Persistence of *Dothistroma septosporum* on Abscised Pine Needles and its Implications for Disease Management

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Abstract

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Dothistroma septosporum is a serious foliar pathogen of pines in Britain. To determine the contribution of spores produced on abscised needles to the *D. septosporum* inoculum load in a forest environment, survival of the fungus was investigated in commercially grown plantations of Corsican pine (*Pinus nigra* subsp. *laricio*) in southern England. The fungus persisted for longer in abscised needles retained in the canopy compared with needles on the litter layer of the forest floor. High relative humidity had a significant negative effect on persistence in the canopy needles, highlighting the effect of moisture on rates of microbial

decay. The median lethal time 50 (LT_{50}) of infective propagules from needles in the litter layer was 20 to 28 days, whereas for needles suspended in the canopy it was 34 to 48 days. Microsatellite markers revealed that the population of *D. septosporum* deemed persistent (i.e., recovered from abscised needles after exposure for 28 to 32 weeks) was similar to the general population of *D. septosporum* in the forest stand (i.e., isolates recovered from attached needles and after exposure for only 4 weeks). Overall, abscised needles with acervuli of *D. septosporum* contribute only moderately and for a limited time to inoculum levels of the pathogen in an infected forest stand.

Dothistroma needle blight (DNB) is a serious and economically important disease of pines that causes premature defoliation, decreased yield, and in extreme cases, tree death (Brown and Webber 2008). Although two fungal species, *Dothistroma septosporum* (Doroguine) Morelet (sexual stage *Mycosphaerella pini*) and *D. pini* Hulbary (sexual stage unknown) cause the disease, *D. septosporum* is the most widely distributed and the only DNB pathogen found in Great Britain. Over 80 species of *Pinus* are affected by the disease (Watt et al. 2009), but Corsican pine (*Pinus nigra* J.F. Arnold subsp. *laricio* (Poir.)) is one of the most susceptible (Gilmour 1967) and an important commercial forestry species in Britain (Brown and Webber 2008). Infection takes place primarily in the spring and early summer via rain-splash dispersed conidia, which form in acervuli on infected needles (Gibson 1972; Karadžić 1989; Mullett 2014; Peterson 1967, 1973; Rogerson 1953). Initial symptoms appear as chlorotic bands but quickly progress to brown necrotic bands, often associated with a brick-red discoloration, with black acervuli forming within the bands (Brown and Webber 2008). Symptoms may be seen by late autumn, but typically the most abundant and clearest symptoms appear the following spring, when acervuli are formed in the needles infected the previous year and produce conidia which infect the new current year's needles (Mullett 2014). Affected needles are predominantly cast in the summer (July), prior to the main shedding of older but uninfected needles in the fall, although infected needles can be cast at any time of year (Karadžić 1989; Mullett 2014). The cast needles contain many acervuli which produce large numbers of infective conidia and have the potential to be a major source of inoculum, particularly if they remain viable for long enough to take advantage of conditions conducive to infection.

Abscised leaves and plant debris are known to be significant sources of inoculum in a number of plant diseases. Ascospores of *Venturia inaequalis* produced on apple leaf litter initiate epidemics of apple scab (Gladieux et al. 2008; MacHardy 1996; Xu et al. 2012). Sanitation measures to remove or destroy the leaf litter in apple orchards can reduce the subsequent incidence of apple scab by up to 57% (Holb 2006). Another example, ascochyta blight of pulse crops, is caused by a number of pathogens including *Mycosphaerella pinodes* and various *Ascochyta* spp., and the primary inoculum source is from infested plant residues from the previous season (Davidson and Kimber 2007). In fact, crop residues are considered the most important source of inoculum for various fungi causing ascochyta blight of faba bean, field pea, lentil, and bean crops in countries as diverse as Canada, Australia, and the United Kingdom (Bond and Pope 1980; Bretag et al. 2006; Carter and Moller 1961; Hawthorne et al. 2004; Morrall 1997). In a forestry environment, the main inoculum source of the pine needle pathogen *Lophodermium seditiosum* comes from fallen needles (Diwani and Millar 1990). Most needles infected with *L. seditiosum* are cast prematurely in early summer, with fruit bodies developing rapidly and producing spores that infect current years' needles a few months later in the late summer and autumn (Diwani and Millar 1990; Strouts and Winter 2000). *D. septosporum* also causes premature defoliation in the summer; however, fruit bodies of the pathogen are present, and spores disseminated, while the needles are still attached (Mullett 2014). Thus it is not known whether fallen needles infected with *D. septosporum* act as a source of inoculum in a similar manner to those of *L. seditiosum* and if so, how much they contribute to the total inoculum load in the stand.

