FOREST HEALTH UNDER CLIMATE CHANGE: EFFECTS ON TREE RESILIENCE, AND PEST AND PATHOGEN DYNAMICS

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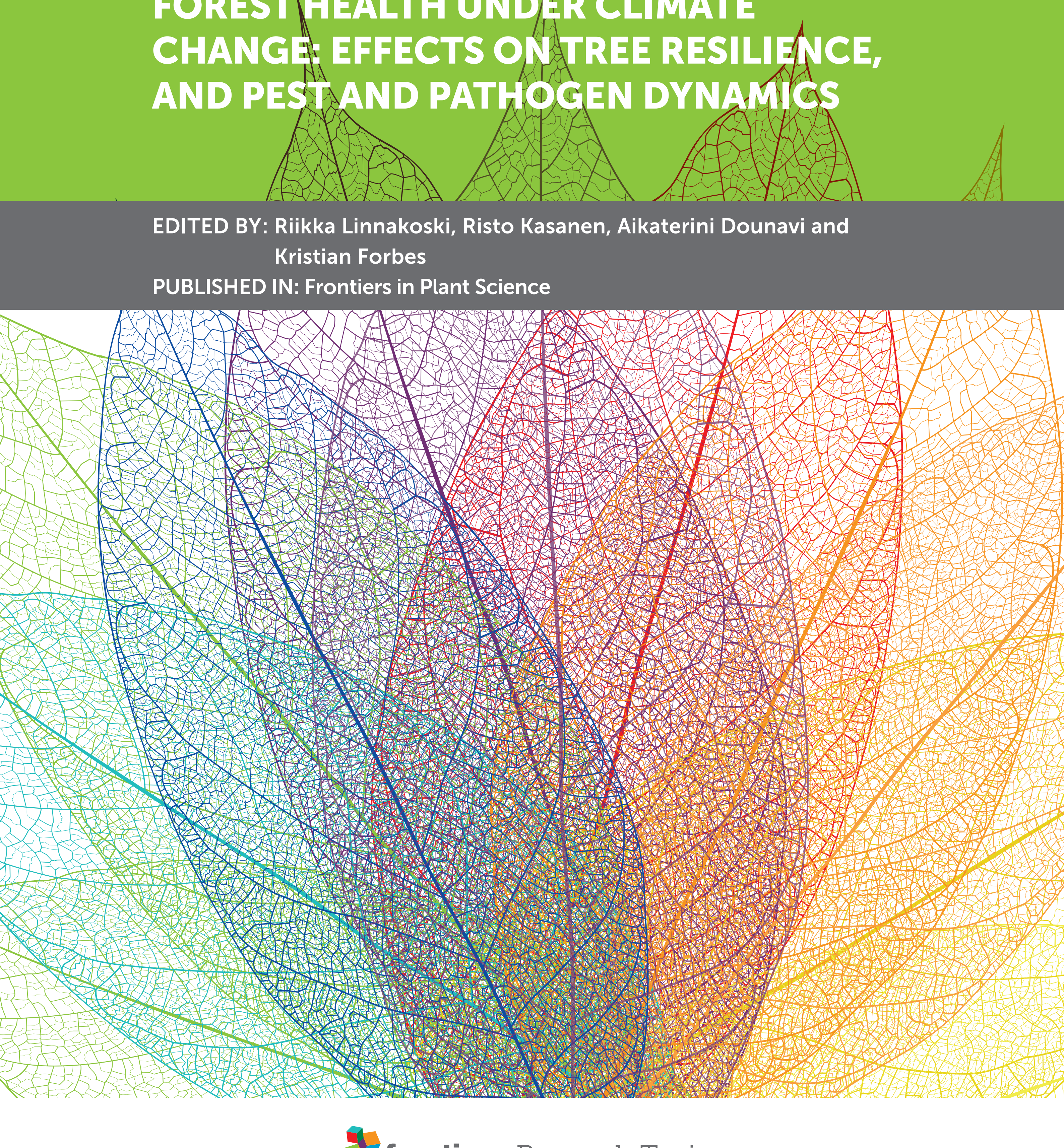

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FOREST HEALTH UNDER CLIMATE CHANGE: EFFECTS ON TREE RESILIENCE, AND PEST AND PATHOGEN DYNAMICS

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Table of Contents

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EDITORIAL
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Editorial: Forest Health Under Climate Change: Effects on Tree Resilience, and Pest and Pathogen Dynamics

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Editorial on the Research Topic

Forest Health Under Climate Change: Effects on Tree Resilience, and Pest and Pathogen Dynamics

INTRODUCTION

Climate change is having important effects on forest dynamics, which can be both positive and negative for natural and managed ecosystems. To date, most empirical and synthesis research has focused on climate change implications for tree species distributions and productivity, while the potential impacts on forest pests and pathogens and the effects on tree population health have been comparatively neglected. These present some of the greatest threats to global forest health under climate change. Climate change is altering the distributions and population structures of forest pests and pathogens, the way they interact with trees, and their evolutionary capacity, while also affecting the capacity of forest systems to resist and tolerate attacks.

Our ability to predict the impacts of interactions among biotic agents and climate change is currently very limited and has important implications for managing increasingly disturbed forests (both natural forest ecosystems and plantation forestry). Accumulating research of environmental effects on interactions among trees, pests, and pathogens suggests that there is an urgent need for systems-based research to investigate both longstanding and novel biotic interactions. More specifically, it is crucial to record changes in forest pest population dynamics, address the factors that trigger their outbreaks, and analyze the development of forest tree disease epidemics, as well as changes in tree susceptibility and resilience under a changing climate. Recent research has started to address these questions, and this Research Topic aims to contribute to the emerging field of climate change research.

In line with this objective, articles in this special issue fall under three broad topics: (1) tree responses to stress associated with climate change; (2) associations among climate, trees, and insect herbivory; and (3) forest pathogen outbreaks and disease spread under a changing climate.

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Tree Responses to Stress Associated With Climate Change

Varsamis et al. tested the adaptive potential of beech seedlings from two provenances in northeast Greece, which vary in the temporal distribution of precipitation. Seedling survival, growth, and leaf phenological traits were analyzed under simulated climate change conditions in a growth chamber; temperature and precipitation levels predicted for the year 2050 were applied for 3 years under two different irrigation schemes. Comparisons were also made between seedlings grown under field conditions. The study found that beech seedlings were generally able to survive under predicted climate change conditions. Differences in growth and leaf phenological traits occurred among treatment groups, but these varied among years and were sometimes absent. In a related study, Kohler et al. investigated the effects of soil liming on the drought tolerance of Norway spruce. Radial increment in relation to drought was compared over 30 years between limed and nonlimed sites. The study found no direct improvement of drought tolerance resulting from liming. However, positive effects were observed in one site treated with wood ash 30 years after the first lime treatment, and growth recovery and resilience after severe drought were marginally enhanced by liming. According to the results, spruce trees growing in limed soils faced a shorter period of stress and are consequently more resilient to pest and pathogen attacks.

Associations Among Climate, Trees, and Insect Herbivory

A study by Whipple et al. used long-term monitoring data to examine interactions among poor soil, severe drought, and moth herbivore susceptibility in a pinyon pine tree species that has suffered extensive climate change–related mortality. Contrary to the authors' hypothesis, they found that chronically stressed and herbivore-susceptible trees had smaller declines in performance during drought years than less-stressed trees. Tree mortality was also higher in finer soils, and moth abundance declined during droughts. The authors concluded that the results support the notion that stressed trees have adapted or acclimatized to harsh conditions, rendering them less sensitive to drought and herbivore effects. In another moth herbivory study, Camarero et al. assessed the effects of climatic stress and European gypsy moth (*Lymantria dispar dispar*) outbreaks on wood characteristics of radiata pine and chestnut tree populations in Spain. Radial growth decreased substantially in both tree species because of defoliation. Climate explained the majority of growth variability in defoliated chestnuts. However, radial growth and wood density were important for radiata pine, and the authors suggest these variables could be used as indicators for susceptibility to defoliation by the gypsy moth. Hentschel et al. performed an extensive analysis of ecological variables related to past outbreaks by three insect defoliators of Scots pine in Germany. They found that the dynamics of these herbivores were influenced by environmental variables in important but different ways. For example, *Lymantria monacha* feeding was mainly dependent on the tree stand surroundings, whereas *Diprion pini* was largely driven by climate, and *Dendrolimus pini* followed a mixed pattern determined by climatic and forest structure parameters.

Three studies examined genetic aspects of host resistance to climate changes and herbivore attack. Stone et al. sought to determine if susceptibility to herbivory influenced the diversity and composition of the extended community of arthropod species, using pinyon pine in the southwestern United States, which shows genetic-based resistance and susceptibility to pinyon needle scale. Among several findings, they reported that during extreme drought, arthropod community composition on scale-excluded trees resembled susceptible trees, indicating composition was strongly influenced by tree genetics independent of tree architecture. However, under moderate drought, community composition on scale-excluded trees resembled resistant trees, indicating traits associated with tree architecture became more important. Creyaufmüller et al. investigated interactions between oak seedlings from two distinct regions in Germany and root herbivory by cockchafer larvae, which can enhance the impacts of drought. Both oak populations were genetically variable, but no clear genetic pattern for feeding preference emerged. Contrary to field observations, larvae preferred seedlings from the source area where severe damage by cockchafer does not occur. Larvae feeding preference was thought to be driven by root-released volatile terpenes and benzenoids, and the authors identified five oak terpene synthase genes with geographically structured expression patterns. Finally, Six et al. tested whether a genetic basis is responsible for certain individual mature white bark and lodgepole pine trees in western North America surviving recent climate-driven outbreaks of the mountain pine beetle. They compared sequence repeats from surviving trees to those of the general tree populations and identified segregation among these tree groups that supports a genetic basis to survivorship.

