



Tree mortality caused by Diplodia shoot blight on *Pinus sylvestris* and other mediterranean pines

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ABSTRACT

Diplodia shoot blight is an emergent forest disease in Europe caused by *Diplodia sapinea*. The short-term impacts of the pathogen on tree physiology are well known, but its capacity to cause mortality has been poorly documented.

We compared the survival of four pine species affected by Diplodia shoot blight following a hailstorm: *Pinus sylvestris*, *P. nigra*, *P. pinea* and *P. halepensis*. In the case of *P. sylvestris*, survival in the hail-affected sites was compared with survival in other sites affected by Diplodia shoot blight with no hailstorm records. Mortality and crown condition were recorded over two years. Dendrochronological analyses were conducted to assess growth responses to drought and to test the influence of radial growth before the outbreak of Diplodia shoot blight on survival. The endophytic community, as well as the abundance of *D. sapinea*, was quantified by metabarcoding and qPCR respectively, and were correlated with crown damage.

Crown damage was the best predictor of mortality across species. *Pinus sylvestris* experienced a much higher rate of mortality than *P. nigra*, *P. pinea* and *P. halepensis*. Two years after the outbreak, *P. halepensis* was the only species that could recover crown condition. Mortality was found to be unrelated to radial growth prior to the outbreak. Drought responses did not correlate with mortality differences across pine species or sites. In the case of *P. sylvestris*, mortality was initially higher amongst diseased trees in areas affected by hailstorms than in sites not affected by hailstorms, however it tended to equalise after two years. Amongst *P. sylvestris* trees, crown damage correlated with a higher abundance of the pathogen. Signs of competition amongst endophytes were observed between non-defoliated and defoliated Scots pine trees following hailstorms.

Our study shows that *D. sapinea* can cause a significant mortality to *P. sylvestris*. The legacy effects of crown damage can last for at least two years after an outbreak. During this time, crown damage is a good predictor of survival, and can help managers decide which trees remove after an outbreak.

1. Introduction

Diplodia shoot blight is a widespread forest disease in Europe (Desprez-Loustau et al., 2006). It is caused by the ascomycete *Diplodia sapinea* (Fr.) Fuckel (Syn: *Sphaeropsis sapinea* (Fr.) Dyko & B. Sutton). *Diplodia sapinea* causes shoot death on major forest species such as *P. sylvestris* L., *P. nigra* Arn. and *P. radiata* D. Don (Ortiz de Urbina et al., 2017), leading to decreased tree growth and reduced timber quality (Desprez-Loustau et al., 2006). *Diplodia sapinea* is a good example of a forest pathogen that has recently expanded its range to northern

latitudes (Brodde et al., 2019). In 2016 and 2018, unprecedented outbreaks affecting northern Scots pine (*Pinus sylvestris*) forests were reported in Sweden (Brodde et al., 2019), following earlier observation of symptomatic trees in Sweden and Estonia in 2012 (Adamson et al., 2015, Oliva et al., 2013). Outbreaks in Northern Europe occurred after a series of first-time reports of the pathogen in pine cones in Estonia in 2007 (Hanso & Drenkhan, 2009), in Sweden in 2012 (Oliva et al., 2013) and in Finland in 2015 (Müller et al., 2019). To date the pathogen has been observed as far north as 68° N in Finland i.e. above the polar circle (Terhonen et al., 2021). The range expansion of Diplodia shoot blight in

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the last decades has been linked to warmer winter conditions (Fabre et al., 2011). It is expected a shift in terms of pathogenicity and range expansion into higher latitudes will continue under a warming climate scenario. On a continental scale, the associated risk to Scots pine is of greatest concern as it is the second-most abundant species in Eurasian boreal forests and a major tree species in the mountain forests of Central and Southern Europe (Houston Durrant et al., 2016), where outbreaks are recurrently observed (Luchi et al., 2014, Zlatković et al., 2018).

Predicting the effects of the current range expansion has been impaired by our limited knowledge on the long-term impacts of the disease. *Diplodia sapinea* is considered a facultative pathogen, as it requires stress in the host to cause damage (Slippers & Wingfield, 2007). *Diplodia sapinea* is a common endophyte and can be usually found in asymptomatic tissues (Stanosz et al., 1997). However, both water deficit (Stanosz et al., 2001, Chhin & O'Brien, 2015) and mechanical damage, such as that caused by hailstorms (Zwolinski et al., 1990a), can activate the pathogen and cause disease. *Diplodia sapinea* is considered a shoot pathogen, where it causes necrosis of the cambium. Necrosis can progress from the current year's shoot into the previous year's growth, causing an almost complete defoliation of the crown (Oliva et al., 2021). Conidia (asexual spores) develop on pycnidia (fruiting bodies) protruding from infected needles and shoots. The pathogen can also infect the cones, which can serve as additional sources of inoculum (Munck et al., 2009). Shoot damage causes crown malformations and latewood growth losses (Brodde et al., 2019). *Diplodia* shoot blight can also cause tree mortality. However, aside from work on *P. radiata* plantations in South Africa (Zwolinski et al., 1990a, Zwolinski et al., 1990b), mortality processes caused by *Diplodia* shoot blight in other areas and affecting other pine species have been poorly documented.

The management of a *Diplodia* shoot blight outbreak is a challenging task for foresters. In a matter of weeks, the needles in the crown of seemingly healthy trees can dry out as result of the death of the cambium in the shoots. In forest regions prone to forest fires, the presence of dry needles in the crown may be perceived as an increased risk for crown fire. Managers may react to *Diplodia* outbreaks by performing sanitation cuttings (Brodde et al., 2019, Zwolinski et al., 1990a), despite some trees still having the potential to restore their crown and survive (Zwolinski et al., 1990b). An accurate prediction of which trees will die is needed. Crown damage could be used as predictor of mortality. A connection between defoliation and survival was established for *P. radiata* at plot level, i.e. plots with defoliated trees experienced higher mortality rates than unaffected plots (Zwolinski et al., 1990b). However, information about the connection between crown damage and mortality at a tree level is still missing.