When needles are shed prematurely, they either become lodged in the dense network of branches and hang suspended above the ground, or they land on the litter layer of the forest floor. These positions present different threats for infection, with spores from suspended needles a threat to both canopy needles and regeneration on the forest floor while spores from needles in the litter are predominantly a threat to regeneration or new plantings. The position of abscised needles will also present very different conditions for the needles and accompanying fungi both in terms of moisture and temperature, as well as the presence of competing saprophytic microfungi. For instance, needles caught in branches are exposed to drying breezes and are not in contact with the heavily colonized litter layer of the forest floor.

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*The e-Xtra logo stands for "electronic extra" and indicates that one supplementary table is included in the online edition.

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Knowledge of how long *D. septosporum* propagules survive in abscised needles should help inform management decisions for infected stands. For example, the frequency or timing of thinning and pruning regimes could be altered if suspended needles in the canopy proved to be a significant source of inoculum. Thinned and pruned stands have fewer branches to trap falling needles and provide increased air flow, thereby reducing the likelihood of infection by promoting conditions less suitable for spore dispersal and infection. Knowledge of survival times of the fungus in the litter layer might also affect the length of time before replanting a site and has implications for nursery sanitation measures. Therefore, this study aimed to i) investigate whether abscised needles produce substantial quantities of inoculum compared with attached needles, ii) determine how long *D. septosporum* can survive in detached needles both when caught in the canopy and when part of the litter layer, iii) assess if weather conditions play a role in the longevity of the fungus in abscised needles, and iv) examine, using microsatellite analysis, whether any particular *D. septosporum* multilocus haplotypes are better suited to persisting in abscised needles.

Materials and Methods

Trial design. An infected stand of unthinned 19-year-old *P. nigra* subsp. *laricio* in the south of England (Abbotts Wood Compartment 68C, planted 1992, 51°09'32" N, 0°50'14" W) was chosen for the experiment as it represented a typical closed-canopy stand where many needles are caught in the canopy on abscission. However, needles were too inaccessible for regular collection, so needles still attached to twigs and naturally infected with *D. septosporum* were collected from a nearby 9-year-old stand of *P. nigra* subsp. *laricio* (Compartment 75B, planted 2002, 51°09'09" N 0°50'13" W), with a fruit body of the pathogen present on at least one needle in each fascicle. Collection of needles was carried out every 4 weeks for 2 years, beginning in the first week of February 2011 and ending in January 2013, in order to ensure needles and fruit bodies from

different seasons and physiological stages were tested. At each of the 26 collection dates (i.e., run replicates) seven fascicles were placed into each of 16 nylon mesh bags (Agralan Enviromesh, bags approx. 18 × 10 cm, mesh size 2 × 2 mm, see Fig. 1) and laid out in the 19-year-old stand. Eight of the mesh bags were tied to branches approx. 3.5 m above the ground (Fig. 1A and B). The remaining bags were laid out approximately 1.5 m apart in a line on the surface of the litter layer (Fig. 1C and D).

Every 4 weeks, one of the eight mesh bags with suspended needles and one of the eight mesh bags from the litter layer from each needle collection date (i.e., run replicates) were taken back to the laboratory and acervuli assessed for viable *D. septosporum* propagules. At the same time, a sample of freshly collected infected needles was also assessed. Thus, bagged needles were assessed for viable propagules on the week they were collected (i.e., fresh, at time 0), and after 4, 8, 12, 16, 20, 24, 28, and 32 weeks exposure in both positions (the litter or suspended in the branches). Needle assessments were carried out every 4 weeks for 2 years (from February 2011 to August 2013) to determine if survival time of the fungus varied with season and the actual weather conditions the needles were exposed to. Therefore, 26 run replicates were carried out; each run replicate consisted of assessing freshly collected needles as well as needles exposed to each of the eight exposure lengths (see Supplementary Table 1 for timings of needle collections and assessments).