Tree Pathogen Outbreaks and Disease Spread Under a Changing Climate

Two studies evaluated pathogen outbreaks in northern Europe, one of the areas where climate change effects are expected to be most pronounced. Brodde et al. reconstructed the development of the first large outbreak of *Diplodia* tip blight in Scots pine in northern Europe, which occurred in Sweden in 2016. They found that isolated attacks began approximately 10 years earlier and culminated in 90% of trees being attacked in 2016. Limited damage occurred in surrounding stands, suggesting the pathogen was a new introduction. Warm temperatures were associated with greater tree damage and slow growth, while cold and rainy conditions supported tree growth and impaired attacks. Significantly, the study identified no restrictions to *Diplodia* tip blight becoming a serious pathogen of northern European forests. In a similar study, Hietala et al. examined the increase of propagule pressure of *Hymenoscyphus fraxineus*, a fungus from Asia causing the dieback of European ash, by monitoring its sporulation in a newly infested stand in Norway. Interactions with a competing nonpathogenic native fungus, *Hymenoscyphus albidus*, were also investigated. The authors found that infection pressure was strongly influenced by summer temperatures (higher temperatures favor population growth) and that *H. fraxineus* populations grew exponentially during years with favorable local conditions.

In southern Italy, Colangelo et al. investigated the effects of drought and *Phytophthora* on populations of two declining oak species (*Quercus cerris* and *Quercus pubescens*) using comparisons with nearby nondeclining trees. The growth rate of both tree species decreased with drought and warming temperatures and was most pronounced in *Q. cerris*. No effects of *Phytophthora* on tree performance were identified, with the fungus being more prevalent in nondeclining trees. Pan et al. assessed the virulence of three blue-stain fungal associates of pine shoot beetles on Yunnan pine. These fungal species are associated with *Tomicus* shoot beetles, which often kill Yunnan pine trees in China. Their results show that all three blue-stain fungi (*Leptographium wushanense*, *Leptographium sinense*, and *Ophiostoma canum*) are potentially pathogenic to Yunnan pine, with *L. wushanense* and *L. sinense* more virulent than *O. canum*. The authors stated that the two *Leptographium* species may facilitate bark beetle colonization and contribute to their aggressiveness and, given sufficient spread, could cause significant damage to Yunnan pine forests across southwest China. Lastly, Linnakoski and Forbes extended this concept in an opinion article broadly discussing the role of fungal symbionts of wood-boring insect pests and their ability to both inflict their own damage on trees and seedlings and to enhance the severity of insect damage. These implications are likely to change and potentially be amplified under climate change as insect vectors invade new areas. The authors highlight how little we understand the effects of fungal pathogens in comparison to their insect vectors and outline gaps in knowledge to help advance this topic, including the need for baseline information of insect-fungal associations in their native ranges and for assessment of fungi roles in tree declines that are usually attributed to their more conspicuous insect vectors.

Overall, our understanding of the effects of climate change on forest pest and pathogen dynamics is in its infancy, with generalizations extremely difficult due to the huge amount of diversity in study systems and climate change effects across geographical regions. Forests are critical ecosystem components across many global regions, and a human capacity to predict and respond to pest and pathogen outbreaks in these habitats as environments continue to change is clearly required. We hope that the work presented in this Research Topic will help to both consolidate the field and motivate researchers to address the many gaps in knowledge.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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ORIGINAL RESEARCH
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Are Survivors Different? Genetic-Based Selection of Trees by Mountain Pine Beetle During a Climate Change-Driven Outbreak in a High-Elevation Pine Forest

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Increased mortality of forest trees, driven directly or indirectly by climate change, is occurring around the world. In western North America, whitebark pine, a high elevation keystone species, and lodgepole pine, a widespread ecologically and economically important tree, have experienced extensive mortality in recent climate-driven outbreaks of the mountain pine beetle. However, even in stands experiencing high levels of mortality, some mature trees have survived. We hypothesized that the outbreak acted as a natural selection event, removing trees most susceptible to the beetle and least adapted to warmer drier conditions. If this was the case, genetic change would be expected at loci underlying beetle resistance. Given we did not know the basis for resistance, we used inter-simple sequence repeats to compare the genetic profiles of two sets of trees, survivors (mature, living trees) and general population (trees just under the diameter preferred by the beetles and expected to approximate the genetic structure of each tree species at the site without beetle selection). This method detects high levels of polymorphism and has often been able to detect patterns associated with phenotypic traits. For both whitebark and lodgepole pine, survivors and general population trees mostly segregated independently indicating a genetic basis for survivorship. Exceptions were a few general population trees that segregated with survivors in proportions roughly reflecting the proportion of survivors versus beetle-killed trees. Our results indicate that during outbreaks, beetle choice may result in strong selection for trees with greater resistance to attack. Our findings suggest that survivorship is genetically based and, thus, heritable. Therefore, retaining survivors after outbreaks to act as primary seed sources could act to promote adaptation. Further research will be needed to characterize the actual mechanism(s) of resistance.

Keywords: ***Pinus albicaulis*, *Pinus contorta*, *Dendroctonus ponderosae*, whitebark pine, climate adaptation, climate change, natural selection**

INTRODUCTION

The capacity of forests to adapt to rapid climate change is not known. Their ability to adapt will vary greatly depending upon tree species, amount and type of genetic variation existing within and among populations, type and degree of change required, strength and type of selection pressure, heritability of desirable traits, and the timeframe over which selection is able to act. Many long-lived sessile organisms, including trees, are unlikely to be able to track shifting conditions through migration (Kremer et al., 2012). This is especially true for those restricted to montane ecosystems where movement higher in elevation ends at the top of the mountain and poleward migration is blocked by competitors, valleys, and development (Jump and Penuelas, 2005; Aitken et al., 2008; Dullinger et al., 2012). For many tree species and forests, adaptation will need to occur in place if they are to persist into the future (Aitken et al., 2008).

Bioclimatic envelope models used to predict range expansions and contractions of forest trees treat species as clones, with all individuals exhibiting identical responses (Mimura and Aitken, 2007). While these models are useful to provide estimates of shifts in habitat suitability, they can mask the high genetic diversity and geographic differentiation of most tree species (Mimura and Aitken, 2007; Thuiller et al., 2008; Reed et al., 2011). Likewise, most management focuses primarily on increasing forest resilience through manipulating stand structure and composition while ignoring genetic diversity, natural selection, and the potential for adaptation (Churchill et al., 2013; O'Hara and Ramage, 2013; DeRose and Long, 2014).

Except for highly fragmented or relictual populations, forest trees possess moderate to high levels of standing genetic variation and often exhibit considerable local adaptation and within and among population diversity (Austerlitz et al., 2000; Hamrick, 2004; Savolainen et al., 2007; Alberto et al., 2013). Adaptation of forests to climate change will depend upon the outcome of interactions between existing genetic diversity, phenotypic plasticity, and selection pressure over a relatively short period of time. However, adaptation in trees can be slow due to long generation times and low mortality of older, well-established, but increasingly maladapted trees that continue to contribute to the gene pool (Savolainen et al., 2007; Kuparinen et al., 2010). Long generation times can result in considerable genetic load with long lags between mean optimal genotype and existing climate (Kuparinen et al., 2010). Additionally, while phenotypic plasticity may allow some genotypes to maintain high fitness over a broad range of environmental conditions and aid in resilience to climate change in the short-term, it may slow down or hinder adaptation and persistence in the longer-term (Valladeres et al., 2014).

Adaptation in trees may be accelerated when new conditions or agents lead to high levels of mortality and directional selection in favor of heritable traits associated higher fitness and survival. For example, Kuparinen et al. (2010) used computer simulations to investigate rates of adaptation to longer thermal growing seasons and found that mortality of established trees was the key factor regulating the speed of adaptation with dispersal ability and maturation age having substantially lesser effects. Disturbances caused by agents that use selective behaviors in choosing individual trees, such as herbivorous insects that respond positively to tree stress, can elicit rapid microevolution even in slow-growing tree species (Petit and Hampe, 2006). Such agents may benefit forests in the long-term by increasing mortality of poorly adapted trees, enhancing the reproductive potential of surviving better-adapted trees, and reducing genetic lag loads in affected populations (Kuparinen et al., 2010; Pedlar and McKenney, 2017).

The mountain pine beetle (MPB) (*Dendroctonus ponderosae*) is a highly selective insect that chooses hosts based on a complex array of chemical cues whose production by the tree is influenced by both tree condition and genotype (Emerick et al., 2008; Blomquist et al., 2010). Secondary metabolic chemicals produced by the tree are used by MPBs to distinguish among tree species as well as to assess the relative strength of defenses of individuals (Blomquist et al., 2010; Raffa et al., 2017). Such chemicals also likely signal adequacy of nutritional content for brood production given that beetles avoid hosts of very poor quality (Taylor et al., 2006; Dooley et al., 2015). Using such cues, a MPB will decide whether or not to enter a particular tree and initiate a mass attack. Once in the tree, the insect converts some terpenes to pheromones important in initiating and sustaining the mass attack required to kill the tree (Blomquist et al., 2010). When MPB populations are low to moderate in size, weakened trees with poor defenses that require fewer beetles to overcome defenses are most often attacked (Boone et al., 2011). However, during outbreaks, MPBs may switch to attacking healthier trees that, although better defended, possess thicker phloem and higher nutritional contents for brood development (Boone et al., 2011). Interestingly, some trees escape attack even when MPB populations are present in high numbers and suitable hosts become increasingly scarce.

In this study, we investigated whether trees that survive MPB outbreaks are genetically different than those that are selected for colonization and killed. Our overarching hypothesis was that surviving trees do not escape by chance, but rather possess genetically based characteristics that confer resistance. The basis for resistance, whether it is the ability to tolerate warmer drier conditions without a reduction in defenses, a chemical profile that negatively affects MPB host location or selection, or some other phenotypic trait, is likely to be under genetic control (González-Martínez et al., 2006; Keeling and Bohlmann, 2006).

