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Diplodia sapinea as a contributing factor in the crown dieback of Scots pine (*Pinus* sylvestris) after a severe drought

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ABSTRACT

The frequency and impact of drought on global ecosystems have increased within the last century, while drought has affected tree health in many regions. *Diplodia sapinea* is a widespread, opportunistic pathogen infecting most conifers, causing Diplodia tip blight, thriving on hosts impacted by stress such as drought, heat, or mechanical wounding. In summer of 2018, a large-scale drought was recorded all over Europe. In late summer, pine trees all over Gotland showed crown dieback, where necrotic twigs and needles were found, especially in the upper part of the crowns. Symptoms were consistent with a potential outbreak of *D. sapinea*. Effects of the combination of drought and Diplodia tip blight on mortality or recovery of Scots pine in Nordic conditions are unknown. This study confirmed the presence and potential contribution of *D. sapinea* in the observed damages of Scots pine. Shoot blight and drought led to crown defoliation which was observed one year post-drought, while trees showed a clear recovery of newly grown shoots within the second year. Severely affected pines (>70% of the upper third of the crown with shoot blight) showed increased mortality. Recovery of the surviving trees was independent of previous dieback levels. *Diplodia sapinea* was most abundant in twigs with shoot blight of the symptomatic trees compared to healthy-looking twigs from the same trees and asymptomatic trees in affected and healthy pine stands. Sampling on affected and healthy sites showed possible endophytic infections with low abundance within healthy-looking twigs. Spore deposition of *D. sapinea* was monitored on healthy and affected sites for two consecutive years after crown damages occurred to confirm the presence of the opportunistic pathogen in the affected region. Spore deposition was observed during all seasons and correlated with high precipitation during sampling. Our observations provide insights into the emergence of Diplodia tip blight in the Northern countries and underline the potential impact of *D. sapinea* on tree health in the course of a changing climate.

1. Introduction

The frequency and thereby impact of drought on global ecosystems have increased within the last century (Chiang et al., 2021). Drought can significantly affect tree growth and vitality (Anderegg et al., 2015; Camarero et al., 2018). Tree growth could be partitioned in needle and leaf elongation, or shoot and stem growth, which are all influenced by climate, especially by temperature and water availability (Dobbertin et al., 2010). Drought-exposed Scots pines (*Pinus sylvestris* L.) react with closing stomata and loss of needle biomass (Dobbertin et al., 2010; Galiano et al., 2010), which can be related to reduced canopy development (Poyatos et al., 2013) and low growth rates (Galiano et al., 2011). Estimates of crown vitality can serve as a proxy for tree vigour (e.g. Rebetez and Dobbertin, 2004). Different measurements of crown vitality can provide distinct angles on the health status of a tree. Evaluating crown dieback involves estimating the ratio of deceased branches in relation to the entire healthy crown. This measurement serves as an early indication of reduced vigour and growth potential due to recent stresses or damage, including severe defoliation caused by factors like drought or defoliating agents. Crown transparency, on the other hand, quantifies the extent of a tree's healthy crown that becomes absent due to early needle loss. This transparency can be influenced by a range of causes, including diseases, environmental stresses, declining tree vigour, and decreased needle retention resulting from events like insect

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outbreaks or drought. Foliage transparency is a dynamic variable, rapidly changing within tree crowns and acting as a measure of defoliation or stress (Schomaker, 2007). By combining these approaches, a more comprehensive understanding of the tree's health, performance, and recovery trajectory can be achieved.

Pines are particularly plastic in their patterns of biomass allocation to leaves (Delucia et al., 2000; Martínez-Vilalta et al., 2004; Poyatos et al., 2013). Premature shedding of needles reduces whole tree transpiration (Whitehead et al., 1984; Martínez-Vilalta et al., 2014) and is presumably a last-chance mechanism to avoid hydraulic failure (Wolfe et al., 2016; Nadal-Sala et al., 2021). Crown dieback and associated symptoms can therefore be considered as indicators of reduced or poor vigour in Scots pine trees exposed to drought stress. Once drought stress is relieved the canopy can recover even in trees with relatively high rates of defoliation (Dobbertin et al., 2010; Eilmann et al., 2013).

In summer 2018, a large-scale drought was recorded all over Europe (Peters et al., 2020), and Scandinavia was among the regions that showed the highest temperature anomalies (Moravec et al., 2021). In Sweden, 2018 showed the warmest recorded mean temperatures in May. A third of the days from May to August were significantly warmer than the average since 1756 (Wilcke et al., 2020). The anomalies in temperature in combination with precipitation deficits impacted soil moisture, while increased evapotranspiration might have been the driving force in the drought observed during 2018 (Moravec et al., 2021). Although summer droughts are frequent for the calcareous island of Gotland (Lindroos, 2001), the drought of 2018 had a larger impact on the flora and fauna of the island than usual (Johansson et al., 2022). Scots pine dominates both production- and natural forests on the island, and in late summer of 2018 large areas of Scots pine showing crown dieback were found all over Gotland. Trees were showing an overall discolouration of needles, mixed with symptoms of shoot blight in the upper parts of the crowns. The symptoms were consistent with a potential outbreak of Diplodia tip blight caused by *Diplodia sapinea* (Fr.) Fuckel (syn. *Diplodia pinea* (Desm.) Kickx., *Sphaeropsis sapinea* (Fr.: Fr.) Dyko & Sutton).