Weather data, in the form of air temperature, rainfall, relative humidity, wind speed, and photosynthetically active radiation (PAR) were collected from a weather station (Delta-T) in the stand from which the needles were collected.

Assessment of viable *D. septosporum* propagules. Three needles from each bag were assessed at each assessment time interval (i.e., needle replicates), with the remainder of the needles placed into gripseal plastic bags and stored at -70°C. Three freshly collected needles were also assessed for each collection date (i.e., run replicate).

Fig. 1. Persistence trial layout. **A** and **B**, Bagged needles suspended in the canopy to simulate shed needles caught among branches. **C**, Spacing of bagged needles on the forest floor. **D**, Close up of bagged needles on the forest floor.

Each needle was wiped with a tissue soaked in industrial methylated spirits (IMS) and an individual acervulus from each needle excised under a dissecting microscope (30×). The excised acervulus was placed in a 2.0 ml microcentrifuge tube (MCT) along with an IMS sterilized and air dried 6 mm steel ball and 200 µl sterile distilled water (SDW). Samples were then ground on a bead mill (Retsch, Haan, Germany) for 2 min at 30 Hz. This allowed release of all conidia into the water while causing minimal damage to the spores. Some hyphal fragments are also likely to have also been released from the acervulus into the suspension, although they were not observed directly. Two spread cultures, each containing 80 µl of the resulting suspension, were made on 90 mm petri dishes of dothistroma medium (Bradshaw et al. 2000) supplemented with 20 ml of a 1% streptomycin sulfate salt (Sigma-Aldrich, MI, USA) solution per liter of media.

Spread cultures were incubated in the dark at 20°C for 9 days after which the number of *D. septosporum* colonies was counted. *D. septosporum* colonies from each needle were then subcultured onto dothistroma sporulation medium (Bradshaw et al. 2000) bijou slopes and stored at -20°C.

Microsatellite analysis. Microsatellite markers were used to investigate whether particular *D. septosporum* multilocus haplotypes (MLHs) were more prevalent in abscised needles than other MLHs. Isolates recovered from abscised needles exposed to field conditions for 24, 28, and 32 weeks (termed the 'persistent' group) were compared with the overall population, represented by isolates recovered from freshly collected needles and from those exposed to only 4 weeks of field conditions (termed the 'general population' group). DNA extraction, mating type determination, and microsatellite alleles at 11 loci were determined using the methods detailed in Mullett et al. (2015). Briefly, DNA was extracted from cultures using a Kingfisher Flex magnetic particle processor and Kingfisher Plant DNA Extraction kits (Thermo Scientific, MA, USA). Species-specific mating type primers (Groenewald et al. 2007) and microsatellite markers Doth_DS1, Doth_DS2, Doth_E, Doth_F, Doth_G, Doth_I, Doth_J, Doth_K, Doth_L, Doth_M, and Doth_O (Barnes et al. 2008) were used for multilocus haplotyping.

Statistical methods. Initial viable propagule counts varied greatly across the run replicates of the study, with time series analysis failing to identify any monthly, seasonal, or yearly components that might explain this variation. As the viable propagule counts between individual needle replicates within each run replicate exhibited even greater initial variability, replicate needle and run data were pooled in a first attempt to model persistence rates. Therefore, for an overview of propagule decay patterns in abscised needles, data were pooled for all litter and suspended assessment periods over the two years: i.e., all litter needles from the 4-week exposure period were pooled regardless of the specific month they were exposed, all suspended needles from the 4-week assessment period were pooled, etc... Mean decay rates of *D. septosporum* propagules were then calculated using a generalized linear model with a Poisson distribution implemented in GENSTAT 14.1 (VSN International 2011). The median lethal time (Lethal Time₅₀ or LT₅₀) was calculated from the model. This is the median time required for half of the propagules to become nonviable. The time needed for 90% of the propagules to become nonviable, the LT₉₀ value, was also calculated as these are potentially more informative for forest management purposes.