MPB outbreaks are triggered by extended periods of warm weather and drought (Meddens et al., 2012). The recent MPB outbreak in western North America was a magnitude larger than any recorded in the past and affected millions of hectares of pine forest (Meddens et al., 2012). The outbreak was primarily driven by climate although its severity was intensified in some areas by past logging practices and fire suppression (Taylor et al., 2006; Creeden et al., 2014; Buotte et al., 2017). Climate change also supported movement of MPB further north in British Columbia and eastward across Alberta into naïve forests (those with no prior history of MPB) of lodgepole pine and jack pine (*P. banksiana*, a novel species for MPB) (Burke et al., 2017). While the size and extent of the recent outbreak was far outside the historic norm, outbreaks of MPB are not unusual and have likely occurred for millennia. Selection by MPB during outbreaks, as well as persistent low-level activity during non-outbreak periods, are believed to have been a major force shaping constitutive and induced defenses in host pines (Raffa and Berryman, 1987; Franceschi et al., 2005). MPB activity in naïve forests can be expected to exert especially rapid and strong selection for host resistance because of high levels of susceptibility. Indeed, naïve lodgepole and jack pine forests exhibit lower defenses to MPB attack than those with a co-evolutionary history with the beetle (Clark et al., 2010; Cudmore et al., 2010; Raffa et al., 2013, 2017; Burke et al., 2017).

We focused on two tree species that have suffered high mortality by MPB in the recent outbreak. One is a relatively naïve host, whitebark pine (*Pinus albicaulis*), and the other is a highly co-evolved host, lodgepole pine (*P. contorta*). Whitebark pine is a high elevation tree that is considered a keystone in western subalpine ecosystems of the United States and Canada (Tomback et al., 2016). Historically, outbreaks in whitebark pine appear to have been rare and limited in size (Logan et al., 2010). During warm periods, beetles sometimes moved upslope from lower elevation outbreaks (Bartos and Gibson, 1990) where they killed some whitebark pine, but either did not reproduce successfully due to winter mortality, or completed only one or a few generations before the return of cold conditions once again limited them to lower elevations (Logan et al., 2010). The recent outbreak in whitebark pine has been extensive and has been driven by chronic warm temperatures that allowed the beetle to move into the subalpine and to persist there for an extended period (Buotte et al., 2016, 2017). With climate change, the presence of MPB in high elevation whitebark pine forests is expected to be persistent rather than occasional (Buotte et al., 2016, 2017). Whitebark pine exhibits many of the characteristics of a naïve host, including lower levels of defense chemicals and resin (Raffa et al., 2013, 2017). Reduced snow packs may also result in greater drought stress that may increase susceptibility (Larson and Kipfmueller, 2012). Outbreaks in this tree have been devastating in some areas, including the Greater Yellowstone Area, contributing to the recommendation that it be listed as an endangered species (United States Fish, and Wildlife Service [USFWS], 2011).

The second species studied was lodgepole pine, a co-evolved host that has experienced repeated extensive outbreaks in much of its range, likely over a long evolutionary period. Vigorous lodgepole pine typically exhibits strong constitutive and inducible defensive responses to beetle attack (Burke et al., 2017; Raffa et al., 2017). Outbreaks of MPB in lodgepole pine are considered natural disturbances that, much like fire, help maintain lodgepole pine forests by periodically regenerating new stands free of many diseases, initiating nutrient cycling, and stimulating regeneration, understory productivity, and supporting biodiversity (Dordel et al., 2008; Diskin et al., 2011; Pec et al., 2015).

Our objective in this study was to investigate whether whitebark and lodgepole pine growing in a mixed high elevation stand that survived the outbreak are genetically distinct. If so, this may indicate an increased potential for these pines to persist in the face of the more frequent and extensive outbreaks predicted due to a changing climate. We would expect genetic change at loci underlying beetle resistance but not at a genome-wide scale. Without knowing the basis for resistance in survivors, we chose to use inter-simple sequence repeats (ISSRs) to develop genetic profiles for whitebark and lodgepole pine. ISSRs target highly variable sequences within microsatellite regions (Parasharami and Thengane, 2012). Because ISSR markers can be used to detect high levels of polymorphism and are highly reproducible, they provide a powerful approach for comparing genetic diversity between individuals as well as within and among populations of plants including pines (Mehes et al., 2007; Parasharami and Thengane, 2012; Lucas-Borja et al., 2016). In many studies, ISSR profiles have been useful in marker assisted selection when particular markers were associated with particular traits (REFS). In our screens, we looked for patterns that indicted differences between survivors and susceptible trees.

MATERIALS AND METHODS

Site Description

This study was conducted at Vipond Park, a high-elevation plateau supporting a patchwork of grassland and open forest stands located on the Beaverhead National Forest, Montana, United States (2,501 m elevation, 45.6974°N, 112.9106°W). The site is relatively xeric with an understory of sagebrush and a diverse mixture of annual and perennial forbs. Vipond Park was chosen to take advantage of the high mortality to pines that occurred there during a recent high elevation outbreak of MPB (2009–2013) when approximately 93 and 75% of mature *P. albicaulis* and *P. contorta*, respectively, were killed. The relatively flat topography of the plateau combined with its location at the transition zone between lodgepole and whitebark pine-dominated forests allowed us to study the effects of MPB selection on more than one pine species growing under the same conditions and experiencing the same level of beetle pressure. Although *P. contorta* existed at lower numbers than *P. albicaulis* at the site, they were abundant enough to allow sufficient sampling to make comparisons with whitebark pine. Additionally, white pine blister rust infection incidence and severity were very low reducing the potential for the presence of the disease to influence the choice of individual host trees by the beetle (Six and Adams, 2007).

Transects

Transects were established in 2015 (*P. albicaulis*) and 2016 (*P. contorta*). These were variable length belt transects 2 m in width that started on the edge of a stand and then followed a randomly chosen bearing until another edge was reached at which point a new bearing was adopted to establish a new transect in the same or an adjacent stand. This process was continued until the desired number of trees per species per treatment were measured. When trees occurred in clumps (resulting from seed caching by Clark's Nutcrackers), we restricted measurements and samples to one tree per clump to avoiding sampling trees potentially originating from the same cone/parent.

Determination of the Diameter Distribution of Mountain Pine Beetle-Killed Pines

In initial transects, the diameter at breast height (DBH, 1.4 m above the soil line) of 100 *P. albicaulis* and 45 *P. contorta* killed by MPB were measured to estimate the diameter distribution of MPB-killed trees for each species. This distribution was used to inform our sampling of "survivors" (mature trees that survived the outbreak) so that a similar distribution was achieved, and to determine the diameter below which trees were not attacked.

Collection of Samples for Genetic Analysis

In 2015, transects were established as previously described. Thirty survivor *P. albicaulis* with diameters representative of the diameter distribution of MPB-killed *P. albicaulis* were located on the transects. For each tree, DBH was measured and each was rated for white pine blister rust infection severity using the method of Six and Newcomb (2005). Then, approximately 30 current-year needles were collected and placed in a small plastic bag that was sealed and placed on ice in a cooler. In the lab, needles were placed into silica gel for drying and preservation. In 2016, this procedure was repeated for *P. contorta* ($n = 20$) (except for rust rating) in the same stands sampled the previous year.

The smallest diameters of *P. albicaulis* and *P. contorta* killed by MPB were 12 and 18 cm, respectively. Because beetle-killed trees did not yield DNA, we used this information to choose a second set of living trees for sampling of each species we designated as the "general population." These trees were expected to approximate the genetic structure of the population of each tree species at the site without beetle selection and so should contain a mix of survivor and "susceptible" genotypes. If our hypothesis was correct that survivors were genetically distinct from beetle-susceptible trees, then we expected only a few general population trees would have genotypes matching those of survivors (roughly reflecting the proportion of mature survivors to mature MPB-killed trees at the site). To sample general population trees, we established similar transects as before, but collected needles from trees between 9–11 and 14–17 cm DBH for *P. albicaulis* ($n = 36$) and *P. contorta* ($n = 20$), respectively.

DNA Extraction and Amplification

Needles (3–5) from each sample were ground to a fine powder in liquid nitrogen using a mortar and pestle. DNA was then isolated from each sample using a Qiagen DNeasy Plant Kit (Qiagen, Valencia, CA, United States) following the protocol provided by the manufacturer.

Five ISSR primers were chosen for use (**Table 1**). Not all primers worked equally well for both species of trees. Therefore, we chose three primers for use with *P. albicaulis* and four for *P. contorta*. Two primers overlapped in use for both trees (**Table 1**).

For amplification we used a 25 μl reaction mixture consisting of 12.5 μl Promega Master Mix (Promega, Madison, WI, United States), 2.5 μl RNA-free water, 8 μl of 0.5 M primer and 2 μl of DNA template. Reactions were run individually with one of the five ISSR primers. PCR was conducted with one cycle denaturation at 95°C for 5 min, followed by 42 cycles of denaturation at 95°C for 1.3 min, annealing at 47°C for 2 min, and extension at 72°C for 1 min. A final cycle was conducted at 72°C for 1 min and final products were held at 6°C (Parasharami and Thengane, 2012).

PCR products were visualized in a 1% agarose gel prepared using 1× tris borate buffer (TBE) to which 2 μl ethidium bromide per 100 ml gel was added. A 100 bp ladder (Promega, Valencia, CA, United States) was placed in the first lane of each gel to provide a reference for scoring bands. Amplified DNA was loaded into the remaining lanes with bromophenol blue as a running dye. Each gel was run with 1× TBE as a running buffer at 70 mA until the dye moved ¾ of the length of the gel. Gel images were captured using a UV table. Any sample that gave ambiguous results (no, faint, or smeared bands) was repeated. Approximately 20% of samples were rerun and compared to check for consistency in results. Only samples exhibiting clear bands were included in the final analysis. Bands were scored manually.

Data Analysis

Diameter Distributions

A two-sample *t*-test was used to compare mean diameters among groups (survivor, general population, and beetle-killed) using Statistix 7 (Analytical Software, Tallahassee, FL, United States).