Diplodia sapinea is an opportunistic forest pathogen, causing Diplodia tip blight on conifers. As a latent pathogen, it can be present in asymptomatic *Pinus spp*. (Luchi et al., 2011). Symptoms develop when its host is challenged by stress such as drought, heat, or mechanical wounding (e.g. Stanosz et al., 2001; Smith et al., 2002). It is well known from field observations (e.g. Swart et al., 1987a), as well as greenhouse studies (e.g. Johnson et al., 1997), that symptomatic infections by *D. sapinea* increase in severity under drought stress of the host. The fungus spreads via asexual spores (conidia) (Bihon et al., 2011), which are released from asexual pycnidia in moist weather, and are supposed to be dispersed by wind-driven rain and rain splash (Brookhouser and Peterson, 1971; Swart et al., 1987b). Pycnidia develop on cones, necrotic shoots, and needles in relation to moist conditions (Peterson, 1977). In central Europe, tracking of conidia from closely related *Diplodia* species illustrated a release mainly during the vegetative period with peaks in late summer and early autumn (Kuntzmann et al., 2009). Historically, severe impacts on forestry have been reported from the southern hemisphere (Swart et al., 1985; Swart and Wingfield, 1991; Burgess et al., 2004), whereas an increase of damages by Diplodia tip blight further North were recognised during the last decades (Hanso and Drenkhan, 2009; Oliva et al., 2013; Adamson et al., 2015; Brodde et al., 2019; Blumenstein et al., 2021; Terhonen et al., 2021). Diplodia tip blight is considered as a relatively new disease in the Nordic countries; ten years ago, *D. sapinea* was found for the first time on healthy and single, symptomatic pines in Sweden (Oliva et al., 2013). In 2016, the first outbreak in a commercial Scots pine plantation in Sweden was discovered (Brodde et al., 2019). Diplodia tip blight has been shown to be associated with drought, though effects of the combination of drought and Diplodia tip blight on mortality or recovery of Scots pine in Nordic conditions are unknown.

In this study, we aimed at describing the mortality and recovery of drought-induced crown dieback and the relation with *D. sapinea* in Scots pine. We observed the development of Scots pine displaying crown dieback and apparently healthy trees within affected and healthy areas for two consecutive years after the initial drought in 2018. We also investigated if *D. sapinea* was present locally only in symptomatic trees of the affected sites or also in healthy-looking trees, as well as on both affected and healthy sites. Furthermore, we tested if the presence could be classified as a local or systemic infection within the trees. By local infection, we mean present only in tissues exhibiting Diplodia tip blight symptoms, in contrast to systemic, where the level of *D. sapinea* colonisation is similar in symptomatic and apparently healthy tissues of affected trees. Additionally, the spore dispersal of *D. sapinea* was analysed each season for two years to monitor inoculum on all studied sites.

We investigated the presence of *D. sapinea* in the affected area of Scots pine and hypothesised that drought effects in combination with *D. sapinea* infection limit recovery of the trees, so that i) severely damaged trees (>70%) show higher mortality rates and ii) mildly damaged trees (<30%) show a higher probability of recovery. We also predicted that iii) *D. sapinea* is abundant in symptomatic and asymptomatic trees on affected and healthy sites and that iv) the spore dispersal of *D. sapinea* is associated with the occurrence of dieback of Scots pine at the sample site (affected vs. healthy sites). We could show that a reduction of crown dieback occurred within two years after the drought, even under the presence of *D. sapinea,* where i) severely damaged Scots pine (>70%) showed higher mortality rates two years post-drought, but ii) recovery could be observed independently from initial crown dieback levels. iii) *D. sapinea* was endophytically present with low abundance in healthy twigs of symptomatic and asymptomatic trees on affected and healthy sites and abundant only in necrotic twigs of symptomatic trees. iv) Spore deposition of *D. sapinea* was correlated with precipitation during sampling but not with the health status of the site.

2. Methods

2.1. Experimental sites

All experimental sites are located east and northeast of Visby (Fig. 1) within an area most affected after the drought of 2018 on Gotland. Four sites with Scots pine showing symptoms of crown dieback (Affected 1 to 4) and four sites with healthy-looking Scots pine (Healthy 1 to 4) were selected (representative picture: Fig. S1). The soil type on Gotland is Inceptisol, where the beginning of profile development is visible (Brady and Weil, 2008). All affected sites and site Healthy 3 are located on bedrock, where available growth substrate consists mainly of organic matter in cracks in the rock. The asymptomatic sites Healthy 1 and 4 are located on post-glacial sand/gravel, and Healthy 2 is located on clay till (SGU, 2014). In general, all sites but Healthy 4 had very thin soil layers on top of the rock layers (Soil depths map, SGU 2014, 2017). Groundwater levels measured close to the affected sites during the last 60 years were generally low, while 2018 showed the lowest values during a longer-than-average time in the last 30 years. In 2019, even lower groundwater levels were reached for a short period, while levels recovered to magnitudes recorded pre-drought in 2020 (data obtained from SGI, see Fig. S2).

2.2. Detection of D. sapinea

Visual detection of Diplodia tip blight symptoms on affected sites was confirmed by spore morphology. For this purpose, necrotic shoots in the upper part of the crown and cones infested with pycnidia morphologically matching *D. sapinea*, were collected. Three to nine shoots and six to nine cones were collected on each site. Spores from pycnidia were microscopically examined from three needles of each shoot and two scales of each cone. No presence of other potent pathogens capable of inducing the observed symptoms was identified in symptomatic pine

