29	72.5%	46	10	22%
Total	145	110	75.8%	159	104	65.4%

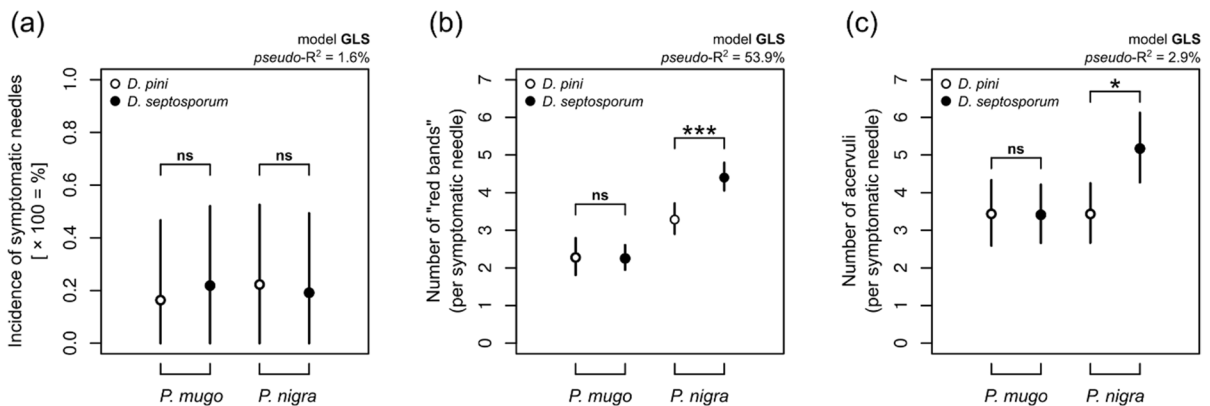


Fig. 3 Pine species (*Pinus mugo* or *P. nigra*) and pathogen species (*Dothistroma septosporum* and *D. pini*) effects on symptoms of Dothistroma needle blight: **a** incidence of symptomatic needles, **b** a number of red bands, **c** number of acervuli. Points and vertical lines represent estimated mean

values with corresponding 95% confidence intervals for each host×fungi pair. Intervals overlap indicates the absence (ns) of a significant difference. Asterisks indicate level of statistical significance: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$

Table 4 Minimum, maximum and mean number of red bands per needle from *Pinus mugo* and *P. nigra* showing symptoms of Dothistroma needle blight after natural infection with *Dothistroma septosporum* and *D. pini*. N=number of needles evaluated

Locality/ <i>Dothistroma</i> species	Number of red bands per needle							
	<i>Pinus mugo</i>				<i>Pinus nigra</i>			
	Min	Max	Mean (CI ^{95%})	N	Min	Max	Mean (CI ^{95%})	N
Jahodná / <i>D. pini</i>	0	11	2.3	261	0	21	3.3	551
Mlyňany 1 / <i>D. pini</i>	0	9	(1.8–2.8)	329	0	14	(2.9–3.7)	298
Kálnica / <i>D. septosporum</i>	0	12	2.3	1381	0	20	4.4	1279
Mlyňany 2 / <i>D. septosporum</i>	0	4	(2.0–2.6)	83	0	3	(4.0–4.8)	25

Table 5 Minimum, maximum and estimated mean number of acervuli counted per needle from *Pinus mugo* and *P. nigra* showing symptoms of Dothistroma needle blight after natural infection with *Dothistroma septosporum* and *D. pini*. N = number of needles evaluated

Locality / <i>Dothistroma</i> species	Number of acervuli per needle							
	<i>Pinus mugo</i>				<i>Pinus nigra</i>			
	Min	Max	Mean (CI ^{95%})	N	Min	Max	Mean (CI ^{95%})	N
Jahodná / <i>D. pini</i>	0	8	3.5	114	0	16	3.5	195
Mlyňany 1 / <i>D. pini</i>	0	12	(2.6–4.3)	149	0	23	(2.7–4.3)	131
Kálnica / <i>D. septosporum</i>	0	19	3.4	200	0	41	5.2	200
Mlyňany 2 / <i>D. septosporum</i>	0	5	(2.7–4.2)	86	0	12	(4.3–6.1)	24

Virulence of *D. septosporum* and *D. pini*

No significant difference was observed in any of the three symptoms (incidence of symptomatic needles, number of red bands and number of acervuli per needle) of DNB on *P. mugo* regardless of the *Dothistroma* species (Fig. 3). However, with *P. nigra*, there were significant differences in both the number of acervuli and the number of red bands per needle observed for *D. septosporum* and *D. pini*, respectively.