In general, predicting tree mortality is problematic since the impact of different stressing events on the health of trees is difficult to quantify, and often have long-lasting interacting effects (Manion, 2003). This issue was well-illustrated in the case of *Gremmeniella abietina*, which is another shoot pathogen present in Europe. The survival of pines following such an outbreak was largely affected by the amount of crown damage in a previous outbreak that had happened years before (Oliva et al., 2016). Legacy effects are difficult to measure because annual forest health inventories at a stand level are lacking. Legacy effects may be particularly important in the case of facultative pathogens, such as *D. sapinea*, which require stress in the host to cause disease. However, information on the role of tree vigour and previous stressing events on the likelihood of surviving a *D. sapinea* attack is scarce. In a previous study, we showed that different pine species harboured different biomass of the pathogen in asymptomatic tissues (Oliva et al., 2021). Pine species also vary on their response to drought (Camarero et al., 2015). Therefore, the capacity of different tree species and populations to cope with environmental stressing events, such as drought, as well as variation in interspecific resistance to the pathogen render to different mortality patterns across pine species (Munck et al., 2009).

As a facultative pathogen, competition between *D. sapinea* and other endophytic species can also influence the occurrence of disease (Oliva

et al., 2021). Competition for N-rich metabolites such as proline (Sherwood et al., 2015) or antagonistic interactions (Blumenstein et al., 2021) could play an important role in the interplay amongst endophytes and the pathogen. Despite trees producing N-rich metabolites as a stress response to both drought and hail, these and other eventual stressing events can vary considerably in terms of intensity and duration, and therefore affect the shoot-inhabiting fungal community. In order to advance in our understanding of the disease, we need more comparative studies of the fungal community associated with *Diplodia* shoot blight triggered by different stressors.

In this study, we investigated the survival of *P. sylvestris* damaged by *D. sapinea* in the vicinity of the Pyrenees (NE Spain). Mortality was studied in some sites in which symptoms of disease appeared after a hailstorm and in sites where symptoms were unrelated to hail damage. The hail event affected mixed pine stands, and therefore we could compare the survival of *P. sylvestris* with that of *P. nigra*, *P. pinea* and *P. halepensis* growing in the same plots. We hypothesised that survival would depend (i) on the amount of crown affected by the pathogen and (ii) on the tree vigour. The crown condition and tree survival were followed for two years after the outbreak. The dendrochronology pattern of trees before and after the hail-storm was reconstructed. The cumulated radial growth in the 10 years before the outbreak was considered as a proxy of tree vigour. We assumed that any precondition with a potential to influence the capacity of trees to survive a *D. sapinea* attack would be reflected in the radial growth. In order to understand mortality differences across species and across sites, the sensitivity to drought was studied by correlating radial growth with the Standardised Precipitation and Evapotranspiration Index (SPEI) from Vicente-Serrano et al. (2010) which measures drought severity. The responses of *D. sapinea* and the accompanying shoot mycobiome in hail and no-hail areas were compared by quantitative qPCR and metabarcoding of the ITS 2 region. Finally, the stress response of the trees was assessed by measuring the proline content in the same tissue from where the fungal community was studied.

2. Material and methods

2.1. Study sites and field measurements

The study area is located in the Southern Pyrenees in NE Spain. During the summer of 2018, eight mixed pine stands were affected by summer hailstorms in the vicinity of the village of Oristà (41° 55' 58.4" N, 2° 3' 35.7" E, 468 m a.s.l.) (Oliva et al., 2021). The damaged areas were covered by mixed pine forests of *P. sylvestris*, *P. nigra*, *P. pinea* and *P. halepensis*. Two to three weeks after the hailstorm, trees showed severe symptoms of *Diplodia* shoot blight. Six months after the outbreak, thirty trees per species were marked. Trees were selected as having high (>70%), medium (30–70%) and low (<30%) crown defoliation caused by *D. sapinea* (Table 1). Defoliation was visually assessed by the percentage of necrotic shoots from the current year. In general, more trees in the most severe defoliation classes were marked, as it would facilitate the study of mortality among severely diseased trees. The marked trees were distributed as evenly as possible between locations. After marking, crown condition was monitored 12, 18 and 28 months after the outbreak. At every crown evaluation, trees were assessed as alive or dead. Trees were considered dead when no green needles could be observed in the crown, and the cambium was dead underneath the bark. Three *P. sylvestris* stands were also selected in an area located sixty kilometres north from the hail-affected area near the St. Quirze de Besora town (42° 06' 12" N, 2° 13' 13" E), where trees showed severe symptoms of *Diplodia* shoot blight. Despite the conspicuous symptoms of disease, there were no records of recent hailstorms affecting these stands, and therefore disease was regarded to be triggered by other causes than hail damage. These stands were located on south exposed slopes with thin soils. Trees in these stands were marked and monitored in the same way as trees in the area that had been affected by hail.

Table 1

Predicted probabilities of mortality after one and two years for pine trees affected by *D. sapinea* depending on the level of defoliation caused by the pathogen in sites affected by hail or none affected by hail in the Pyrenees. Values of radial growth (BAI, basal area increment) before the outbreak, and the overall number of trees studied is shown (N). Average age and average diameter of the studied trees is also shown. High, medium and low defoliation classes include defoliation values of > 70%, 70–30% and < 30%, respectively.

Sites	Tree species	Defoliation class	N	Age 1.3 m (yr)	Diameter (cm)	10 year-BAI prior outbreak (cm ²)	Mortality	
							1 yr	2 yr
No-hail	<i>Pinus sylvestris</i>	High	22	43	25	6.3	0.09 (0.02–0.22)	0.49 (0.26–0.69)
		Medium	13	36	26	9.3	0.01 (0–0.12)	0.26 (0.07–0.5)
		Low	17	42	23	8.0	0 (0–1)	0 (0–1)
Hail	<i>Pinus sylvestris</i>	High	15	34	21	6.4	0.3 (0.16–0.5)	0.83 (0.64–0.93)
		Medium	10	39	20	6.0	0.01 (0–0.06)	0.1 (0.02–0.34)
		Low	7	42	21	9.5	0.01 (0–0.07)	0.08 (0.01–0.4)
	<i>Pinus nigra</i>	High	28	50	23	5.9	0.02 (0.01–0.07)	0.18 (0.07–0.37)
		Medium	15	53	27	8.3	0 (0–0.01)	0.01 (0–0.04)
		Low	10	51	25	8.5	0 (0–0.01)	0 (0–0.04)
	<i>Pinus pinea</i>	High	30	65	32	5.7	0.01 (0–0.06)	0.1 (0.02–0.34)
		Medium	20	66	32	7.6	0 (0–0)	0 (0–0.03)
		Low	16	63	37	12.2	0 (0–0)	0 (0–0.03)
<i>Pinus halepensis</i>	High	6	34	20	8.1	0.03 (0–0.16)	0.25 (0.06–0.64)	
	Medium	10	39	20	7.9	0 (0–0.01)	0.01 (0–0.07)	
	Low	15	42	26	9.8	0 (0–0.01)	0.01 (0–0.07)	

Besides Diplodia shoot blight no signs or symptoms of other pests and diseases were observed in any of the studied sites. In total, 234 trees were monitored for two years (101, 68 and 65 trees in the ‘high’, ‘medium’ and ‘low’ crown defoliation classes respectively).