To investigate the influence of particular weather conditions on decay rates, a multiplicative model was fitted to the data to test for the effect of assessment week and exposure time. The model was:

$$\text{Number of viable propagules}_t = \text{number of viable propagules}_{t-1} \times \alpha(\text{week}_t) \times \beta(\text{time}_j)$$

Where t = the time of assessment of propagule viability, in 4-week increments; α = a parameter for the effect of week t , the week of the year the assessment was carried out; and β = a parameter for the effect of time j , the number of 4-week exposure periods that the needles were out in the field. The term $\log(\text{number of viable propagules at time } t-1)$ was set as an offset in the model.

A series of regression models were fitted to identify relationships between the resulting parameter estimates for every assessment period (t) and meteorological metrics recorded during the preceding 4-week period. The meteorological metrics were a summary of the weather the needles had been exposed to during the 4-week period before they were assessed. The metrics were composed of mean temperature, mean windspeed, mean relative humidity, mean PAR (photosynthetically active radiation), and total precipitation over the 28 days prior to assessment.

Molecular statistical methods. Mating type and 11 microsatellite markers were used to assign isolates an MLH. Isolates with the same allele at all 12 loci were considered the same MLH (i.e., a clone). Two methods were used to compare the persistent and general population groups. Firstly, an AMOVA was conducted in GENALEX 6.5 (Peakall and Smouse 2012) to investigate if the two groups were significantly differentiated. Secondly, the χ^2 value of the contingency table with the counts of each MLH was calculated. χ^2 values are unreliable when expected frequencies are low (i.e., <1, or $\leq 20\%$ of values <5) as is often the case for genotype or multilocus haplotype data (Cochran 1954; Roff and Bentzen 1989), and many infrequent MLHs were observed in the dataset. Therefore, a distribution of χ^2 values, if the null hypothesis of no differentiation was true, was generated using the Monte Carlo method as suggested by Roff and Bentzen (1989), and compared with the χ^2 value of the original dataset. The number of times the χ^2 value of the randomized datasets equaled or exceeded that of the original dataset allowed calculation of the probability of the null hypothesis being true. The method was implemented in the CHIRXC program using 1,000 simulations (Zaykin and Pudovkin 1993). Additionally, indices of genetic variation were calculated using methods outlined in Mullett et al. (2015). Briefly, haplotypic diversity was calculated using MULTILOCUS 1.3b (Agapow and Burt 2001), gene diversity using ARLEQUIN 3.5 (Excoffier and Lischer 2010), allelic richness and private allele richness using ADZE (Szpiech et al. 2008), and clonal fraction using the method of Zhan et al. (2003).

Results

Decay of *D. septosporum* propagules and median lethal times.

Viable *D. septosporum* propagules could still be detected in needles exposed for 32 weeks in the field (the maximum time of exposure), although only at very low levels. For needles placed in the litter layer, fewer than 4% of all needles assessed harbored viable *D. septosporum* after 32 weeks, while for suspended needles, 16% harbored viable propagules after the same time. However, in almost all cases this was limited to one or two viable *D. septosporum* propagules per needle (i.e., per acervulus).

The number of viable propagules declined rapidly with increasing time of exposure in both litter and suspended needle samples (Fig. 2). For the analysis all assessment times and 4-week exposure periods were combined, giving a mean decay rate of 0.42 for the number of viable propagules in acervuli for the litter needles and 0.61 for the suspended needles (Table 1). The two decay rates were significantly different ($F_{1, 482} = 30.8$, $P < 0.001$) with propagules from litter needles declining at a faster rate.