Discussion

The results suggest the existence of pine stands in Slovakia with only a single species of *Dothistroma* present under conditions of natural infection. Based on the symptoms (number of red bands and acervuli), the results further indicated no difference in the virulence of *D. septosporum* and *D. pini* on *P. mugo*. However, on *P. nigra*, the symptoms differed depending on the pathogen species. The results of our study demonstrate that the virulence of the two pathogens is affected by the pine host species.

The similar prevalence of infected needles on both host species of pine indicates similar infection capability of *D. septosporum* and *D. pini*. Infected needles typically had red bands due to the presence of a mycotoxin, dothistromin. Kabir et al. (2015) confirmed that the ability to produce dothistromin is not needed for the fungus to cause disease but found that dothistromin does affect the symptom severity. Without dothistromin, the pathogen colonised a smaller area of the needle, produced significantly smaller lesions, and importantly, produced fewer spores per lesion, indicating that dothistromin is a virulence factor. In our experiments, the red band symptom was used to estimate the virulence of DNB pathogens. However,

the characteristic red bands may be present at a very low level, or even absent with DNB (Schwelm et al., 2009). Our results support differences in red band number, in this case due to differences between the two pine host species. The number of red bands was significantly lower on *P. mugo* needles than on *P. nigra*. The difference was commonly observed in the field as a low presence or absence of red bands on *P. mugo*. The low presence of red bands on *P. mugo* may explain why no difference was found between *D. septosporum* and *D. pini* on that species, while a highly significant difference was observed between *D. septosporum* and *D. pini* on *P. nigra*.

Acervuli, the reproductive sources of inoculum, were also assessed. Acervuli number differed between *D. septosporum* and *D. pini* on *P. nigra*, with more acervuli formed by *D. septosporum*. The greater number of acervuli associated with *D. septosporum* infections on *P. nigra* corresponds to the wider and more frequent occurrence of this *Dothistroma* species in Slovakia. Furthermore *P. nigra* is one of the most common and widespread hosts of DNB and causes severe disease in Slovakia (Jánošíková-Hečková et al., 2018, Adamčíková et al., 2023). Evaluation of the symptoms, number of red bands and acervuli per needle, which reflect the disease development, showed a difference in virulence between *D. septosporum* and *D. pini*, but only in the case of *P. nigra*. These initial results indicate that the virulence of the two pathogens could be affected by pine species, due to differences in susceptibility to *D. septosporum* and *D. pini*.

Under field conditions, Fraser et al. (2015) observed variability in the susceptibility of pine to DNB between sites and years. Therefore, to explore the virulence of each pathogen we evaluated the pathogens on two hosts at three sites. Several factors other than virulence may explain the variation between sites, including *Dothistroma* population

diversity, or uneven inoculum pressure, and there are no reports of previous experiments investigating the virulence of different *Dothistroma* genotypes or populations (Fraser et al., 2015). Recent research describing the population structure and genetic diversity of *D. septosporum* suggested that the pathogen had been established in Slovakia for a long time (high genetic diversity), while the population structure and genetic diversity of *D. pini* (high clonality) indicated a recent introduction into the country (Adamčíková et al., 2021a, 2021b; Jánošíková et al., 2021). In most cases, introduced pathogens are highly virulent towards a new host species in a new environment. Variation in DNB susceptibility of genetically different populations were observed by Fraser et al. (2015) in Scots pine population. Fraser et al. also suggested that *Dothistroma* population characteristics, as genetical diversity and clonality, may affect host relative susceptibility and infection rate, but the effect of site factors may be more important than *Dothistroma* population.