2.2. Pathogen and proline quantification

In each of the no-hail stands, shoots of three non-defoliated trees and three defoliated trees were collected. Tree shoots were processed as previously described in Oliva et al. (2021), and corresponded to the same tissue/year so they could be compared. Sampling targeted the asymptomatic section of the shoot corresponding to 2017 i.e. the year before the hailstorm as in Oliva et al. (2021). Once in the laboratory, needles were removed and the bark was scraped to reduce contamination from the bark. Shoot samples were divided for proline analysis and qPCR analysis. For the proline analysis, the sample was immediately placed at –20 °C. Proline quantification was later conducted by the Serveis Científicotècnics (SCT) at the University of Lleida (Spain) by LC-MS using a Xevo TQ-S Triple Quadrupole detector, and quantified with Multiple Reaction Monitoring (MRM) with five points of external calibration. The rest of the sample was surface sterilised, lyophilised at –54 °C and ground with liquid nitrogen. DNA was extracted following Oliva et al. (2017) and using the commercial kit NucleoSpin® Plant II protocol by Macherey-Nagel (2018). The amount of *D. sapinea* in the studied tissues was quantified by the Taqman protocol from Luchi et al. (2005). Standard curves were constructed using serial dilutions from 10⁶ to 10⁰ using PCR product as a template. Proline content and copy numbers were used as a variable to compare non-defoliated and defoliated *P. sylvestris* trees growing in hail and no-hail sites.

2.3. Dendrochronology analyses

In 2020, two years after the outbreak, two stem cores were taken at 1.3 m using Pressler increment borers (Haglöf, Sweden) to quantify changes in radial growth. A total of 204 trees were sampled from the four pine species. Some of the original 234 trees had died in the first year of monitoring, and extracting cores was no longer possible because they were in an advanced stage of decay by 2020. Other trees had been removed. For each tree, two wood cores were extracted to the pith and, where possible, perpendicular to the slope to avoid stem eccentricity caused by a wood reaction. Each core was then prepared according to standard dendrochronological procedures. Cores were air dried, glued

onto wooden supports and sanded until the tree rings were clearly visible. Then, these samples were visually cross-dated (Fritts, 1976). The ring widths of cross-dated samples were measured to a 0.01 resolution using scanned images (resolution 2400 dpi) and the CDendro software (Larsson & Larsson, 2018). The cross-dating was checked using the COFECHA software (Holmes, 1983), which calculated moving correlations between the individual series and the mean site series of each species. Tree-ring widths were converted into basal area increment (BAI), assuming a circular shape for the stems since BAI reflects growth changes better than tree-ring width. The BAI was calculated by subtracting cumulative, annual basal area values. Then, the mean BAI series for each species was used to group them according to: species, damage type (hail vs. no-hail) or tree status (dead vs. surviving tree). To detrend and standardise BAI series, we used the ARSTAN program (Cook & Krusic, 2008), which allows eliminating the influence of changes in tree size and age or disturbances on radial growth. We fitted cubic smooth splines with a length of 67% of the series. Then, the average was calculated from the individual BAI series for species within each site by using bi-weight robust averages. Finally, tree age at 1.3 m was estimated by counting the maximum number of rings in the two cores extracted per tree.

To assess drought-growth relationships we correlated species' series of BAI indices and monthly values of the SPEI, which was used to characterise drought severity at temporal resolutions from 1 to 24 months (Vicente-Serrano et al. 2010). SPEI values were obtained at 0.5° spatial resolution for the two study areas (Oristà and St. Quirze de Besora) from the global SPEI database available at <https://spei.csic.es/database.html>. Then, we calculated Pearson correlations between BAI indices and SPEI values for the period 1970–2019. We focused on the temporal scale and month showing the maximum correlation between growth and SPEI. The growth responsiveness to drought was assessed as the slope of SPEI-growth linear regression for that specific resolution and month.

2.4. Metabarcoding and bioinformatics analysis

The fungal community inhabiting shoots in the areas not subjected to hail was analysed following Ihrmark et al. (2012). The methods were equivalent to those used in Oliva et al. (2021) to allow for comparisons with shoot samples collected from hail-affected areas. Briefly, the ITS2 region was amplified by ITS4- and ITS7- tagged primers. PCRs were run in triplicates for 30–32 cycles at an annealing temperature of 58 °C. The

three triplicates of each sample were pooled and purified with magnetic beads following the NucleoMag® NGS commercial protocol. The DNA concentration of each sample was assessed with Qubit™, so the same quantity of PCR product from each sample could be included in the library. Libraries were sequenced with Illumina-MiSeq in Stab Vida (Lisbon, Portugal). Samples were quality filtered, clustered and demultiplexed using the bioinformatics SCATA pipeline (scata.mykopat.slu.se) (Lindahl et al., 2013). Sequences smaller than 150 bp were discarded. Sequences were clustered in operation taxonomic units (OTUs) with a threshold of 98.5%. Only OTUs appearing in more than three samples, and having at least 10 reads were retained. OTUs belonging to plants or non-fungal organisms were excluded based on a Protax (Somervuo et al., 2016, Abarenkov et al., 2018) classification with 0.5 as threshold value (plausible classification). Non-fungal OTUs, according to Protax, were then run through MEGAN (Huson et al., 2007), which uses a phylogenetic approach to ensure that no fungal OTU is excluded. A final curation with the ‘lulu’ package in R (Frøslev et al., 2017) was

used to eliminate duplicate OTUs originating from sequencing errors.