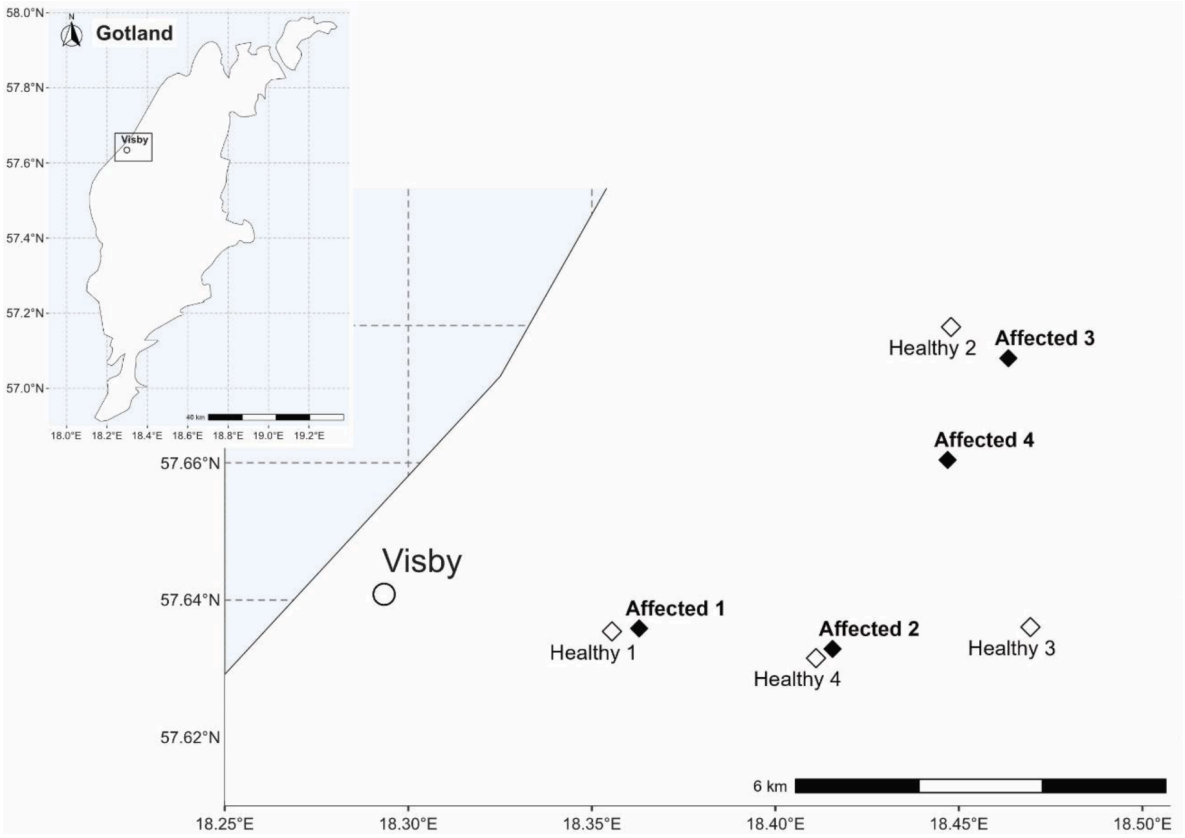

Fig. 1. Location of experimental sites within an affected region of Scots pine showing crown dieback after a severe drought in summer 2018 on Gotland, Sweden. Severely affected stands (Affected 1–4) were located near healthy-looking stands (Healthy 1–4). Map source: rnaturalearth (Massicotte and South, 2023).

twigs at the sites (Brodde, 2023).

2.3. Crown dieback estimation

Crown dieback of 20–27 Scots pines was estimated on each affected site for the first time in December 2018 (Table 1). The level of crown dieback was estimated in 10% steps as the proportion of twigs with shoot blight and dead twigs in the upper third of the living crown in relation to a completely healthy tree in the same population. Selection of trees was based on the level of crown dieback, with the aim of including trees covering a span from very low (<30%) to very high crown damages (<70%). Selected trees showed a minimum of 5% and a maximum of 100% crown dieback. Measured trees were revisited in October 2019 and November 2020. In 2019, shoot blight and loss of needles (crown transparency) were difficult to distinguish and estimated as one overall measurement of crown dieback as carried out in 2018. By 2020, crown transparency had increased to such a high level that two separate estimations were carried out; one overall estimate of crown dieback as carried out in 2018 and 2019, and one estimate of dieback focused on the shoots grown post-drought, not considering the defoliated, transparent parts of the crown. Five asymptomatic trees on each healthy site were randomly selected and measured for crown dieback in 2018, 2019, and 2020. For all trees selected at affected sites, height, diameter at breast height (DBH, 1.3 m), and bifurcation of the stem (absence or presence) were recorded in 2018.

Table 1
Overview of experimental site properties. Soil type SUG, 2014; soil depth SUG, 2017.

Site	Coordinates (SWEREF99 TM)	Soil type	Measured trees [n]	Mean tree height [m] ± 1SD
Affected 1	57.63587, 18.36288	bedrock	20	5.1 ± 1.6
Affected 2	57.63289, 18.41557	bedrock	24	9.7 ± 2.2
Affected 3	57.67524, 18.46347	bedrock	25	9 ± 1.7
Affected 4	57.66042, 18.44692	bedrock	27	8.3 ± 1.2
Healthy 1 *	57.63544, 18.35548	post-glacial sand/ gravel	5	8.2 ± 0.6
Healthy 2	57.67979, 18.44784	clay till	5	11.4 ± 2.4
Healthy 3	57.63607, 18.46947	bedrock	5	9.3 ± 1.4
Healthy 4	57.63154, 18.41107	post-glacial sand/ gravel	5	12.1 ± 1.5

* Site destroyed in 2020.

2.4. Sampling of Scots pine twigs and spore traps

Scots pine twigs were collected in December 2018. Twigs were sampled with a telescopic pruner (5 m long) as high as possible, aiming for light-exposed twigs at the upper half of the crown. Three healthy twigs were collected from all sampled trees. If a tree was symptomatic, three additional twigs showing tip blight were sampled. On each healthy site, three trees were randomly chosen. On affected sites, three symptomatic and three asymptomatic trees were selected based on the crown dieback estimations. Healthy trees showed ≤ 10%, and symptomatic trees showed ≥ 20% crown dieback.

Twigs were transported at < 4 °C and stored at −20℃ within five days. Surface sterilisation was carried out according to Bußkamp (2018) with minor modifications. In brief, needles were removed, stems were brushed under running tap water and then surface-sterilised under a sterile hood. Twigs were incubated for 1 min in 70% ethanol, 5 min in

3% NaOCl, and 1 min in 70% ethanol. A final washing step in ddH2O for ca. 15 sec was added to the protocol. A surface print of a sterilised twig on 2% malt extract agar was carried out each day of sample preparation to check the efficiency of surface sterilisation; fungal growth was observed in 5% of the cultures (n = 20). Sampling of stem tissue was carried out with a sterile scalpel. Each sample contained 1 cm twig tissue which was either healthy (asymptomatic twigs) or from the border of an infection, including necrotic and healthy tissue (symptomatic twigs). Healthy twigs could be sampled for the 2018 year's growth, while symptomatic twigs were sampled at the growth year where the infection border was localised, reaching from year 2008 to 2017. Twig samples were cut into thin sections, transferred into a 2 mL screw cap tube containing one 5 mm and two 2 mm glass beads, and stored at −20 °C until lyophilisation. Freeze-dried samples were homogenised using the Precellys® 24 Tissue homogenizer (Bertin Instruments), 2x 25 s at 5000 rpm. DNA extraction was carried out according to instructions with the E.Z.N.A® SP Plant DNA Kit (Omega Biotek).