The 95% confidence interval for the litter needles' LT₅₀ was 20 to 28 days (i.e., 0.7 to 1.0 of a 4-week period), while the 95% confidence interval for the suspended needles' LT₅₀ was 34 to 48 days (i.e., 1.2 to 1.7 4-week periods). For the litter and suspended needles' LT₉₀, the 95% confidence interval was 63 to 90 days (i.e., 2.25 to 3.2 4-week periods), and 113 to 157 days (i.e., 4.05 to 5.6 4-week periods), respectively. This is equivalent to the number of viable *D. septosporum* propagules reducing 56 to 59% faster when infected needles were in the litter layer compared with suspended in the tree canopy.

Correlations with weather variables. The regression analysis between the parameter estimates for each 4-week assessment period (t) and the meteorological metrics revealed a significant negative effect of humidity ($P < 0.01$) on the viability of *D. septosporum* propagules in suspended needles. Lower humidity levels were therefore associated with higher propagule survival in the suspended

needles. However, none of the meteorological variables were found to have a significant effect on the viability of *D. septosporum* in the litter needles.

Microsatellite analysis. The AMOVA did not support any significant population differentiation (d.f. = 1, $P = 0.820$), and the analysis of the contingency table and its randomizations confirmed the null hypothesis of no difference between the two groups (general population group versus persistent group) ($\chi^2 = 60.3095$, d.f. = 63; P -value = 0.634 [95% confidence interval 0.604 to 0.664]). Therefore, both tests endorsed the lack of any distinctive population or MLHs persisting on needles. All groupings of the data had similar levels of haplotypic and gene diversity, as well as comparable clonal fractions (Table 2), further supporting the lack of a distinct population grouping of haplotypes able to persist in needles for extended periods.

Discussion

Abscised needles are clearly not the ideal habitat for *D. septosporum* over a period of several months. Survival declined rapidly once needles were shed from the tree. Although a higher number of infective propagules were recovered from needles suspended above the forest floor compared with needles in the litter, for both the number of viable propagules was halved one to two months after abscission and dropped to 10% within six months. Thus, shed needles contribute only slightly to the overall inoculum load in the stand with still attached needles contributing the majority of inoculum. Furthermore, isolates from the ‘persistent population’ were not differentiated genetically from the general population of isolates.

Many aspects, ranging from environmental conditions to microarthropod communities, differ in the tree canopy when compared with the forest floor. The canopy is subject to more extreme climatic conditions with greater exposure to wind, sunlight, and more frequent and extreme wet/dry cycles (Lindo and Winchester 2006). Relative humidity is also lower in the canopy and decreases with increasing canopy height (Cardelús and Chazdon 2005; Unterseher and Tal 2006). The reverse is true of temperature during the day, with higher temperatures occurring in the canopy (Bohlman et al. 1995), and microarthropods such as mites and collembolans are more numerous on the forest floor than in the canopy (Lindo and Winchester 2006, 2007). These conditions all affect the rate of microbial colonization and activity.

Microarthropods are known to eat fungi, in some cases selectively feeding on particular fungal species (Mitchell and Parkinson 1976; Parkinson et al. 1979) and such selective feeding can alter the composition of the microbial community (Mitchell and Parkinson 1976). In this study, microarthropods were not observed systematically, yet casual observations indicated more microarthropods in the older litter needles with many *D. septosporum* fruit bodies ‘missing,’ as evidenced by blackened depressions with rough edges within the red bands when observed under a dissecting microscope. Presumably the fruit bodies were being consumed by microarthropods and it may be that *D. septosporum* is a particularly palatable, or just a highly concentrated, food source. Some arthropods are good dispersers of propagules (both fungal and bacterial) both in their feces and on their bodies, potentially leading to higher rates of microbial colonization where they are numerous (Coulis et al. 2013; Lilleskov and Bruns 2005). Furthermore, Kaneko et al. (1998) and Setälä et al. (1988) reported higher levels of microbial activity from litter with increased levels of microarthropods.