2.5. Statistical analyses

The probability of mortality following the outbreak in the hail-affected area and in the no-hail area was adjusted with a parametric survival function in JMP (JMP® Pro 15.2.0, SAS Institute Inc.). Censoring accounted for trees that did not die or that were removed during the sampling. In the parametric model, the effect of tree species, initial defoliation and mean BAI in the 10 years prior the outbreak, and their interactions were tested. The best distribution function was selected based on the Bayesian Information Criteria (BIC) value. Tree species was a significant factor, so differences between species were tested using a Cox Proportional Hazard model. The temporal development of mortality risk was adjusted for hail and for no-hail by selecting the best distribution according to the BIC value. Comparisons of *D. sapinea* abundance and proline concentration were carried out with a

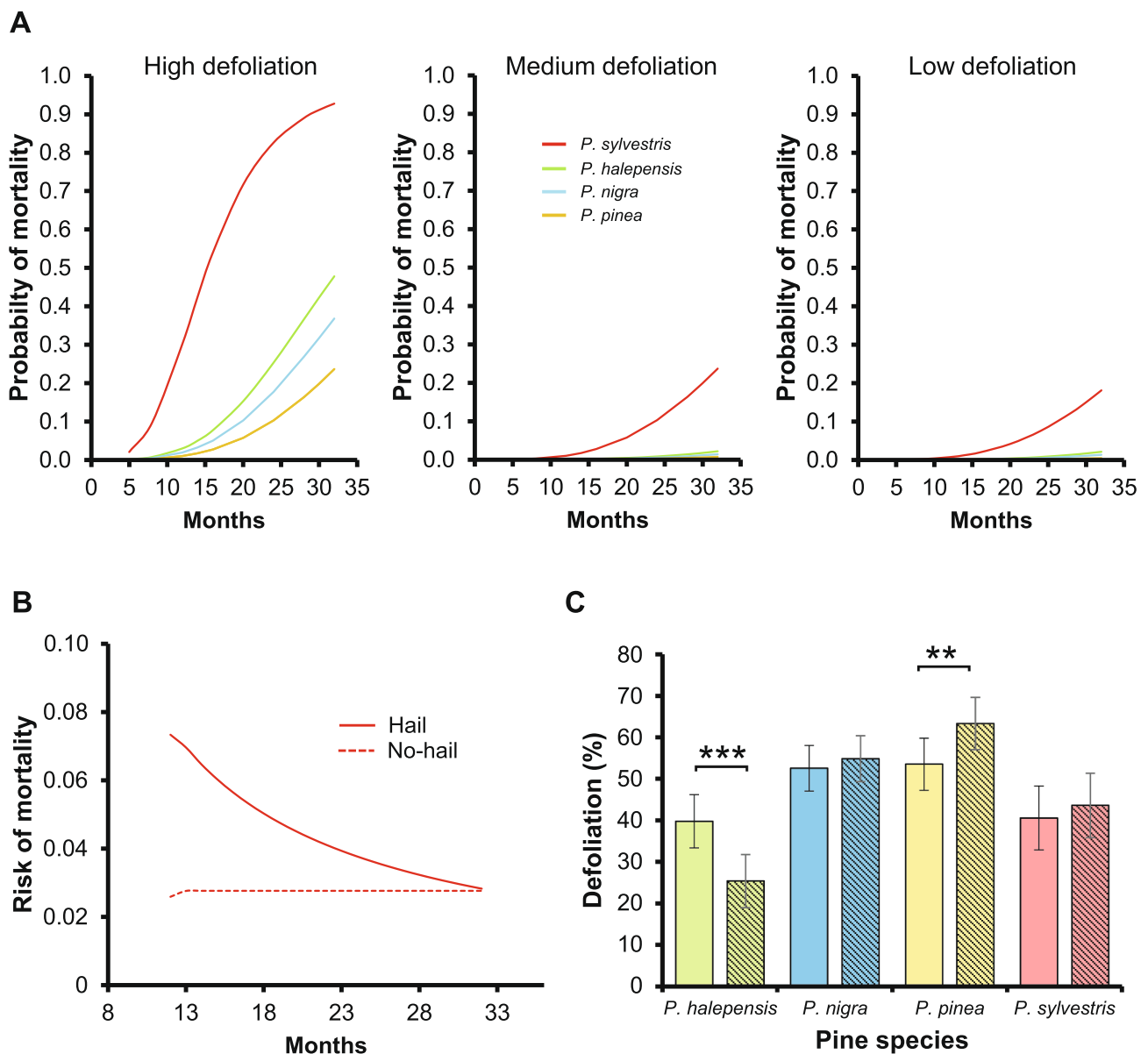


Fig. 1. (a) Two-year probability of mortality and crown condition based on defoliation of different pine species affected by *D. sapinea* following a hail event in the Pyrenees. The same amount of high, medium and low defoliated trees were included at the start of the experiment. (b) Risk of mortality for *P. sylvestris* following a hail event compared to the risk of mortality in an area not subjected to hailstorms (c) Mean defoliation of surviving trees before (empty fill) and after disease outbreak (pattern fill). “***” and “****” indicate significant differences at $p < 0.01$ and $p < 0.0001$, respectively, following a Fischer-protected least significant difference test.

generalised linear model using a ‘quasipoisson’ distribution and logarithm as link function. The effects of the different factors were assessed by the likelihood ratio test.

Alpha-, and beta-diversity analyses were run in a rarefied OTU database at 250 reads per sample. Asymptomatic and defoliated trees in hail- and no-hail affected areas were compared in terms of OTU richness, and both Shannon and Simpson diversity indices. The same comparison was made in terms of community structure via a PERMANOVA test with the *adonis2* function in the package ‘vegan’ (Oksanen, 2011). Composition was visualised with a principal coordinate analysis (PCoA) with Bray-Curtis distances. Segregation analyses were run with the *cooc_null_model* function of the ‘EcoSimR’ package using 10^5 simulations (Gotelli & Ellison, 2013). C-score simulations were calculated on binary presence/absence data. Only OTUs present in more than two samples were included. Observed C-score values higher than the 95% upper quantile of the simulated values showed a higher segregation than expected at random, and were interpreted as indicative of competition. Indicator species analysis was run on OTUs with a relative abundance higher than 0.1% to find associations with non-defoliated and defoliated *P. sylvestris* trees growing in hail and no-hail areas.