To monitor *D. sapinea* spore release, four spore traps were placed in the centre of each site (Table 2). One healthy site was lost at the beginning of the second sampling year due to destruction of the whole site. The traps consisted of one horizontally fixed filter paper (Munktell, Ahlström; 90 mm diameter) treated with 4x TE buffer (for detailed description, see Zhang et al., 2022) at the height of 1.2–1.5 m. Filter papers were exposed during two consecutive seven-day sampling periods per season from January 2019 to October 2020. The sampling periods were chosen to capture potential seasonal variations in spore release. Filters were collected in 50 mL Falcon tubes, kept on ice during transportation, and stored at −20 °C within 4 hrs and until DNA extraction.

For spore DNA extraction, 20 mL SDS buffer (0.05 M Tris pH 8, 0.05 M EDTA pH 8, 0.104 M SDS, 1 M NaCl, dissolved by incubation at 60 °C for two days) was added to the Falcon tubes containing the filter papers followed by incubation at 65 °C for 90 mins. After removal of the filter papers, 20 mL 2-propanol was added to the SDS extract. The samples were mixed thoroughly and incubated at RT overnight. The following day, the samples were centrifuged at 7000 rpm for 10 mins at RT before the supernatant was removed. The pellet was resuspended in 700 µL lysis buffer PL2 (Macherey-Nagel, Düren, Germany) and transferred to 2 mL screw-cap tubes containing ~ 130 mg 0.2 mm glass beads, ~200 mg 0.4 mm glass beads, ~200 mg 3 mm glass beads, and ~ 4 mg diatomaceous earth (powdered siliceous sedimentary rock). The samples were lysed for 30 s at 5000 rpm using Precellys® 24 Tissue homogenizer (Bertin Instruments). Extractions were performed using the Macherey-Nagel NucleoSpin Plant II kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's protocol, with increased volumes of buffer PL3 and buffer PC proportionally to the volume of lysis buffer PL2 used for resuspension of the pellet.

Table 2
Spore trap sample weeks (time points) and number of successfully analysed filter papers per sample week. Samples with failed qPCR (no signal) were excluded (in total n = 14). Filter papers were left on site for seven consecutive days. Year 2019: four filter papers per week on four affected (n = 16) and four healthy sites (n = 16). Year 2020: four filter papers per week on four affected (n = 16) and three healthy sites (n = 12); site "Healthy 1" lost by destruction at beginning of 2020.

Week	Start	Season	Year	Analysed samples (n)
1	21.01.	winter	2019	31
2	29.01.	winter	2019	31
3	10.04.	spring	2019	32
4	17.04.	spring	2019	27
5	15.07.	summer	2019	32
6	22.07.	summer	2019	31
7	14.10.	autumn	2019	31
8	21.10.	autumn	2019	30
9	13.01.	winter	2020	28
10	20.01.	winter	2020	28
11	09.04.	spring	2020	28
12	16.04.	spring	2020	28
13	06.07.	summer	2020	27
14	13.07.	summer	2020	26
15	15.10.	autumn	2020	28
16	22.10.	autumn	2020	28

2.5. Quantification of *D. sapinea* DNA from twigs and spore traps

The amount of *D. sapinea* DNA from twigs and spore traps was quantified using qPCR. A standard curve was produced by PCR amplification on DNA template from a *D. sapinea* isolate and the PCR product's desired length (79 bp) was confirmed through gel electrophoresis. DNA from the PCR product was precipitated, quantified using a Qubit Fluorometer, and the highest concentration replicate was chosen for serial dilutions to generate a standard curve. The primers used for PCR and qPCR, and the probe used for qPCR, were designed by Luchi et al. (2005). The qPCR master mix was prepared following their methodology with the following modifications: 1x SsoAdvancedTM Universal Probes Supermix (BioRad), 250 nM each of forward and reverse primer, and 200 nM probe. Reactions were set up in triplicates, including the standard curve from 1×10^6 to 1×10^2 copies per µL and non-template controls (nuclease-free water). Each reaction consisted of 15 µL master mix and 5 µL 1:1 dilutions of the spore trap DNA extractions or 5 µL twig DNA (diluted so that the total amount of DNA was 5 ng per reaction). The qPCR program was 2 min at 95 °C followed by 40 cycles of 10 s at 95 °C and 15 s at 60 °C. iQ™5 Optical System software (BioRad) was used to analyse the qPCR data. Samples were excluded if the triplicate cycle threshold (Ct) value's standard deviation (stDev) was > 0.5. Samples below the linear detection limit (37 Ct) of the assay were included in the analysis with SQmean = 0.01 even if the stDev was > 0.5.

Sixty-four of 466 DNA samples extracted from spore trap filter paper were below the detection limit for *D. sapinea*, and 17 of 466 were excluded due to non-reliable reads. Nine of 141 DNA extractions from twigs were below the detection limit.

2.6. Statistical analysis

All analyses were performed using R Statistical Software (v4.1.1; R Core Team 2021). Crown dieback estimations per year and qPCR data from twig samples were compared by ANOVA using AICcmodavg R package (v.2.3–1) followed by post hoc Tukey's honest significance test. Effects of initial dieback, tree height, and bifurcation on crown dieback in new growth, as well as the overall dieback in the upper third of the crown of pines in 2020, were tested in a linear mixed-effects model using the lme function of the nlme R package (v.3.1.157). Site and tree identity were used as random factors to account for repeated measurement of the trees and within-site variability. The effect of low (<30%), medium (40–70%) or high (>70%) initial crown dieback on recovery (<-10% difference in dieback), stagnation (+/-10%), or decline (>10%) of the pines was tested by a pairwise *t*-test using the rcompanion R package (v.2.4.15).

Differences in *D. sapinea* DNA quantifications from spore traps between affected and healthy sites during the two sampled years were analysed with a repeated measures linear regression model using the lme function of the nlme R package (v. 3.1.157). Fixed effects were site type (healthy/affected) and sampling week (time point, 1 to 16). Mean values were computed for each site type and sample week in combination to analyse the development of *D. sapinea* quantity over time. Repeated measurement of the same traps was accounted for by the correlation of the error term by sample week for each trap. Site number and spore trap ID were treated as random factors. Post hoc pairwise comparisons of *D. sapinea* DNA quantities per site type were carried out by sample week using the emmeans R package (v. 1.7.3).