Genetic Analysis

Bands were scored as present (1) or absent (0) to develop a binary matrix combining data for all primers by tree species. The matrices were analyzed in Popgene v. 1.32 (Yeh et al., 1997) (assuming each group was in Hardy-Weinberg equilibrium) to calculate percent polymorphism, the Shannon information index (I), Nei's gene diversity index (h), total genetic diversity (H_T), genetic diversity within groups (survivor, general population) (H_S), and evidence for deviations from neutrality (selection) with an overall Ewens–Watterson test for neutrality. Population genetic structure was investigated using STRUCTURE v. 2.3 (Pritchard et al., 2000). The *admixture* model was used with a 10,000 burn-in period and 10,000 Markov Chain Monte Carlo replications. Twenty runs were performed with each value from 1 to 10 to estimate the optimal number of clusters (K) using the ΔK statistic (Evanno et al., 2005).

For each tree species, we examined genetic variation between groups using analysis of molecular variation (AMOVA) in GenAlEx 6.5 (Peakall and Smouse, 2006). We then conducted

TABLE 1 | Primers used for ISSR amplification.

Primer ID	Sequence	Tree species
HB12	CAC CAC CAC GC	*Pinus albicaulis*
17899A	GTG TGT GTG TGT CA	*P. albicaulis, P. contorta*
17901	CAC ACA CAC ACA AG	*P. contorta*
UBC 807	AGA GAG AGA GAG AGA GT	*P. albicaulis, P. contorta*
UBC 811	GAG AGA GAG AGA GAG AC	*P. contorta*

a principle coordinates analysis (PCoA) in GenAlEx based on genetic distances between individual trees in the two groups for each species of tree. Genetic distance matrices were developed for each tree species in the *Restml* program and then imported into *Neighbor* in PHYLIP 3.67 (Felsenstein, 2005) to produce an unweighted neighbor-joining tree. The tree was visualized using TreeView 1.6.6 (Page, 1996).

RESULTS

Diameter Distributions and Blister Rust Infection Severity

The mean, median, and range of diameters of beetle-killed and survivor *P. albicaulis* were similar (**Table 2**). The mean diameter was not significantly different between survivor and beetle-killed trees, while the diameter of general population trees, as expected, differed significantly from both groups (**Table 2**). The same was true for *P. contorta* (**Table 2**). Similarly, mean diameters of MPB-killed and survivor *P. albicaulis* and *P. contorta* did not differ from one another. However, the minimum size of tree attacked by the beetle differed by tree species resulting in the choice of different diameter distributions for sampling general population trees (**Table 2**). Blister rust infection severity was overall very low at the site, but significantly lower in survivors (mean = 1.3, *SD* = 1.8) than in general population trees (mean = 1.7, *SD* = 2.4; *F* = 1.63, *df* = 65, *P* = 0.013; potential range 0–18).

Genetic Analyses

Pinus albicaulis

Three primers (17899A, HB12, and UBC807) resolved well for *P. albicaulis* and were used for ISSR analysis. A total of 28 loci (bands) were resolved using the three primers (**Table 3**). Mean percent band polymorphism (BP) for all primers for all trees (general population and survivors) combined was 96.4% and this value was similar to the BP for each group individually. The Shannon information index and Nei's gene diversity was lower in general population trees compared with survivors (**Table 2**). Nei's unbiased measure of genetic identity between the survivor and general population trees was 95% while genetic distance was a corresponding 5%.

H_T, the total genetic diversity between the two study groups, was 0.26, and the diversity within groups, H_S, was 0.24. Seven of 28 loci (25%) exhibited significant differences between observed and expected frequencies of bands between the two groups (data not shown). However, no bands were unique to either group. The Ewens–Watterson test for neutrality detected only one marginally non-neutral locus. AMOVA indicated 87% of the variation exhibited existed within groups and 13% existed between groups.

The neighbor-joining tree resolved most general population trees together in the basal clades while one major terminal clade contained all survivor trees as well as eleven general population trees that were distributed throughout the clade (**Figure 1**). The results of Bayesian clustering using STRUCTURE indicated that the optimal *K*-value was 3 with the general population dominated by one cluster (red, **Figure 2**) and survivors dominated by the other two (blue and green, **Figure 2**). The eleven general population trees that clustered with survivor trees in the neighbor-joining tree exhibited predominantly blue and green profiles in the STRUCTURE bar graph (shown with asterisks) indicating similarity to survivors (**Figure 2**). In the PCoA, the first two principle coordinates explained a total of 33% of the variation associated with the two groups. Adding the third, 43.55% was explained. In general, the eleven general population trees that clustered with survivors in the neighbor-joining tree resolved separate from other general population trees and with survivors in the PCoA (**Figure 3**).

Pinus contorta

Four primers resolved well for this species (17899A, UBC807, UBC901, and UBC811). Using these primers, we were able to resolve a total of 85 bands. The mean percent BP across all primers and groups was 98.82. This was considerably higher than BP for the general population (89.4%) and survivor (88.2%) trees (**Table 2**). The mean number of effective alleles was slightly lower than the mean number of observed alleles. Shannon's information index was similar within and across groups while Nei's gene diversity was lowest in survivors and highest for both groups combined (**Table 2**). Nei's unbiased genetic identity and diversity between the two groups was 93 and 7%, respectively.

H_T was 0.26 and H_S was 0.25, similar to values for whitebark pine. Allele frequencies were significantly different between survivors and general population trees at 12 of 85 loci (14%) (**Table 3**). No bands were unique to either group. The Ewan–Watterson test for neutrality indicated that six loci in the general population and nine loci in the survivors were outside the 95% CI indicating non-neutrality. All had positive *F*-values greater than the upper bound indicating a potential for directional selection. AMOVA indicated that 89% of variation occurred within groups while 11% occurred between groups.

The neighbor-joining tree partitioned general population and survivor trees into several clades (**Figure 4**). Most (55%) general population trees resolved in one clade. The remainder resolved into two clades interspersed with survivors (**Figure 4**). The general population trees that resolved with survivors in the neighbor-joining tree shared clusters with survivor trees in the STRUCTURE analysis (**Figure 2**) and also partitioned with survivor trees in the PCoA (**Figure 3**). The first two principle coordinates in the PCoA explained 21.5% of the

TABLE 2 | Summary statistics for diameter breast height (cm) of *Pinus albicaulis* and *P. contorta* by group.

Tree	Group	N	Mean (*SD*)	Median	Minimum	Maximum
P. albicaulis	Beetle-killed	75	24.5 (5.3)^a	24.2	12.0	37.3
	Survivor	30	25.0 (5.2)^a	24.1	17.0	37.3
	General	36	10.0 (0.6)^b	10.0	9.0	11.0
P. contorta	Beetle-killed	45	26.7 (5.0)^a	26.4	17.5	36.8
	Survivor	20	27.5 (5.4)^a	29.9	18	37.2
	General	20	15.3 (0.9)^c	15.2	13.9	16.8

Different letters following means denote statistically significant differences, α = 0.05.

TABLE 3 | Percent band polymorphism (BP), number of observed (N_a) and effective (N_e) alleles, Shannon's Information Index (*I*), Nei's gene diversity (*h*), and diversity between (H_T) and within groups (H_S), presented by tree species and group.

Tree species	Group	N	%BP	N_a	N_e	*I*	*h*	H_T	H_S
P. albicaulis	Survivor	30	96.58	1.97 (0.19)	1.39 (0.25)	0.40 (0.19	0.22 (0.14)		
	General	36	96.55	1.97 (0.19)	1.32 (0.27)	0.36 (0.14)	0.25 (0.14)		
	Combined	66	96.43	1.96 (0.19)	1.41 (0.19)	0.42 (0.18)	0.26 (0.14)	0.26 (0.10)	0.24 (0.01)
P. contorta	Survivor	20	88.24	1.88 (0.32)	1.40 (0.33)	0.25 (0.17)	0.39 (0.23)		
	General	20	89.41	1.89 (0.31)	1.40 (0.31)	0.26 (0.16)	0.40 (0.22)		
	Combined	40	98.82	1.90 (0.11)	1.41 (0.29)	0.27 (0.14)	0.42 (0.18)	0.27 (0.02)	0.25 (0.02)

Means are accompanied by standard deviations in parentheses.

variation between the two groups. Adding the third component explained 31%.

DISCUSSION

Knowledge of the effects of bark beetle outbreaks on host tree population genetic structure and resistance to attack will be increasingly valuable as climate change drives more frequent outbreaks and facilitates the movement of beetle species into naïve forests. Outbreaks of MPB seldom kill all mature trees despite high beetle numbers during population peaks. Our results suggest that surviving trees possess a wealth of information that can be used to inform our understanding of the genetic and phenotypic bases for resistance and to develop management approaches that support forest adaptation.