The different climatic conditions in the canopy have a strong effect on the microbial community. While solar radiation, particularly UV radiation, is known to kill fungal spores, *D. septosporum* conidia are likely to be well protected inside an acervulus, as has been shown for other fungi (Rotem and Aust 1991), yet any competing colonizing fungal spores on the surface would be slightly more exposed to radiation, potentially slowing their colonization of the needles. However, the higher solar radiation and greater exposure to wind in the canopy also leads to increased drying. Many studies have reported that moisture is a key factor influencing microbial activity in plant litter (Butenschoten et al. 2011; Lee et al. 2014; Riutta et al. 2012; Schimel et al. 1999; Witkamp 1966; Zelles et al. 1991). Higher microbial activity is known to result in faster breakdown

Table 1. Parameter estimates from the generalized linear model of *D. septosporum* propagule decay

Parameter	Estimate	Standard error	t(482)	t. pr.	Antilog of estimate
Constant	5.5219	0.0849	65.07	<0.001	250.1
Litter	−0.8695	0.0778	−11.18	<0.001	0.4191
Suspended	−0.4877	0.0405	−12.05	<0.001	0.6140

Fig. 2. Mean number of viable *D. septosporum* propagules recovered from litter needles and suspended needles in 4-week exposure periods in the field. Fitted lines are estimated values from the generalized linear model of mean propagule numbers from pooled data ($n = 26$ for each position).

and decomposition of the shed plant material (Witkamp 1963, 1966). Baldrian et al. (2010) reported that microbial biomass increases with litter moisture and that fungi were more affected by moisture than were bacteria. Additionally, it has been suggested that the stress effect of repeatedly wetting and drying the canopy litter has a negative effect on microbial activity (Bohlmann et al. 1995; Schimel et al. 1999). The combined effect of these factors generally results in lower microbial activity and thus reduced rates of decomposition in the canopy litter than in the forest floor litter (Cardelús 2010; Li et al. 2014). The lower microbial activity means less competition against *D. septosporum* for needle resources, allowing the fungus to persist for longer in the canopy than in the forest floor litter. The significant negative effect of relative humidity on persistence of *D. septosporum* in the canopy emphasizes the importance of moisture on microbial activity. In contrast, the forest floor litter layer rarely dried out, thereby sustaining high levels of microbial activity and rapid degradation of *D. septosporum*. Thus the decay was so rapid in the continuously damp forest floor litter needles that relative humidity played no significant role here. High relative humidity and moisture may also promote *D. septosporum* conidial release, allowing loss of propagules to the wider environment.

D. septosporum has been considered a weak saprophyte, easily outcompeted on dead needles by saprophytic fungi (Gadgil 1970; Gibson et al. 1964; Shishkina and Tsanova 1966). Shishkina and Tsanova (1966) noted that *D. septosporum* was quickly overrun by *Lophodermium* on fallen *P. brutia* var. *pityusa* needles in Georgia, while Gibson et al. (1964) reported the rapid decline in the proportion of *D. septosporum* conidia in litter needles when compared with those of saprophytes, in particular *Pestalotiopsis* spp. The results of this study are in agreement with these authors. Schwelm et al. (2009) demonstrated that dothistromin, the toxin produced by *D. septosporum*, is not needed for infection of pine needles by the fungus but it did confer a competitive advantage against other pine needle fungi in vitro. This suggests that even with live host material, the fungus is an inherently weak competitor and dothistromin may be needed to help exclude other fungi. It may not be surprising, therefore, that on dead and dying host material, the fungus is quickly outcompeted by saprophytic organisms better adapted to utilizing dead host material. Needles falling onto the forest floor will be in direct contact with previously shed needles that are already colonized by saprophytes, thereby facilitating the colonization of newly arrived needles by saprophytes. Conversely, in the canopy, needles are often not in direct contact with many other older needles, slowing rate of colonization by saprophytes.

Regardless of the vertical position of the abscised needles in the forest stand, persistence of the fungus is limited. Some fungal propagules are thought to survive over 25 years, although evidence is often anecdotal, e.g., *Urocystis cepulae* and *Synchytrium endobioticum* (Putnam and Sindermann 1994; Thaxter 1890). Other fungal propagules can enter a dormancy phase, much like plant seeds, enabling them to survive periods of many years (Bruns et al. 2009; Nara 2009).