3. Results

The exceptional drought of 2018 was followed by severe crown dieback of Scots pine on Gotland. The observed symptoms were consistent with a potential outbreak of *D. sapinea* (Fig. 2a). The presence of *D. sapinea* on the affected sites could be confirmed by spore morphology and on average ca. 60% of the examined shoots and cones from the affected sites carried fruiting bodies of *D. sapinea* (Table S1).

3.1. Crown dieback development

In 2018, Scots pine trees in the affected sites showed an average of 25% crown dieback (Fig. 3a). Mean crown dieback of the trees doubled from 2018 to 2019, from ca. 25% to > 50% (Fig. 3a). No significant shift in overall crown dieback occurred during the second year, from 2019 to 2020 (Fig. 3a). However, in 2020 shoots grown after 2018 became clearly visible when necrotic and senescent needles from the previous years were shed (Fig. 2d, Fig. 3a). The assessment of dieback in shoots that developed post-drought revealed a reduction in the proportion of impaired shoots. This decline was in line with the levels of crown dieback observed in 2018 when the initial damages occurred, highlighting the cumulative defoliation and subsequent foliage recovery dynamics (Fig. 3a).

Scots pine on healthy sites showed no crown dieback in 2018 (Fig. 3b), with a slight increase of mean dieback to ca. 10% in 2019 and ca. 17% in 2020. Shoots grown after 2018 showed lower crown dieback than the overall estimate, with a mean value of ca. 12%.

Fig. 4 visualises the crown dieback development of single trees in 2019 and 2020 in relation to their previous year's crown dieback. Trees falling on the plotted line of equality (x_1, y_1 = (0, 0), x_2, y_2 = (100, 100)), +/-10% in × and y stagnated in their level of crown dieback between the two compared years. Most pines showed increased crown dieback of 20–50% within the first year after the drought of summer 2018 (Fig. 4a). An evident decrease in crown dieback compared to the conditions observed in 2018 was recorded in only two cases. The remaining pines displayed a stagnation in crown dieback (within the +/-10% blue area in Fig. 4a). By 2020, two years after the drought, dieback levels of the overall crown stagnated or changed within +/-20% compared to 2019 for the majority of the pines (Fig. 4b). Crown dieback estimation of shoots grown post-drought (Fig. 4c) showed a strong reduction of symptomatic shoots. Most pines exhibited<40% dieback in their new shoots. The extent of crown dieback in 2018 had a notable impact on both the overall crown's dieback and the dieback observed in the newly grown shoots by 2020 (Table S2). Bifurcation and tree height in 2018 had no significant effect on crown dieback development until 2020. Notable were five trees with a crown dieback level of 90% in 2019, which showed recovery in the newly grown shoots at the last estimation in 2020 (Fig. 4c).

Classes of trees with initially low (<30%), medium (40–70%), or high (>70%) crown dieback showed no significant difference in frequencies of recovery, stagnation, or decline by 2020 considering the overall crown estimation (Table S3a). The highest frequency of recovery was recorded in newly grown shoots of initially medium-damaged trees but with no significant difference to highly damaged trees (Table S3b).

Taken together, the observed crown dieback of Scots pine in the affected sites started with severe needle losses, which doubled during the first year. Surviving trees showed clear recovery in 2020 compared to 2019, independently of their initial crown dieback.

Among the trees classified as having medium- and low levels of crown dieback, total mortality observed was low (5–10%, Fig. 5). However, in severely damaged trees (>70% crown dieback) total mortality exceeded 50%. Almost all mortality among the trees in the study was recorded during the first year of the study whereafter only limited additional mortality was recorded (Fig. 5). Trees were more likely to die when crown dieback exceeded 70% and within the first year after the drought event.

Fig. 2. Representative pictures of different Scots pine trees on Gotland. Crown dieback estimation started after a severe drought in 2018; trees were revisited in 2019 and 2020. a) 2018: A Scots pine tree with 70% crown dieback of upper third of living crown (2018). Browning of needles was found in apical shoots (shoot blight), consistent with symptoms of Diplodia tip blight. b) 2019: Scots pine tree with 40% crown dieback, measured as shoot blight and loss of needles (defoliation), as they were difficult to distinguish. c) 2020: Scots pine tree with 30% dieback, including defoliation. d) 2020 new growth: Scots pine tree with total crown dieback of 70% vs. 20% dieback when taking shoots grown post-drought into account, not considering the defoliated, transparent parts of the crown.

3.2. Quantification of D. sapinea DNA in Scots pine twigs.

To localise symptomatic as well as asymptomatic infections of *D. sapinea* in declining Scots pine, we quantified *D. sapinea* DNA in symptomatic and asymptomatic twigs of symptomatic and asymptomatic trees on affected sites and asymptomatic twigs of asymptomatic trees on healthy sites (Fig. 6). Significantly higher amounts of *D. sapinea* DNA were found in symptomatic twigs from symptomatic trees on affected sites compared to asymptomatic twigs. Healthy-looking twigs contained similar low amounts of *D. sapinea* DNA, whether they originated from symptomatic or asymptomatic trees on affected or healthy sites.

Overall, *D. sapinea* was most abundant in symptomatic twigs, while detection in asymptomatic twigs was independent of the tree's or site's health status.

3.3. Quantification of D. sapinea DNA in spore traps.

Diplodia sapinea spore deposition was sampled on affected and healthy sites to monitor inoculum levels for two years after the initial crown dieback occurred. *D. sapinea* DNA was detectable in filter paper spore traps throughout the two years on affected and healthy sites. The quantity of *D. sapinea* DNA showed neither a seasonal pattern nor an increase by the second year (Fig. 7a, b). A significant correlation was found between *D. sapinea* DNA quantity and high mean precipitation during spore trap exposure ($R^2 = 0.66, p = 0.005$), but not between DNA quantity and mean temperature or mean wind speed during sampling (Fig. 7c, Table S5).