3. Results

In 2018, a series of hailstorms triggered a severe *Diplodia* shoot blight outbreak which affected several mixed pine stands in Southern Pyrenees. Two years after the outbreak, mortality mainly affected *P. sylvestris* (Fig. 1a). *Pinus sylvestris* was 5.1 times more likely to die than *P. nigra*, 8.8 times more likely to die than *P. halepensis*, and 11 times more likely to die than *P. pinea*, assuming proportional hazard ratios across species. Mortality differed between tree species ($p < 0.0001$) and depended on the amount of crown that was affected by *D. sapinea* ($p < 0.0001$). In the case of highly defoliated *P. sylvestris* trees, the predicted probability of mortality in the first year was 30.2% and increased to 82.6% in a two-year time frame. For *P. nigra*, another well-known host of *D. sapinea*, the probability was 1.9% and 17.7%, respectively. In the case of *P. nigra*, a higher growth rate in a 10 year-period prior the outbreak correlated with higher chances of dying. No effects were observed for the other tree species.

For *P. sylvestris*, the overall mortality was slightly higher in the areas affected by hailstorms than in the sites not affected by hail (40% vs 30% mortality, in a two-year time frame), however the difference was not significant ($p = 0.14$). In both hail and no-hail sites, mortality of *P. sylvestris* followed a sharp decrease in terms of radial increment, which in the case of the hail-affected sites, it appeared unrelated to the cumulated growth before the outbreak (Fig. 1b, 2b). The temporal evolution of mortality risk following a *D. sapinea* outbreak was different between trees subjected to hailstorms compared to those that were not damaged by hail. In the case of the no-hail sites, the best fit was obtained with a gamma-generalised function giving a constant mortality risk over time. In contrast, the best fit for the risk of mortality in the sites affected by hailstorms followed a log-gamma generalised function, implying a decrease of the mortality risk with time (Fig. 1b). After 24 months, the risk of mortality tended to equalise across hail and no-hail sites.

The development of crown condition in surviving trees differed

amongst pine species ($p < 0.0001$). After two years, the crown condition of surviving *P. pinea* worsened ($p = 0.004$), while the crown condition of *P. halepensis* improved ($p < 0.0001$) (Fig. 1c). No significant changes were observed for *P. nigra* and *P. sylvestris*. The results concerning *P. sylvestris* should be interpreted with caution as most of the highly-defoliated trees died during the study.

Drought responses did not explain mortality differences across species or sites. In the hail-affected sites, growth responsiveness to drought was similar across species (Table 2, Fig. S1) although mortality affected mainly *P. sylvestris*. Even showing a similar mortality rate, *P. sylvestris* growth responsiveness to drought was stronger in the hail-sites than in the no-hail sites (Table 2).

In the years following the outbreak, some trees had arrested radial growth and no longer produced a tree ring in 2018, 2019 or 2020. Trees classified as dead based on crown condition represented the 55% of trees that arrested radial growth. By reconstructing their dendrochronology, we found that arrested radial growth was more likely to occur on trees that had suffered higher crown damage ($p < 0.0001$). Arresting radial growth was in general not correlated to cumulated growth prior to the outbreak ($p = 0.30$) (Fig. 2a, Table 1). Only in the case of *P. pinea*, trees with arrested radial growth had cumulated less growth in the 10 years before the outbreak than those that did not arrest radial growth ($p = 0.015$). Among trees that were considered dead according to their crown condition, 5% had still produced a ring in 2019 and 2020.

In the case of *P. sylvestris*, trees with severe crown damage had a higher abundance of *D. sapinea* than non-defoliated trees (Fig. 3a). Proline content was unrelated to crown damage. However, trees in the area not affected by hail showed a higher proline content than in the area subjected to hail damage (Fig. 3b).

The endophytic composition (beta-diversity) following a *D. sapinea* outbreak was different between the hail and no-hail sites ($p = 0.0002$). No beta-diversity differences were observed between non-defoliated and defoliated trees ($p = 0.48$) (Fig. 4a). However, significant differences in terms of alpha diversity were observed between defoliated and non-defoliated trees in the hail-affected area (Fig. 4b). In the area not affected by hailstorms, defoliated trees tended to have a more even community than asymptomatic trees. However, the opposite trend was found in the hail-affected area, where defoliated trees tended to have a significantly less even community than asymptomatic trees ($p = 0.018$) (Fig. 4b). Species richness, inverse Simpson and Shannon indices were lower in the hail-affected area than in the area not affected by hail ($p < 0.05$). Species co-occurrence patterns also differed between hail-affected sites and sites not affected by hailstorms. While endophyte communities appeared more segregated than expected by chance in hail-affected areas, no signs of segregation could be identified in the no-hail sites (Fig. 4c). The distribution of the ten-most abundant endophytes was similar in hail and no-hail affected areas (Table S1). Within the no-hail area, indicator species analysis identified four OTUs typical for asymptomatic trees and nine for defoliated trees (Table S2). From those, all but one OTU belonging to an unidentified fungal cluster named “Ascomycota_8” had relative abundances lower than 1%. No indicator species associated with crown damage in the hail-affected area were identified.

Table 2

Summary of correlation analyses between site and species series of growth indices and the SPEI drought index. Correlations were calculated for the common period 1970–2019.

Sites	Species	Maximum Pearson correlation of growth indices with SPEI			Slope of SPEI-growth linear regression
		Correlation	SPEI resolution (months)	Month	
No-hail	<i>P. sylvestris</i>	0.35	5	July	0.08
Hail	<i>P. sylvestris</i>	0.57	7	August	0.12
	<i>P. nigra</i>	0.50	11	July	0.11
	<i>P. pinea</i>	0.57	9	July	0.12
	<i>P. halepensis</i>	0.51	6	July	0.13