A detailed study of *D. septosporum* persistence in fallen needles was also undertaken by Gadgil (1970). He assessed needles in the litter and canopy of both thinned and unthinned *P. radiata* stands in New Zealand and also found a rapid decrease in inoculum, with slightly higher persistence in canopy needles compared with needles on the forest floor. Gadgil (1970) found no viable conidia after 6 months, with the maximal survival time being between 4 and

6 months (i.e., viable at 4 months but not at 6 months, the subsequent assessment period). This result is in broad agreement with the current study although the pathogen was still found to be viable at 6 months in some cases in our study. The difference may be due to a different host species (*P. radiata*), location and climate (New Zealand), and/or assessment method. Gadgil (1970) damp-chamber incubated needles recovered from the field and then picked off any exuded conidia with a loop and streaked them onto agar plates. This method allowed assessment of conidia only, but also possibly omitted conidia that might have been released with further incubation. The method used in the current study allowed recovery of all infective propagules in a fruit body regardless of whether these were conidia or mycelial fragments. Both are known to cause infection; in fact, germinating mycelial fragments have been reported to penetrate the host cuticle directly while germinating conidia must enter the needle via stomata (Gadgil 1967). Although potentially more infective, mycelial fragments have not been shown to be dispersed along with conidia; therefore, the current study may slightly overestimate the quantity of easily dispersible inoculum. In Japan, Ito et al. (1975) reported viable *D. septosporum* conidia from abscised *P. thunbergii* and *P. densiflora* needles after 6 months of outdoor winter storage, whereas Shishkina and Tsanova (1966) reported conidia viability as between 3 and 5 months from *P. brutia* var. *pityusa* needles stored as herbarium specimens. Gibson et al. (1964) also recovered viable conidia from *P. radiata* foliage that was kept in a dry incubator at 30°C for 5 months. They also found live conidia on foliage kept at room temperature for 11 months, the maximum reported in the literature (Gibson et al. 1964).

Investigation using microsatellite markers did not reveal any particular MLHs that were associated with longer persistence in abscised needles. This is consistent with the view that *D. septosporum* has limited survival on abscised needles, so any selection pressures are likely to be acting much earlier in the pathogen's life cycle, before needles are shed. It would seem that persistence of the fungus is governed primarily by the environmental conditions the abscised needles are subjected to with particular MLHs being of little consequence in this respect. It is likely that needles harboring the fungus many months after abscission were subjected to lower moisture levels, slower colonization by saprophytes, lower levels of microbial activity and degradation, and that the *D. septosporum* fruit body escaped microarthropod predation.

In conclusion, shed needles infected with *D. septosporum* contribute only slightly, and for a limited time, to the overall pathogen inoculum levels in a stand. Replanting a felled infected area with a susceptible pine species carries risks, but where this is to be undertaken, any inoculum in the litter will be reduced by 90% after 3 months. If replanting is undertaken after 6 or 12 months, inoculum from the site is likely to be negligible, although not absent. Adjacent infected stands are likely to pose a much greater risk. Although shed needles caught in the canopy of a standing forest can be a threat for slightly longer than those reaching the forest floor, inoculum levels were also found to decrease rapidly in this environment, halving in under 2 months. However, to minimize the number of needles caught in the canopy and thus reduce the inoculum they will subsequently contribute, it is recommended that thinning, pruning, and brashing operations are carried out prior to the main needle shedding periods of late summer and autumn.

Table 2. Summary of isolate numbers and genetic variability of *D. septosporum* used in this study^a

Group	Number of isolates	Number of different multilocus haplotypes	Haplotypic diversity	Clonal fraction	Gene Diversity	A _R ± standard error	PA _R ± standard error
All isolates	241	64	0.942	0.734	0.542	n.a.	n.a.
Fresh needle isolates	179	54	0.939	0.698	0.546	4.472 ± 0.606	0.571 ± 0.007
Persistent isolates	62	27	0.950	0.565	0.512	4.364 ± 0.560	0.463 ± 0.124

^a Haplotypic diversity calculated using the non-clone-corrected dataset. Gene diversity, allelic richness (A_R), and private allele richness (PA_R) calculated using the clone-corrected dataset. A_R and PA_R were standardized to the smallest clone-corrected sample size of 27.

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