We found that surviving mature trees in a high elevation forest of whitebark and lodgepole pine were genetically distinct from "general population" trees that were assumed to represent the genetic structure of the population pre-outbreak and without selection by the beetle. In line with our hypothesis, a low percentage (<10%) of "survivor" genotypes were identified within the general population. The proportion of these survivors roughly mirrored the proportion of mature trees that survived the outbreak at Vipond Park. The neighbor-joining tree, the PCoA and the STRUCTURE analyses each indicated strong differentiation between survivors and "susceptible" individuals and identified the same trees as survivors within the general population. In the STRUCTURE analysis for both whitebark and lodgepole pine, susceptible trees belonged to one cluster while survivor trees belonged to two other clusters. This separation can also be seen in the PCoA. Further research will be needed to

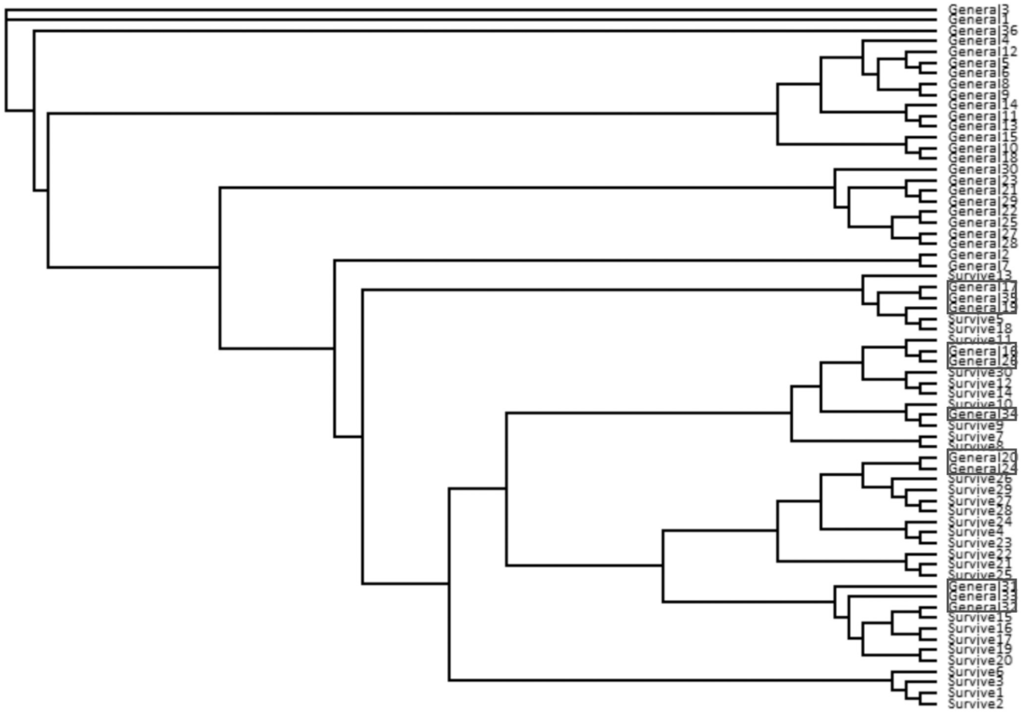

FIGURE 1 | Neighbor-joining tree from ISSR data for *Pinus albicaulis*. General = general population trees (with no *Dendroctonus ponderosae* selection). Survive = mature trees surviving *D. ponderosae* outbreak. Trees in boxes correspond to trees with arrows in **Figure 2** and in ellipses in **Figure 3**.

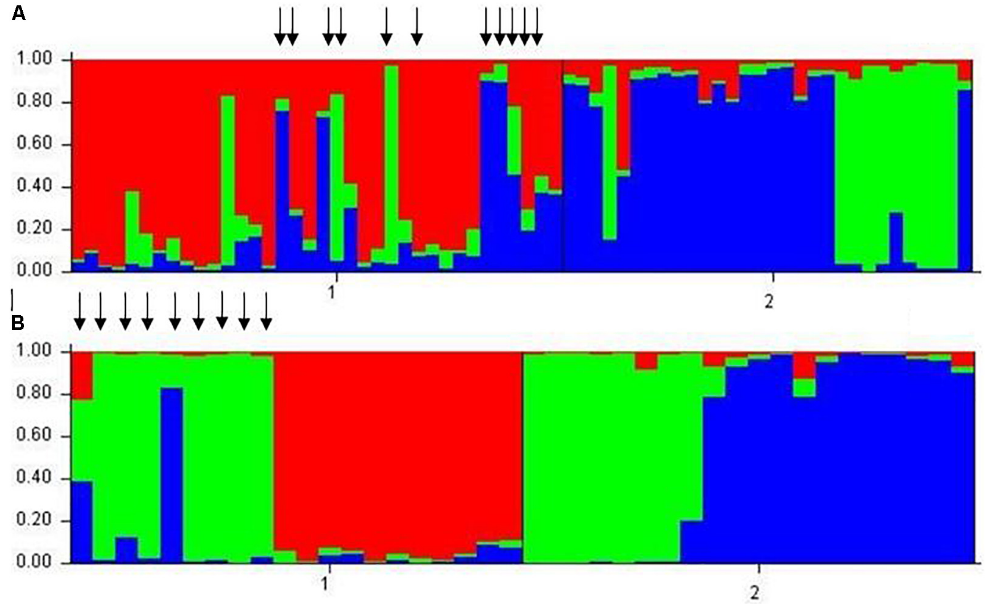

FIGURE 2 | Results of Bayesian clustering using STRUCTURE. Individual trees are represented by vertical bars. Colored segments represent the tree's estimated proportion similarity to each of the three clusters (red, blue, and green) optimally defined by STRUCTURE. **(A)** *Pinus albicaulis*. Arrows denote general population trees that resolved with survivors in neighbor-joining tree in **Figure 1**. **(B)**. *Pinus contorta*. 1 = general population trees. 2 = survivor trees.

determine whether the patterns we detected are indeed indicative of resistance, and if so, whether there are multiple or overlapping factors that account for survivorship.

We found surprisingly high levels of differentiation between survivor and general population trees in both species of pine. For whitebark pine, Nei's genetic distance between survivor and general population whitebark pines was 5%, a value that would indicate moderate differentiation if these comparisons had been made between tree populations. Likewise, AMOVA indicated 13% of the genetic variation present existed between groups. Considering that the trees in this analysis were not from different populations, but rather grew intermixed at the same site, these values seem strikingly high. Likewise, for lodgepole pine, Nei's genetic distance was 7%, and AMOVA indicated 11% of variation occurred between the groups.

These results indicate the presence of genetically based resistance in both pine species and that trees with resistant genotypes are not selected for attack. It has been thought that once MPB achieve high population levels during outbreaks, the selection of individual trees based on tree-produced compounds and condition becomes swamped by high levels of aggregation pheromone production and competition for increasingly rare hosts (Safranyik and Carroll, 2006). However, our results suggest that beetles remain selective even as outbreaks peak and collapse.

We chose ISSR profiling as a first step to determine whether survivors were different than trees chosen by MPB for colonization. This PCR-based method detects high levels of polymorphism, is highly reproducible, and allows the screening of a large number of trees relatively rapidly and economically. Unfortunately, this method cannot tell us why survivors are different, only that they are. Further study will be needed to further investigate whether survivors are indeed highly resistant and, if so, to determine the actual basis behind resistance. Ongoing studies are investigating correlations among genetic profiles of survivor and "susceptible" trees with phenotypic traits including defensive chemistry and growth rates in relation to climate. Genomic approaches will also be extremely useful to elucidate the basis of resistance.

This study corroborated the findings of other studies that found that MPB colonizes smaller diameter whitebark pine than lodgepole pine during outbreaks (Dooley et al., 2015). The mortality of younger whitebark pine trees indicates a more severe impact of MPB outbreaks on whitebark pine forests, at least in the short term, because advanced regeneration is killed along with large trees. However, the loss of large and mid-diameter trees may serve to open areas for nutcracker caching of seeds from the remaining resistant trees, potentially increasing the frequency of those genotypes and phenotypes at the site and within the larger population.

In a previous study, Six and Adams (2007) found that as infection severity increased so did the likelihood of attack by the beetle. However, while we found that white pine blister rust infection severity was significantly higher in general population trees than survivors, the mean level of infection severity at the site was very low and the size effect between means for survivors and general population trees was very small. Therefore, we feel it is unlikely blister rust played a significant role influencing beetle dynamics at the study site.

A caution is in order in interpreting our results. We were unable to amplify DNA from MPB-killed trees which forced us to use smaller diameter "general population" trees as a substitution

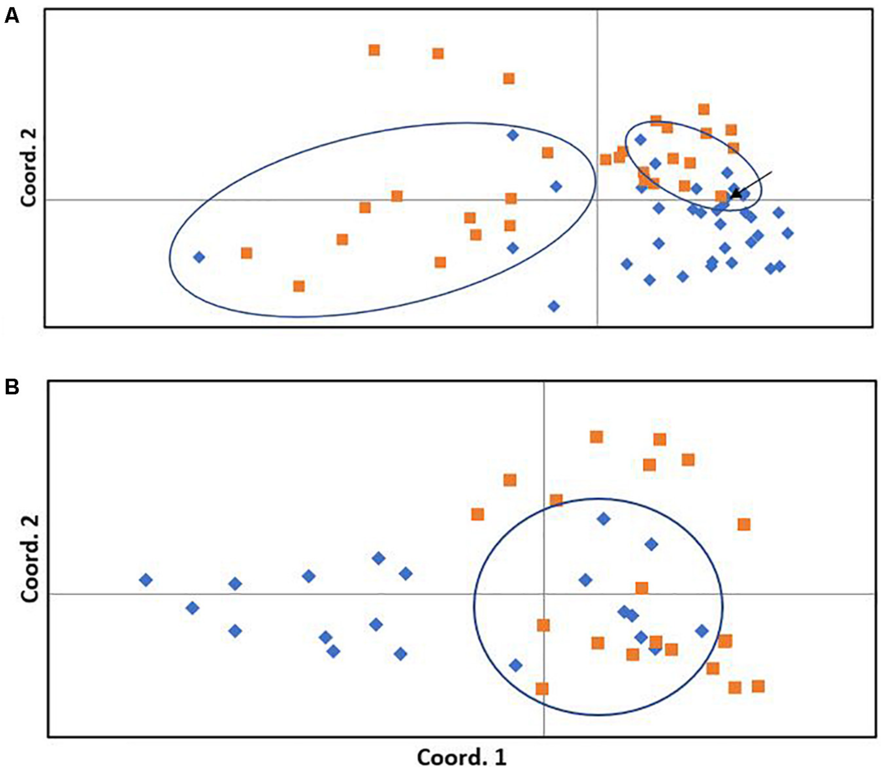

FIGURE 3 | Principle coordinates analysis of general (blue diamonds) and survivor (orange squares) trees. **(A)** *Pinus albicaulis.* The first and second coordinates explain 19.29 and 13.67% of the variation among trees, respectively (total 33%). **(B)** *Pinus contorta*. The first and second coordinates explain 10.98 and 10.55% of the variation among trees, respectively (total 21.5%). Ellipses surround general population trees that clustered with survivors in the neighbor-joining tree (**Figure 1** for *P. albicaulis*, **Figure 4** for *P. contorta*) and correspond to trees marked with an arrow in the STRUCTURE analysis (in this figure). Arrow indicates one general population tree within the ellipse that did not cluster with survivors in the neighbor-joining tree.