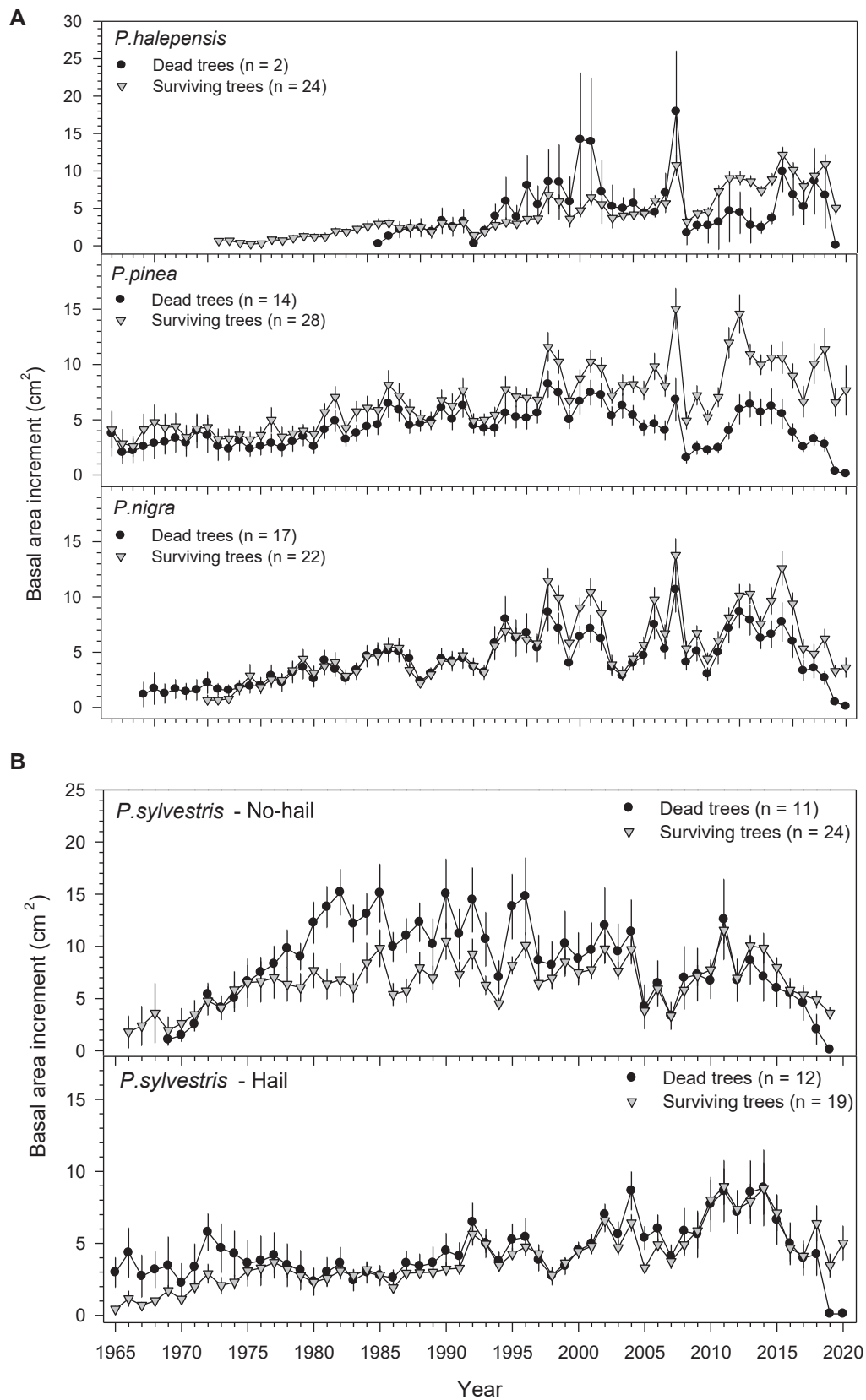


Fig. 2. (a) Radial growth patterns of trees that had sustained radial growth (surviving) and had arrested radial growth (dead) after the *D. sapinea* outbreak following a hailstorm in 2018 in the Pyrenees. Radial growth is shown as basal area increment values \pm SE (b) Radial growth of *P. sylvestris* trees affected by *D. sapinea* and growing in a hailstorm-affected area during 2018 or in an area not affected by hailstorms. “n” indicates the number of trees per group.

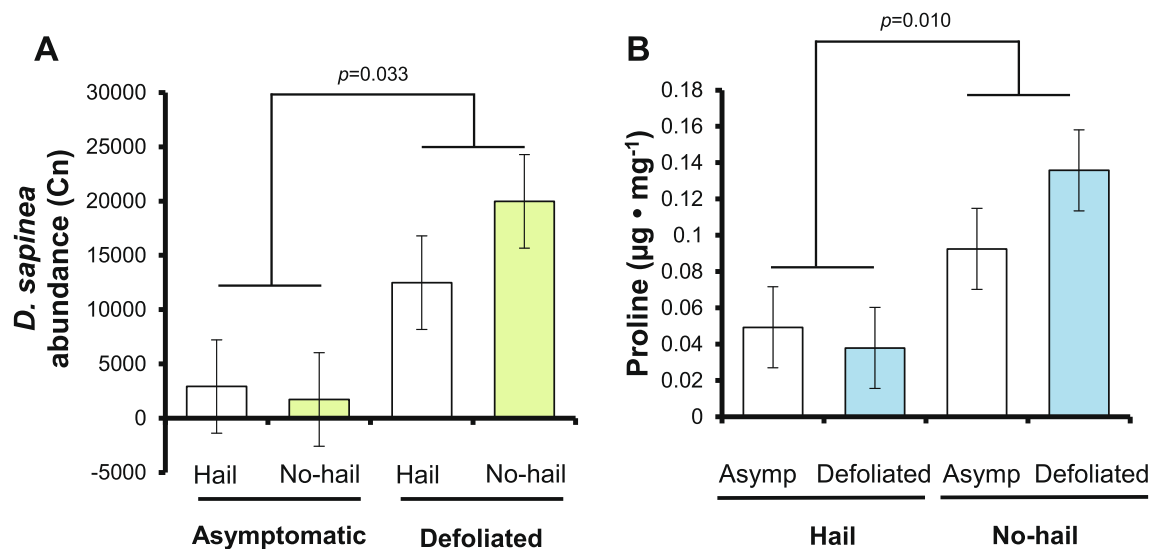


Fig. 3. Abundance of (a) *D. sapinea* and (b) proline content in shoots of *P. sylvestris* trees subjected to hail or not subjected to hail and developing or not symptoms of disease (defoliated and asymptomatic, respectively). Values are means \pm SE.