We observed a trend of higher DNA quantities at affected compared

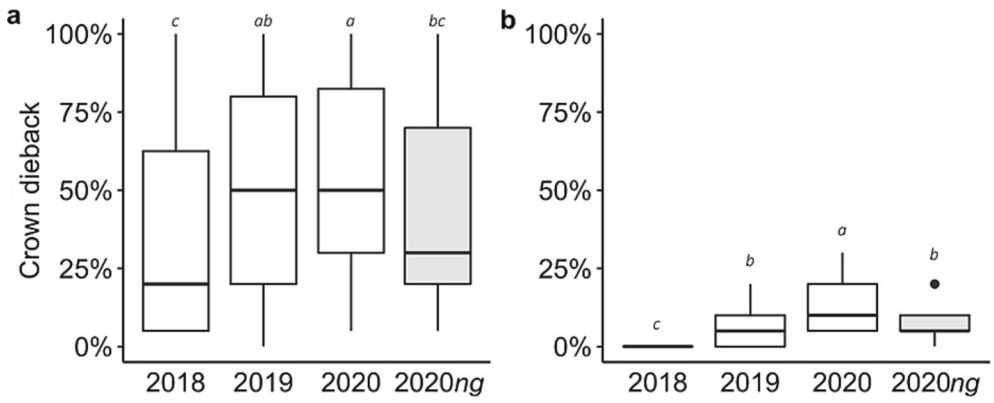

Fig. 3. Crown dieback of Scots pine on a) four affected sites (97 trees) and b) four healthy sites (20 trees) during the exceptional drought of 2018 and the two following years, 2019 and 2020, on Gotland. D. sapinea was confirmed to be present on the affected sites. Crown dieback was assessed by estimating the percentage of symptomatic shoots in the upper third of the living crown at the end of each vegetation period. 2020 ng refers to the estimation of dieback observed in shoots newly grown (ng) post-drought. Significances indicated by different letters: (ANOVA, post hoc Tukey's honest significance test).

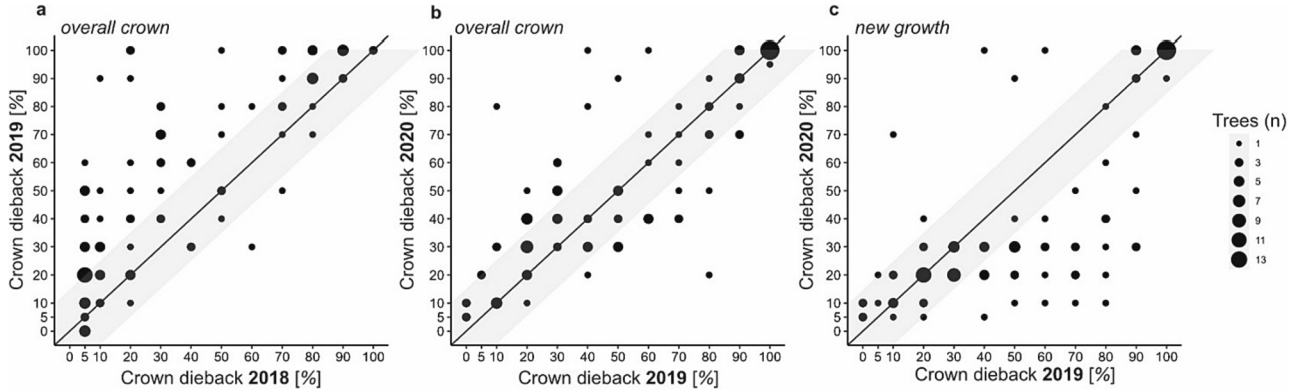

Fig. 4. Crown dieback development of Scots pine on Gotland. Shown are comparisons of crown dieback level of the crowns of single trees between years a) 2018–2019, b) 2019–2020 overall, c) 2019–2020 new growth. Trees on the line of equality (solid black line), within the +/-10% blue area, did not change in crown dieback. Trees above the blue area showed increased crown dieback and trees below showed less crown dieback in the latter year. Initial crown dieback of 2018 was observed after extreme drought and heat during summer 2018. Overall crown dieback was measured in % of symptomatic shoots in the upper third of the crown. Crown dieback of newly grown shoots of 2019 and 2020 (new growth) was measured in % of symptomatic shoots in the upper third of the crown. Scots pines (n = 97) were distributed among four affected sites.

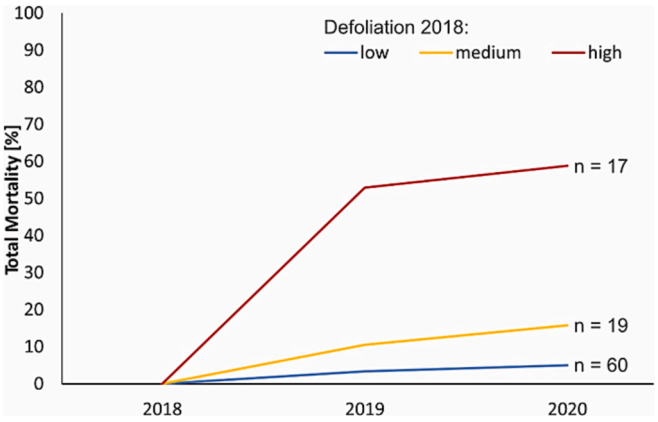

Fig. 5. Total mortality of Scots pine in two years (2019–2020), after initial crown dieback in Scots pine, 2018. Trees were categorised in low (<30%; blue), medium (40–70%; yellow), and high (>70%; red) crown dieback based on the initial estimation. Number of selected trees indicated per category, total n = 96. Pines located on four affected sites near Visby, Gotland.

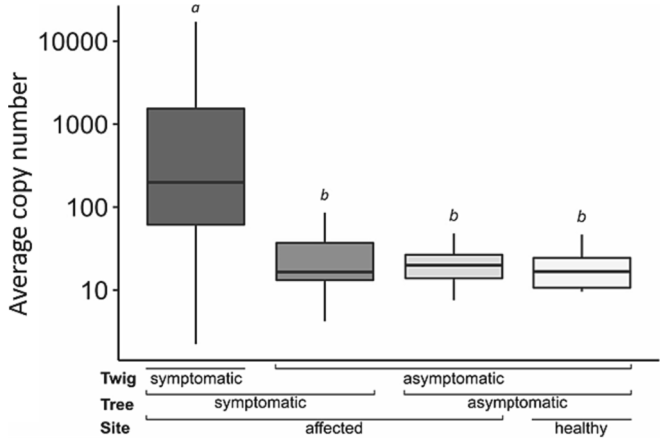

Fig. 6. Quantification of D. sapinea DNA (qPCR) in Scots pine twigs. The scale is logarithmic. 1) symptomatic and 2) asymptomatic twigs from symptomatic trees on affected sites; 3) asymptomatic twigs from asymptomatic trees on affected sites; 4) asymptomatic twigs from asymptomatic trees on healthy sites. n = 12 trees for each category; analysed were three twigs per tree. Same letter above box indicates no difference between twig category (ANOVA; α = 0.01).