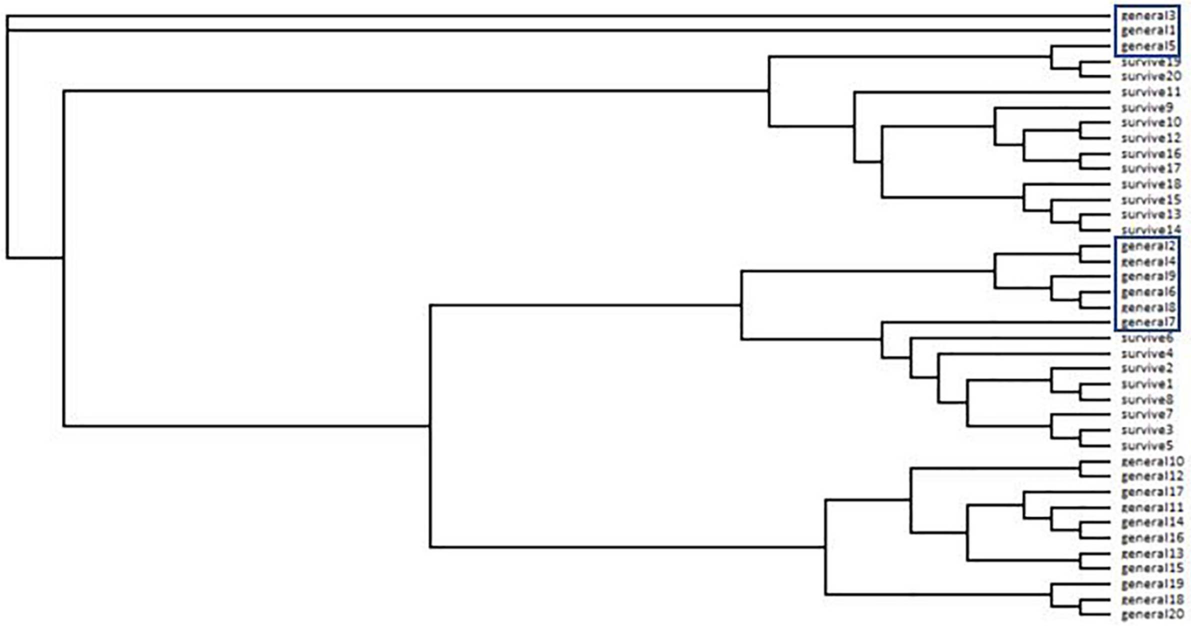

FIGURE 4 | Neighbor-joining tree from ISSR data for *Pinus contorta*. General = general population trees (with no *Dendroctonus ponderosae* selection). Survive = mature trees surviving *D. ponderosae* outbreak. Trees in boxes correspond to trees with arrows in **Figure 2** and in ellipses in **Figure 3**.

for "susceptible" trees. These trees were mature reproductive trees and only slightly smaller than trees selected by the beetle for colonization; however, some or all may constitute a cohort that regenerated under different environmental conditions resulting in a genetic structure unrepresentative of the larger trees that were available for selection by the beetle. However, the proportional distribution of survivor and "susceptible" trees in the neighbor-joining trees, PCoAs and STRUCTURE analyses indicate that the general population samples were likely appropriate proxies.

With climate change supporting the invasion of aggressive bark beetles into naïve forests, and predictions of more frequent and severe outbreaks, it is increasingly important to understand the capacity of trees to adapt and persist (Millar et al., 2007; Ramsfield et al., 2016). While the massive mortality of pines in western North America in recent years is cause for concern, we should also look at these hard-hit forests as opportunities to learn. In almost all cases, affected forests are not completely dead–they retain many living large diameter trees. If these trees are genetically different than those selected and killed by the beetles as our study suggests, these trees may aid in *in situ* adaptation and persistence. They may also be key to developing management and trajectories that allow for forest adaptation. For example, retaining surviving trees as a primary seed source, rather than removing them during salvage operations could support *in situ* adaptation. In contrast, the effects of natural selection in these stands could be instantly negated by clearcutting or replanting with general seed stock.

Supporting forest adaptation is critical in this time of rapid change (Millar et al., 2007). Given the great expanses of forest that are being affected by climate change and the fact that most will need to adapt *in situ*, it is imperative we begin to move past structural approaches to consider the genetic capacity of forest trees to adapt. The high degree of standing genetic variation found in most forest trees indicates many will have considerable ability to adapt. We need to be cognizant of adaptation that is occurring so that our management approaches act to support rather than hinder natural selection for traits needed under future conditions.

AUTHOR CONTRIBUTIONS

DS conceived of the project, participated in field work, conducted data analysis, and wrote the manuscript. CV conducted lab work on whitebark pine and participated in field work and data analysis. MC conducted field and lab work on lodgepole pine and contributed to data analysis.

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Propagule Pressure Build-Up by the Invasive *Hymenoscyphus fraxineus* Following Its Introduction to an Ash Forest Inhabited by the Native *Hymenoscyphus albidus*

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Dieback of European ash, caused by the ascomycete *Hymenoscyphus fraxineus* originating from Asia, has rapidly spread across Europe, and is threatening this keystone tree at a continental scale. High propagule pressure is characteristic to invasive species. Consistently, the enormous production of windborne ascospores by *H. fraxineus* in an ash forest with epidemic level of disease obviously facilitates its invasiveness and long distance spread. To understand the rate of build-up of propagule pressure by this pathogen following its local introduction, during 2011–2017 we monitored its sporulation at a newly infested ash stand in south-western Norway characterized with mild winters and cool summers. We also monitored the propagule pressure by *Hymenoscyphus albidus*, a non-pathogenic native species that competes for the same sporulation niche with *H. fraxineus*. During the monitoring period, crown condition of ash trees had impaired, and 20% of the dominant trees were severely damaged in 2017. *H. fraxineus* showed an exponential increase in spore production between 2012 and 2015, followed by drastic decline in 2016 and 2017. During 2011–2013, the two *Hymenoscyphus* species showed similar sporulation level, but thereafter spores of *H. albidus* were no longer detected. The data suggest that following local introduction, the population of *H. fraxineus* reaches rapidly an exponential growth stage if the local weather conditions are favorable for ascomata maturation across years. In the North Atlantic climate, summer temperatures critically influence the pathogen infection pressure, warm summers allowing the population to grow according to its biotic potential, whereas cold summers can cause a drastic decline in propagule pressure.

Keywords: ash dieback, climate, invasive species, population size, r/K selection

INTRODUCTION

The invasive fungus *Hymenoscyphus fraxineus* (syn. *H. pseudoalbidus*, anamorph *Chalara fraxinea*) (Kowalski, 2006; Queloz et al., 2011; Baral et al., 2014) is threatening the future existence of European ash (*Fraxinus excelsior*) in Europe (Pautasso et al., 2013; Mitchell et al., 2014). This ascomycete has probably been introduced to Europe from Asia, where it is regarded as an endophyte with some parasitic capacity in leaves of Manchurian ash (*F. mandshurica*)

(Drenkhan et al., 2017), a close relative of European ash. The dieback of European ash represents a showcase scenario of forest damage that can follow from exposure of evolutionary naïve trees to invasive alien pests that have a high level of adaptation to congeneric tree species in their native range. Since the first European record of ash dieback in Poland in the early 1990s (Przybył, 2002), the disease has spread across Central, Northern, Eastern and Western Europe (Timmermann et al., 2011; McKinney et al., 2014) and currently only populations at the southern and eastern range margins of European ash remain disease free (Solheim and Hietala, 2017).

In Europe, ash dieback has shown an annual spread rate of 50–75 km (Gross et al., 2014; Hamelin et al., 2016; Solheim and Hietala, 2017). The natural spread of *H. fraxineus* takes place by windborne ascospores released from ascomata formed on previous-year ash-leaf-litter. Typical to foliar pathogens, the ascospores of *H. fraxineus* are released during early morning during summer months (Timmermann et al., 2011; Hietala et al., 2013). While Steinböck (2013) and Chandelier et al. (2014) showed that the ascospore amount of *H. fraxineus* in air reaches a low plateau already a few hundred meters away from an infested stand, the effective dispersal distance of the pathogen propagules remains to be established. Besides ascospores, *H. fraxineus* produces also conidia that have been proposed to function as spermatia (review by Gross et al., 2014) or have a role in secondary local spread of the fungus (Fones et al., 2016). Particularly during the 1990s and early 2000s, when the causative agent of ash dieback still was unknown, trade and movement of infected ash seedlings (Sansford, 2013) and timber (Husson et al., 2012) may have facilitated the spread of ash dieback.

A study from south-eastern Norway located at the edge of continental climate, with average temperature around 15–16°C during the summer months (June–August), showed that *Hymenoscyphus fraxineus* exerts a high ascospore pressure in the period between mid-July and mid-August (Hietala et al., 2013). In the warmer climate of central Europe, a high pathogen propagule pressure can occur already in early June (Chandelier et al., 2014; Dvorak et al., 2016). The high propagule pressure obviously enables the fungus to overcome leaf defence responses and competition imposed by indigenous endophytes that are at this stage still in a quiescent growth mode characterized by low population density (Cross et al., 2017). There is increasing evidence that propagule pressure is a crucial ecological trait that influences the success of introduction but also the transition of invasive species to the subsequent stages; local establishment followed by spread outside the area of introduction and eventual widespread dominance (Lockwood et al., 2005; Colautti et al., 2006). The aim of the present study was to document the build-up rate of propagule pressure of *H. fraxineus* at an initially healthy ash stand. For the purpose, we installed a volumetric spore sampler in an ash stand at the west coast of Norway in 2011. In this region, the first signs of ash dieback were observed in some forests in 2010. At the experimental stand, we monitored the level of airborne *H. fraxineus* propagules over seven growing seasons. We also profiled the propagule level of *Hymenoscyphus albidus*, a fungus that competes for the same sporulation niche as *H. fraxineus*, but is indigenous to Europe and harmless to European ash.