4. Discussion

Diplodia shoot blight is an emerging disease in Europe, which causes shoot necrosis on major forest species such as *P. sylvestris*, *P. nigra* or *P. radiata*. In this study, we found that shoot damage can have long-lasting effects on tree vitality, and lead to severe tree mortality over a two-year period. After an outbreak, *P. sylvestris* was the species with the highest likelihood of dying. *Pinus nigra*, *P. pinea* and *P. halepensis* showed a much lower mortality rate than *P. sylvestris*. Across pine species, mortality depended on the severity of damage in the crown, irrespective of the growth displayed by the tree before the outbreak i.e. mortality affected both vigorous and weak trees. The mechanism behind the observed mortality is unknown, but appeared to be unrelated to differences in terms of drought response amongst pines species. Further research should be carried out to understand the underlying reasons behind the high predisposition of *P. sylvestris* to die after a *D. sapinea* attack. *Pinus sylvestris* is a widespread forest species in Europe with high economic and ecological importance (Houston Durrant et al., 2016). The current range expansion of *D. sapinea* to higher latitudes, coupled with its capacity to cause mortality, raises concerns about the possible impact of this disease in boreal and mountain conifer forests in a climate change scenario (Brodde et al., 2019).

In the mixed stands, Scots pine was by far the most vulnerable to mortality compared to *P. nigra*, *P. pinea* and *P. halepensis*. In this research, we showed that when controlling for crown damage, *P. sylvestris* was five times more likely to die than *P. nigra* and eleven times more likely to die than *P. pinea*. In a previous study, we showed that following a hailstorm, *P. sylvestris* had a much higher quantity of *D. sapinea* in the shoots than the other studied pine species (Oliva et al., 2021), suggesting a role of the endophytic persistence of *D. sapinea* in asymptomatic tissues on tree mortality. It is possible that in the study area rainfall or temperature conditions were less favourable for *P. sylvestris* than for the other pine species, and therefore explaining the high mortality rates in Scots pine. *Pinus sylvestris* is more drought-sensitive than *P. nigra* and *P. halepensis* (Camarero et al., 2015), and in that case, a stronger impact of the climate on the growth would have been expected for *P. sylvestris* compared to the other pine species. However, the correlations between growth and SPEI did not support a role of drought on the observed mortality differences between pine species. On the one hand, the correlation between SPEI and growth of *P. sylvestris* in the hail-site was similar to the other pine species, while *P. sylvestris* mortality was the highest. On the other hand, the correlation

between SPEI and growth in hail-affected sites was much higher than in no-hail sites, while *P. sylvestris* mortality in these two areas was not significantly different. We cannot discard that other unobserved factors, such as root rot pathogens, could explain the observed mortality. Root rots cause long-term losses in growth (Cherubini et al., 2002), and if involved in this process but undetected in our survey, we would have expected them to have an impact on tree vigour and therefore be reflected in the associations between radial growth and mortality.

Crown damage was the best predictor of mortality across species. Crown defoliation has been previously reported as a predictor of mortality following pathogen outbreaks (Oliva et al., 2016) or insect damage (Kulman, 1971), but also following abiotic stressing events, such as fire (Catry et al., 2010) or drought (Michel et al., 2020). Diplodia shoot blight defoliation has an immediate impact on latewood growth (Brodde et al., 2019); however, we found that growth losses may span for years and lead to the death of the tree. These long lasting effects contrast with the growth recovery usually observed after insect defoliations (Jacquet et al., 2012), suggesting that shoot damage is probably more harmful to pines than needle loss (Oliva et al., 2016). The physiological mechanism leading to tree mortality following shoot necrosis is unknown, but we speculate it could relate to the high repair costs involved in restoring the crown (Oliva et al., 2014). Having lost the newest apical buds and shoots, recovery of the crown might have to rely on dormant buds present in older whorls. Although not assessed in the study, mortality could also be determined by indirect effects of shoot blight on mycorrhiza. Crown defoliation can reduce carbon uptake and negatively impact root-associated fungi possessing long exploration hyphae with an essential role in water and nutrient uptake (Castaño et al., 2020).

Combining symptom measurements with dendrochronological analyses showed that crown mortality did not necessarily couple with cambial mortality. Tree ring analyses allowed us to detect trees that had arrested radial growth, although some parts of the crown were still alive. For all species except *P. pinea*, growth losses leading to a total arrest started after the outbreak in 2018, irrespective of the growth displayed by the trees before the hailstorm. These results together with mortality assessments based on crown condition, suggest that *D. sapinea* can cause tree mortality to both vigorous and weak trees, and that mortality is mainly determined by the amount of crown affected by the pathogen. In the case of *P. pinea* trees, we did find that trees with arrested radial growth had also been growing less prior the outbreak. *Pinus pinea* was also different compared to the other species, as it showed a worsening in crown condition with time, while the crown condition of other species