In the Northern Hemisphere, the increasing prevalence of DNB has been linked to changing climatic conditions, notably higher temperature and changes in precipitation conducive for disease development (Drenkhan et al., 2016). Differences in environmental requirements could exist for the two pathogens. Besides the host species and origin of the pathogen species, the site conditions may affect disease development, which could contribute to differences observed in the present study. During the years 2014 to 2017 temperature and relative humidity were recorded at two of the experimental sites (Kálnica and Jahodná). A comparison of the average monthly temperatures showed statistically significant differences between them, although the optimal relative humidity and temperature for disease spread and development (temperature 15–20 °C, relative humidity > 90% (Dvořák et al., 2012)) occurred from May to the end of September at both sites (Ondrušková et al., 2020). To eliminate the effects of site and weather conditions, it would be useful to supplement the present study with artificial inoculation experiments under controlled conditions, despite prior observations that susceptibilities of pine to DNB via artificial inoculation with *Dothistroma* under controlled conditions can be different to that observed in the field (Fraser et al., 2015).

The experiments confirmed the susceptibility of local *P. mugo* provenances to DNB, even though DNB was not confirmed in natural or naturally regenerated stands of *P. mugo* in Slovakia. Localities of natural populations (or of autochthonous occurrence) of *P. mugo* are at high elevation in mountain ranges in Europe – including the Alps, high mountain ranges of Central Europe, Carpathians, Balkan Mountains, and Abruzzi – and grow above the upper forest line, at approximately 1,430 m or in basins in peatbogs at altitude between 565 (in Germany) and 1040 m (in Czech Republic, Blatný & Šťastný, 1959; Businský, 2008). In Slovakia, *P. mugo* populations occur in the north which has the highest elevation, and forms a separate vegetation zone: specifically in the Tatra, Low Tatra, and Fatra (Blatný & Šťastný, 1959).

DNB was observed on native *P. mugo* in Northern and Central Montenegro where the host grows at altitudes between 1,450 and 1,850 m. DNB symptoms were sporadic on the current season's needles, but more visible on 2, 3-year-old and older needles. Infected needles had red bands and dying needle tips, but acervuli and conidia were not observed (Lazarević et al., 2017). However, in planted stands of *P. mugo*, a different situation was observed. Trees were severely diseased with DNB caused by *D. septosporum* (Lazarević et al., 2017). Similar results were previously reported from Slovakia, where only *P. mugo* shrubs in planted, ornamental stands had DNB, with the disease present at a high incidence and a range of severities; but the *P. mugo* trees in natural, native stands (all trees evaluated were in the Tatra mountains) were DNB free (Ondrušková et al., 2017). Several factors could explain these observations. First are the stand itself and the climatic conditions. In native stands, the trees are likely growing in areas where environmental conditions are most ideal, indirectly maximising plant health and thus resistance to disease, and conversely, those conditions may not favour the *Dothistroma* species. Second, both *Dothistroma* species are known to also be dispersed by humans (Jánošíková et al., 2021; van der Nest et al., 2023), thus, native stands may be less frequently visited or exposed to the pathogens via human activity, limiting infection opportunities.

Conclusion

The study is the first to show differences in virulence of the two related *Dothistroma* species infecting two pine species in Slovakia. The results provide a basis for more detailed and comprehensive research, which will contribute to developing phytosanitary approaches for this important disease. Further related research, including studies on the variability in pathogenicity of different *D. septosporum* and *D. pini* ITS haplotypes, and determination of how environmental conditions affect virulence should be addressed to provide a fuller understanding of the pathosystems of these two *Dothistroma* species.

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Data availability The data that support the findings of this study are available from the corresponding author upon reasonable request.

Declarations

Ethical approval This research did not involve any animal and/or human participants.

Competing interests The authors declare that they have no conflict of interests.

Conflict of interests The authors have no relevant financial or non-financial interests to disclose.

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