MATERIALS AND METHODS

Spore Sampling and Assessment of Tree Health Condition

To monitor the release of ascospores by *H. fraxineus* and *H. albidus*, in 2011 we installed a solar power-driven Burkard 7-day volumetric spore sampler (Burkard Scientific, Uxbridge, United Kingdom) at ground level in the center of a mixed deciduous stand dominated by European ash and located in Fana, 15 km south of Bergen (Bergen municipality, 60° 15′ 59″ N, 5° 20′ 12″ E, 30 m a.s.l.). The sampler was equipped with a standard wind vane that directs the trapping orifice into the wind. This stand was chosen because it was still devoid of ash dieback symptoms in 2011 and *H. albidus* ascomata were present at that time. At an air throughput of 10 l/min, airborne particles were collected on the adhesive-coated (50 ml vaseline, 6 g paraffin wax, toluene), transparent Melinex tape, fastened to a revolving drum which moved past the air intake of the spore sampler at a rate of 2 mm per hour. With the collection tape changed weekly, this provided a continuous temporal record of airborne propagules. In 2011, sampling started July 26th and was continued until September 5th. In the years 2012–2017 spore sampling was repeated, starting already during the second half of June and continuing until the beginning/mid of October, with the exception of 2016 when sampling was terminated September 19th. The spore sampler was kept precisely at the same position in the subjected stand across the seven monitoring seasons.

As reference, weather data were obtained from a meteorological station located at Bergen airport Flesland 7 km northwest of the studied ash stand[1]. Missing data in some of measured meteorological parameters occurred due to technical problems at the station. Degree days were calculated by using a basal temperature of 5, 10, or 15°C.

In summer 2017, a damage assessment using defoliation as the main parameter (Eichhorn et al., 2016) was conducted on 20 dominant and 17 subdominant ash trees, subjectively selected and representative for the stand. Trees were considered to be healthy when they had up to 10% defoliation, slightly damaged when having 11–25% defoliation, moderately damaged when having 26–50% defoliation and severely damaged when the crown was more than 50% defoliated.

DNA Isolation and Real-Time PCR

For two randomly selected days per each sampling week in each year, DNA was isolated from tape segments corresponding to the period from midnight to noon (24:00 – 12:00) with protocol #8 (Isolation of DNA from Mouse Tails) of Easy-DNA^{TM} Kit (Invitrogen, Carlsbad, CA, United States) according to the manufacturer's instructions. The resulting 50-µl-volume DNA samples were stored at −20°C until processed by real-time

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[1] eklima.met.no

PCR. As one spore sampler was employed, each sampling day represents a single biological replicate.

The real-time PCR detection of *H. fraxineus* and *H. albidus* DNA was performed using FAST BLUE qPCR MasterMix Plus w/o UNG for probe Assay Low ROX (Eurogentec, Seraing, Belgium) without any modifications in reagent concentrations. For detection of *H. fraxineus,* we used the forward primer Cfrax-F 5′-ATTATATTGTTGCTTTAGCAGGTC-3, reverse primer Cfrax-R 5′-TCCTCTAGCAGGCACAGTC-3′ and probe C-frax-P5′-FAM- CTCTGGGCGTCGGCCTCG-BHQ1-3′ designed and tested for species specificity by Ioos et al. (2009). For detection of *H. albidus*, we used the primer probe set designed and tested for species specificity by Husson et al. (2011), with the modification of using JOE as the reporter dye instead of YY: forward primer Halb-F 5′TATATTGTTGCTTTAGCAGGTCGC-3′, reverse primer Halb-R 5′-ATCCTCTAGCAGGCACGGTC-3′, and probe Halb-P5′-JOE-CCGGGGCGTTGGCCTCG-BHQ1-3′.

For quantification of *H. fraxineus* DNA, the primer and probe concentrations were 300 nM and 100 nM, respectively, as described by Ioos et al. (2009). For quantification of *H. albidus* DNA, 900 nM concentrations were used for the primers and the probe (Hietala et al., 2013).

To obtain fungal DNA for construction of standard curves, three Norwegian *H. fraxineus* (2009-106/1/2, 2009-107/1/2, 2009-112/1/3) and three *H. albidus* strains (2009-111/1/4, 2009-124/3/2, 2009-124/4/1), deposited at the culture collection of the Norwegian Institute of Bioeconomy Research, were grown for 3 weeks at 21°C on 2% malt extract agar coated by a cellophane membrane, and subjected to DNA isolation with a DNeasy plant mini kit (Qiagen) according to the manufacturer's instructions. The obtained DNA was quantified by using micro-volume spectrophotometer NanoDrop 2000 (Thermo Scientific, Wilmington, DE, United States), and pooled together in equal concentrations to prepare fungal DNA standard curve samples that contained 10, 1, 0.1, 0.01, and 0.001 ng of *H. fraxineus* or *H. albidus* DNA in a volume of 3 μl. To ensure that the cycle threshold values from the experimental samples fell within the standard curves and to investigate the presence of compounds inhibitory to PCR, 3-log dilution series were prepared for all the experimental samples. Each experimental sample had undiluted DNA as the most concentrated, and all log dilutions of a sample were used as templates in real-time PCR. For both the experimental and standard curve samples, 3 μl of the DNA solution was used as the template for each 25-μl PCR reaction. Each reaction was repeated twice. PCR cycling parameters were 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 65°C for 55 s. Fluorescence emissions were detected with an ABI Prism 7700 (Applied Biosystems). The data acquisition and analysis were performed with the Sequence Detection System software package (1.7a; Applied Biosystems). Standard curves were constructed based on the relationship of C_t values and known DNA concentrations: the C_t values were plotted against log-transformed DNA amounts, and linear regression equations were calculated for the quantification of DNA pools by interpolation in unknown samples.

To obtain an estimate for ascospore amount of *H. fraxineus* and *H. albidus*, we divided their real-time PCR based DNA amount estimates by the weight of 0.0634 or 0.0549 pg that correspond to the estimated haploid genome sizes of *H. fraxineus* (62 Mb, McMullan et al., 2018) and *H. albidus* (53 MB, Sønstebø et al., 2017).

Statistical Analyses

For each sampling year, Spearman's rank correlation coefficients (ρ) were calculated for spore levels of *H. fraxineus* and *H. albidus* and weather parameters (mean daily temperature, precipitation, relative air humidity and wind speed), days with missing values either for the fungi or for the weather parameters were excluded from calculations. We also calculated the degree days for each year, by using 5, 10, or 15°C as basal temperatures. The relationship between initiation, peaking and ceasing of sporulation by *H. fraxineus* and degree days across the 7-year-long monitoring period was considered by calculating Pearson product moment correlations (*r*). ANOVA and Tukey HSD were used to consider species- and year-specific differences in maximum sporulation level; log transformation was made to reduce skewness in spore amount data. All the calculations were made by using SPSS 22.0 (IBM Inc., Armonk, NY, United States); correlations between variables and differences between treatments were considered significant at the 0.05 level.

RESULTS

Development of Ash Health Condition

During installation of the spore sampler in 2011, no visible symptoms of ash dieback were detected in the stand and the health condition of the ash trees was generally considered as good, however, a structured assessment was not conducted. The results of the assessment in 2017 showed that 20% of the dominant trees were severely damaged, 35% moderately damaged, 35% slightly damaged and only 10% were still healthy (Supplementary Figure 1). Most of the severely damaged trees showed a large amount of epicormic shoots. Dead trees have so far not been observed in the stand.

Changes in Infection Pressure by *H. albidus* and *H. fraxineus* During the Experimental Period

Spores of *H. albidus* were detected only during the first three seasons of monitoring, while spores of *H. fraxineus* were present in all seven sampling years. In 2011, 2012 and 2013, the mean spore levels of *H. albidus* during the 5 days with most spores detected (TOP_5) were estimated as 217, 19 and 144, respectively. The respective TOP_5 spore levels for *H. fraxineus* were 302 (2011), 120 (2012) and 1384 (2013), the levels in 2012 and 2013 being significantly higher than for *H. albidus*. After 2013, only spores of *H. fraxineus* were detected: the mean TOP_5 spore levels were estimated as 35305 (2014), 437360 (2015), 20786 (2016) and 30 (2017). The increments in TOP_5 spore level of *H. fraxineus* between 2013 and 2014, and between 2014 and 2015 were significant, as were the declines in top spore level between 2015 and 2016, and between 2016 and 2017. The exponential increase in maximum spore level between 2012 and 2015 can be described by the model $f(x) = 6.7504e^{2.7842x}$ ($R^2 = 0.997$), where x is the time in years (i.e., 1, 2012; 2, 2013 etc.).

Within Season Variation in Sporulation Period and Peak by *H. albidus* and *H. fraxineus*

There was considerable variation between different years in the initiation, peaking and ceasing of sporulation by *H. albidus* and *H. fraxineus*. In 2011, when spore sampling was started first in late July and continued only to the beginning of September, the first detection, maximum level (a single day with most spores detected, TOP$_1$) and last detection of *H. fraxineus* spores occurred on calendar days 209, 209 and 246, respectively, and those of *H. albidus* on calendar days 209, 209 and 239, respectively. Between 2012 and 2017, the spore sampling was started in the second half of June. In 2012 the first detection, TOP$_1$ and last detection of *H. fraxineus* spores occurred on calendar days 204, 225 and 233, and those of *H. albidus* on calendar days 187, 197 and 211, respectively. In 2013 the first detection, TOP$_1$ and last detection of *H. fraxineus* spores occurred on calendar days 200, 242 and 267, and those of *H. albidus* on calendar days 172, 176 and 249, respectively. From 2014 to 2017 spores of *H. albidus* were no longer detected, and the first detection, TOP$_1$ and last detection of *H. fraxineus* spores occurred on calendar days 168, 201 and 276 (2014), 184, 240 and 272 (2015), 177, 225 and 260 (2016), 233, 236 and 236 (2017), respectively.