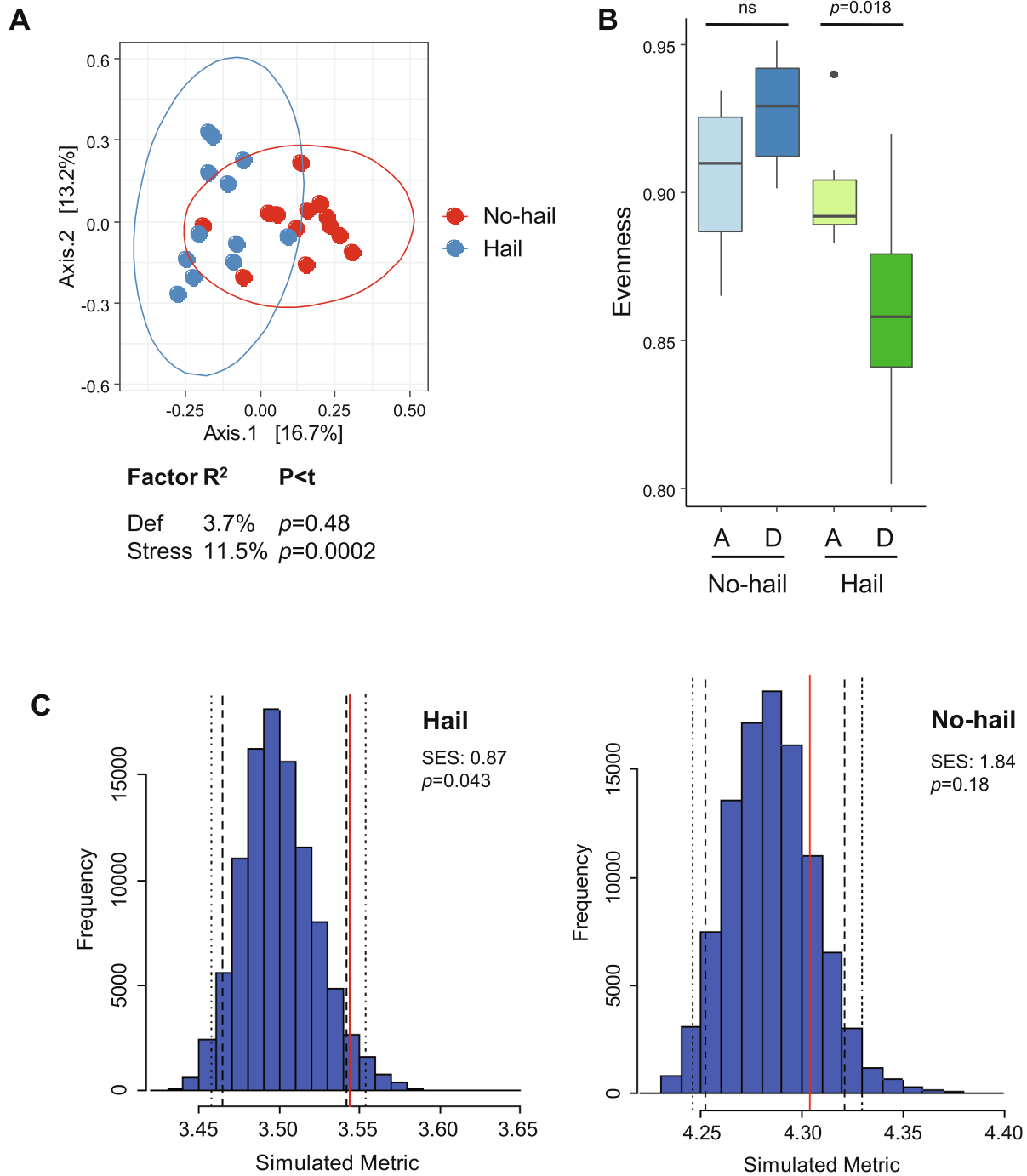


Fig. 4. Endophyte community patterns amongst *P. sylvestris* trees defoliated by *D. sapinea* (D) or with asymptomatic crowns (A) in areas affected by hailstorms and areas not affected by hailstorms in terms of (a) beta diversity, (b) alpha diversity (evenness) and (c) co-occurrence patterns in the Pyrenees. In (a), first two axes of a principal coordinate analysis (PCoA), together with the results of a multivariate analysis of variance considering two factors: defoliation (Def) with the levels “asymptomatic” and “defoliated”, and stressor (Stress) with the levels “hail” or “no-hail”. In (c), SES stands for Standardized effect size (SES) of co-occurrence (C-score).

improved or did not change. It is possible that *P. pinea* was suffering from another stress factor in the study area, as large mortality events have been observed not far from the area of study linked to drought-stress (Tardà et al., 2017).

In *P. sylvestris*, the endophytic community of trees shoots affected by *D. sapinea* was different depending on whether the outbreak had occurred following a hailstorm or not. The most striking differences were found in terms of alpha-diversity, particularly in terms of evenness and segregation. In hail-affected areas, the community was more uneven

and showed more signs of competition amongst endophytes than in trees not subjected to hailstorms. The observed patterns could be explained by the time since the stressing event for disease took place, presumably shorter in the case of hail sites than in no-hail sites, as well as on the intensity, and presumably higher in hail-sites than no-hail sites. Community differences observed within the hailstorm area could be the response to the recent strong and isolated event of mechanical damage. In the area not affected by hail, the endophyte community was perhaps exposed to a constant stress over time, thus diluting the differences

between defoliated and asymptomatic trees. Our results are in line with previous findings that the plant endophytes can be relatively dynamic and responsive to external cues (Naylor et al., 2017); however, further research should be done on trying to understand the causes underlying symptom expression in the no-hail sites before reaching further conclusions.

The quantity of *D. sapinea* and proline was higher in defoliated trees than in asymptomatic trees in both hail-affected and no-hail sites. In contrast, proline levels were much higher in the no-hail area than in the hail-affected sites. Competition amongst endophytes and *D. sapinea* for proline may determine disease (Oliva et al., 2021); however, proline dynamics in pine shoots is still poorly understood (Sherwood et al., 2015). It is difficult to interpret the observed proline patterns without knowing the causes that triggered disease in the no-hail sites.

Mortality was monitored for 28 months after the outbreak was first observed. With the data available, the long-term effects of a *D. sapinea* outbreak occurring after hailstorms are not known. In the case of *P. sylvestris*, we observed that the risk of mortality had a tendency to decrease over time. This contrasted with *P. sylvestris* trees found in areas not affected by hailstorms, where the risk of mortality was constant over time. Interestingly, the risk of mortality following both a hailstorm and in the area not affected by hailstorms tended to equalise after two years, which could suggest that mortality is not likely to increase further in the long term.

In this research, we have shown that (i) following a disease-inciting event such as hail, *D. sapinea* can kill trees depending on the severity of damage in the crown, and regardless the vigour of the tree (in terms of radial growth), and that (ii) *P. sylvestris* has a high likelihood of dying if shoot blight affects >70% of the crown in comparison with other pine species. In terms of management, the amount of crown defoliation is informative of the probability of mortality. Therefore, crown conditions can be used as guide when deciding which trees to keep or which trees to remove after an outbreak. The recommendations that may stem from our data do not consider the epidemiological consequences of leaving or removing affected trees from the stand. For instance, trees with moderately affected crowns would have high chances of survival; however, with the results of our study it is not possible to know whether these trees could act as sources of inoculum and spread the disease to healthy neighbouring trees, or not. While the likelihood of mortality decreases with time after an outbreak, no information is currently available on ways to decrease the vulnerability of stands, mitigate damage or facilitate recovery by silvicultural methods. For now, the best available strategy may be to avoid planting and promoting Scots pine in areas at high risk of hailstorms.

CRedit authorship contribution statement

Maria Caballol: Methodology, Writing – original draft, Writing – review & editing. **Maia Ridley:** Methodology, Writing – review & editing. **Michele Colangelo:** Methodology, Writing – review & editing. **Cristina Valeriano:** Methodology, Writing – review & editing. **J. Julio Camarero:** Methodology, Writing – review & editing. **Jonàs Oliva:** Conceptualization, Supervision, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.foreco.2021.119935>.

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