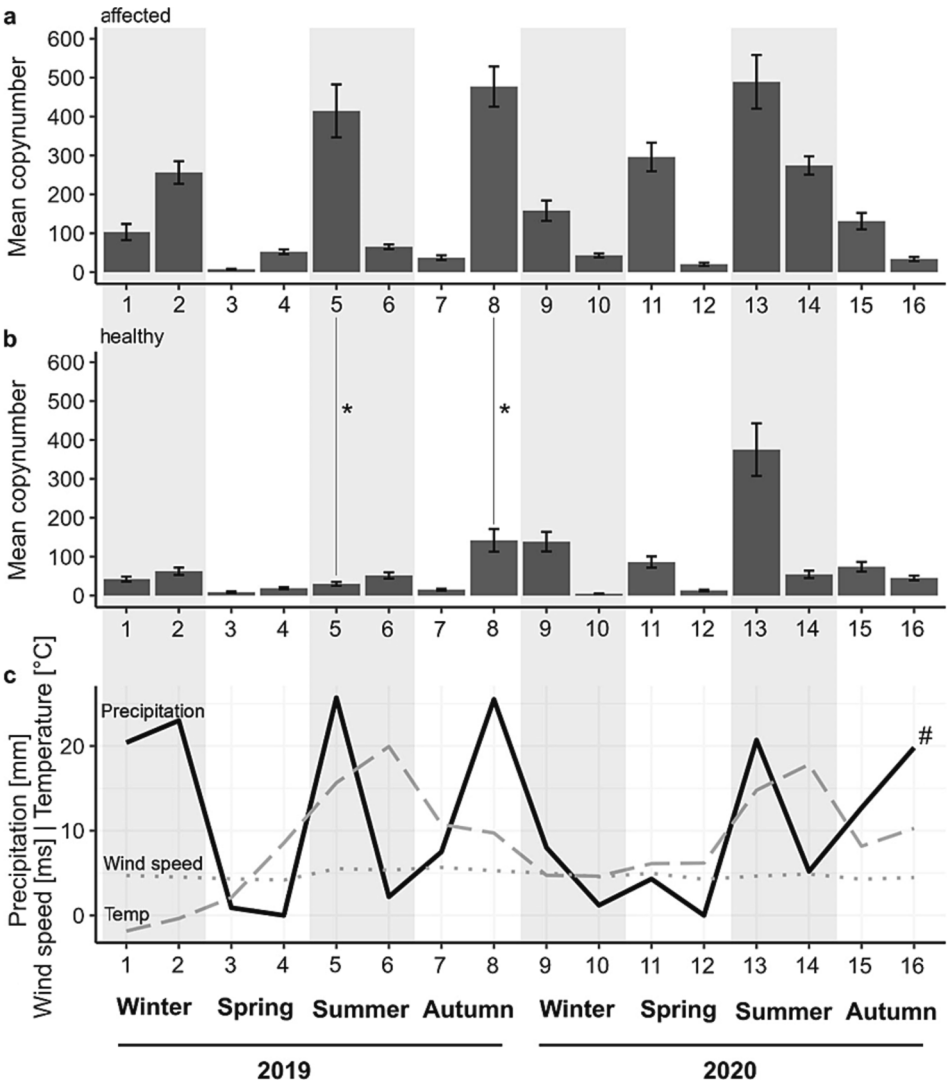

Fig. 7. Mean aerial spore load on the affected sites in relation to climate conditions during a total of 16 sample weeks, 2019 and 2020, on Gotland, Sweden. Mean copy number of D. sapinea DNA (qPCR) extracted from filter paper spore traps. Pairwise comparison of D. sapinea DNA quantity between a) affected and b) healthy sites. Significant difference ($p < 0.05$) labelled with *. Each filter was exposed for one week; four traps were installed on four affected sites per week. c) Total precipitation, mean wind speed, and mean temperature during each sampling week. Significant correlation of precipitation with D. sapinea DNA quantification labelled with # ($R2 = 0.66$, $p = 0.005$).

to healthy sites. However, detected DNA quantities varied greatly between the traps within a site. A statistically significant higher quantity of DNA at affected sites was only found in sample weeks 5 and 8 (Fig. 7, Table S4).

4. Discussion

Diplodia tip blight has raised attention as an emerging forest disease in Fennoscandia with observations of single symptomatic infections in the northern Baltics since 2007 (Adamson et al., 2015; Müller et al., 2019), followed by the first detected outbreak in a Scots pine stand in Sweden in 2016 (Brodde et al., 2019). Given the new occurrence of *D. sapinea* infections in Northwest Europe, the development of affected pines has been one of the central questions since then. In this study, we provide data on how pines affected by a drought event recover and which potential role *D. sapinea* played in crown dieback in that process.

We set up a study of Scots pine in eight sites on Gotland in 2018, during one of the most severe droughts recorded in the last 200 years (Moravec et al., 2021). We studied the effects of drought and Diplodia tip blight on crown dieback and mortality or recovery of Scots pine.

Scots pine trees undergoing drought-induced crown dieback have been shown to be able to recover as soon as water-stress is released (Dobbertin et al., 2010; Eilmann et al., 2013). However, high crown dieback under drought poses a long-term risk for the future performance of the tree, where loss of needles is associated with reduced carbon uptake up to four years post-drought, as well as increased mortality in occurrence of a new drought event (Galiano et al., 2011). Similarly, repeated attacks by the dieback fungus *Gremmeniella abietina* can result in severely reduced crown recovery and increased mortality ten years post the initial outbreak (Oliva et al., 2016).

Crowns of trees at the affected sites had lost about 25% of their foliage during the period of our study. After the first year, the average crown dieback of the trees doubled with limited signs of recovery. It was at this phase that the majority of mortality was observed. Trees with high dieback levels in 2018 showed an increased likelihood of dying in 2019. This is consistent with previous studies that reported a delayed response affecting the trees after a drought event (Rebetez and Dobbertin, 2004; Martínez-Vilalta et al., 2012). Alternatively, observed mortality and increase in crown dieback may have been a consequence of prolonged drought at the sites where the groundwater table was lower

than normal also in 2019, though the intensity of the drought in Scandinavia was lower compared to the drought of 2018 (Moravec et al., 2021; Rakovec et al., 2022).

Two years after the initial drought, a recovery of the new crown was apparent in surviving trees, also in the group of trees classified as severely damaged. In fact, single pines with very severe crown dieback of up to 90% were showing signs of recovery, which is consistent with studies that show that trees even with very high drought-induced crown dieback (>50%) are able to recover once water availability improves (Dobbertin et al., 2010; Eilmann et al., 2013). In general, recovery was not correlated with initial dieback levels. Biases in the method of visual crown dieback estimation cannot be excluded. A likely error rate in the estimates of approximately +/-10% makes differentiating low crown dieback levels (<30%) difficult. Anyhow, recovery independent of initial dieback might be in line with the opportunistic nature of *D. sapinea*. As soon as the major factor stressing the host is released, the impact of *D. sapinea* decreases.

The spore trapping verified that *D. sapinea* was present in the studied region on Gotland, also on healthy sites. In agreement with this observation, pines on healthy sites did develop mild symptoms of Diplodia tip blight during the two years of the study, though disease incidence was substantially lower compared to the affected sites (see Fig. 3a, b). In earlier studies, spore dispersal of *D. sapinea* was shown to be related to precipitation (Brookhouser and Peterson, 1971; Swart et al., 1987b). The observation in the current study, that there is an association with precipitation during sampling, agrees with these studies. However, no significant seasonal pattern in spore dispersal could be observed, in contrast to previous studies (e.g. Kuntzmann et al. (2009)). The absence of such a pattern could be an effect of the limited number of sampled weeks. Alternatively, the sample period might not fully reflect patterns of spore dispersal of *D. sapinea* in Northern latitudes. At least the first monitored year showed atypical weather for Scandinavia.

It is likely that site properties contributed to the contrasting patterns of crown dieback establishment between affected and healthy sites. The soil type has previously been shown to influence the incidence and abundance of *D. sapinea* (Munck et al., 2009). All healthy sites were located on, or on the edge of, soil types with higher water-holding capacities, while all affected sites were located on bedrock. Consequently, it is not impossible that the drought's impact was more severe at the affected sites.

D. sapinea was found to be associated with symptomatic infections in Scots pines showing crown dieback. Endophytic infections of *D. sapinea* could be detected in healthy twigs, independently of the health status of the tree or site. However, the analyses indicated very low levels of endophytic *D. sapinea* colonisation. A comparable study by Oliva et al. (2021) investigated the abundance of *D. sapinea* DNA in *Pinus* spp., which developed Diplodia tip blight symptoms after a hailstorm in summer 2018 in Spain. That study also found the highest amounts of *D. sapinea* in the symptomatic trees on an affected site. Furthermore, asymptomatic trees on the affected and healthy sites showed an endophytic presence of *D. sapinea* with generally lower levels. Nevertheless, asymptomatic trees on the affected site showed significantly higher quantities of *D. sapinea* DNA compared to asymptomatic trees on the healthy sites in the study by Oliva et al. (2021). This might reflect different patterns in colonisation of asymptomatic trees within the affected site studied in Spain compared to the results of the present study. Further studies are needed to investigate colonisation patterns of *D. sapinea* in pines within and outside areas exposed to stress factors. Comparing regions where Diplodia tip blight has been recorded for a long time with regions showing a recent emergence of this stress-related disease might improve damage predictions of forest health.

It is not possible to separate the effect of drought from the impacts of Diplodia tip blight in the observed crown dieback of Scots pine. Nevertheless, the combined effect leading to increased mortality of highly damaged (>70%) trees was comparable to the mortality of *Pinus* spp. in a hail-storm-triggered outbreak of Diplodia tip blight in Spain (Caballol et al., 2022). In the drought-triggered crown dieback, the presence of *D. sapinea* might have made a difference in recovery or mortality in medium-damaged trees, where additional loss of needles caused by *D. sapinea* infection could have pushed a tree over the threshold to mortality.

In the present study, we showed that *D. sapinea* is likely to have contributed to the observed crown dieback of Scots pine after a severe drought. The opportunistic pathogen possibly had an impact on the affected trees in their ability to recover post-drought. Nevertheless, we could show that trees can recover, even if damages by drought and *D. sapinea* infections are high. Predictions of future drought frequencies depend on the respective region, season, and emission scenario (Spinoni et al., 2018), though an increasing impact of drought, not only on tree health, is generally expected as a consequence of climate change (Schär et al., 2004; Bellard et al., 2012). If severe drought frequently reoccurs in already affected regions, e.g. on the island of Gotland, drought could be considered a predisposing, as well as an inciting, factor of a potential further decline of Scots pine. *D. sapinea,* as a contributing factor, could accelerate the potential decline, leading to even higher mortality among pines similar to what was reported for *G. abietina* attacks (Oliva et al., 2016). At the same time, our findings reveal that the trees can recover from severe stress, suggesting that immediate sanitary cuttings of surviving trees in infested stands may not be the most viable management choice for stands on sites with poor site properties, as removal of trees could lead to increased soil erosion. However, when deciding on management options, one also has to take timber quality into account and severely affected trees with dieback of leader shoots may not develop into raw material for high-quality timber. Anyhow, further studies of the spread of endophytic *D. sapinea* infections in areas formerly unaffected by Diplodia tip blight, as well as a better understanding of the mechanisms behind symptomatic infections in the pathosystem *D. sapinea* – (Scots) pine, are needed to contribute to more precise predictions of forest health development in a changing climate.

Author contributions

JO, LB, and JS conceptualised the project. LB, JO, and JS designed the experiments. LB coordinated, and LB and MSÅ carried out field and laboratory work. KW coordinated spore trap sampling. ME supported laboratory work. LB and MSÅ analysed data. LB wrote the first manuscript draft. All authors contributed to writing and reviewing the manuscript.

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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.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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