Correlation of Weather and Sporulation of *H. albidus* and *H. fraxineus*

No significant relationship was generally observed between spore level of the two *Hymenoscyphus* species and temperature (**Figure 1**), precipitation (**Figure 2**), relative air humidity

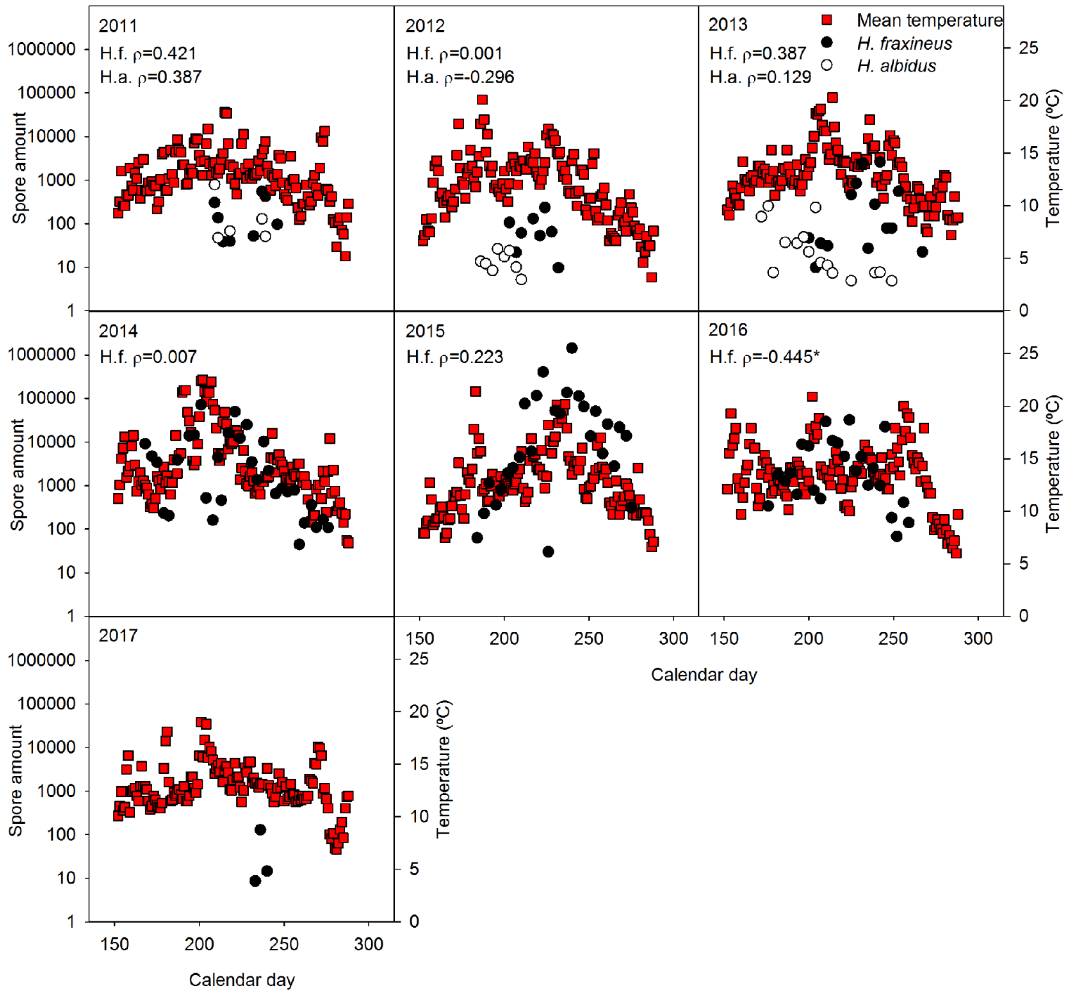

FIGURE 1 | The amount of *H. fraxineus* (H.f.) and *H. albidus* (H.a.) spores 2011–2017 in relation to mean temperature (°C). The spore amount is calculated from amount DNA (pg/12 h) in the day period 12 midnight to 12 noon, each data-point constituting a single biological replicate. The spore data represent two randomly chosen days per each sampling week, whereas temperature data are provided for each day. Spearman correlation coefficient (ρ) between the spore amount and temperature data sets is enclosed, significant relationships being indicated by *.

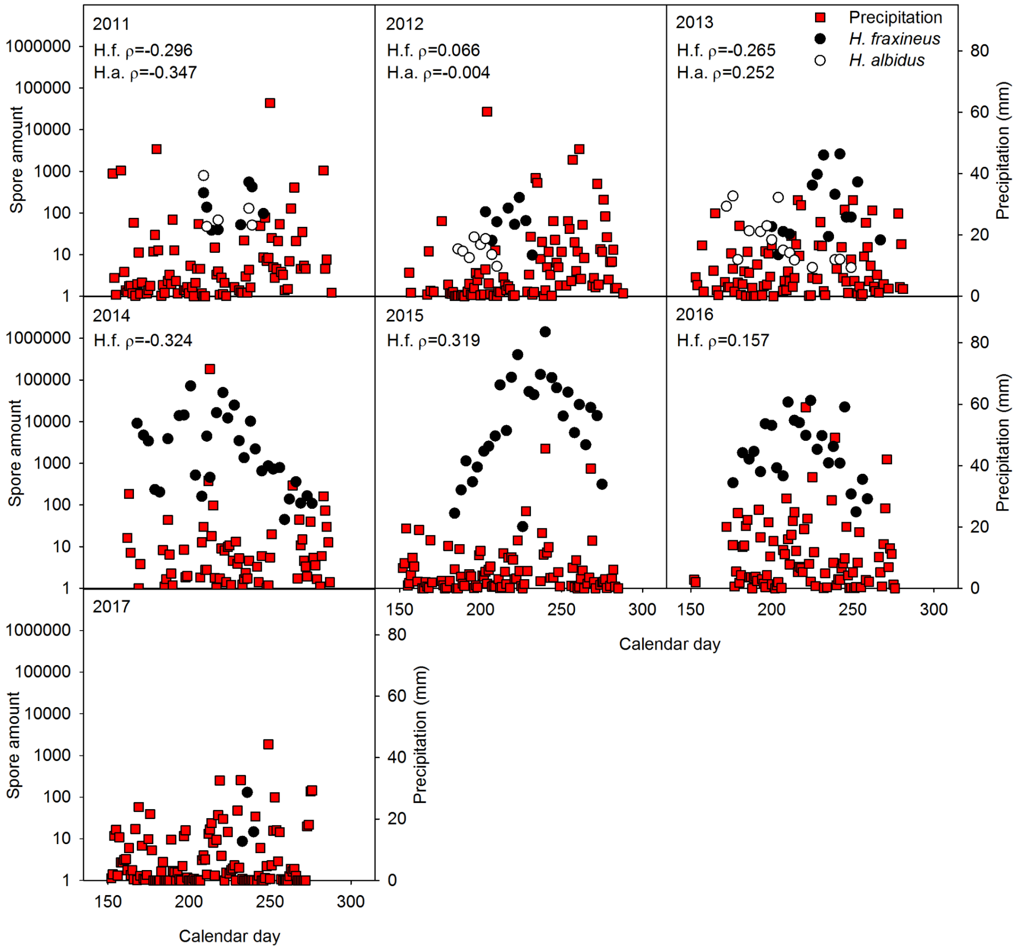

FIGURE 2 | The amount of *H. fraxineus* (H.f.) and *H. albidus* (H.a.) spores during 2011–2017 in relation to precipitation (in mm). The spore amount is calculated from amount DNA (pg/12 h) in the day period 12 midnight to 12 noon, each data-point constituting a single biological replicate. The spore data represent two randomly chosen days per each sampling week, whereas precipitation data are provided for each day. Spearman correlation coefficient (ρ) between the spore amount and precipitation data sets is enclosed, significant relationships being indicated by *.

(**Figure 3**) or average wind speed (**Figure 4**) during the sporulation period. The exceptions were 2011 (ρ between *H. fraxineus* spore levels and average wind speed: 0.58), 2012 (ρ between *H. albidus* spore levels and average wind speed: −0.63) and 2016 (ρ between *H. fraxineus* spore levels and temperature: 0.45).

There were significant relationships between TOP_5 spore level of *H. fraxineus* and the calendar day of first or last spore detection; the corresponding *r* were −0.85 and 0.86. We also calculated the relationship between fungal sporulation and degree days across the seven-year-long monitoring period, using 5°C (T_5), 10°C (T_{10}), or 15°C (T_{15}) as basal temperatures (**Figure 5**). Since *H. albidus* was detected only between 2011 and 2013, this calculation was done only for *H. fraxineus*. The *r* between TOP_1 spore level of *H. fraxineus* and sum of degree days by that calendar day were −0.29 (T_5), −0.12 (T_{10}) and 0.76 (T_{15}), the latter being statistically significant. The *r* between the sum of degree days by August 31st and TOP_5 spore level of *H. fraxineus* in the sampling year were 0.86 (T_5), 0.87 (T_{10}) and 0.82 (T_{15}), all statistically significant. Since ascospore production in a given year is obviously influenced by the success of leaf infection during the previous year, we also calculated the relationship between the degree day sum by August 31st during the previous year and TOP_5 spore level of *H. fraxineus* during the following year (i.e., degree day sum in 2010 vs. spore level in 2011); the corresponding *r* were 0.88 (T_5), 0.90 (T_{10}) and 0.92 (T_{15}), all statistically significant.

DISCUSSION

The infection pressure by ascospores of *H. albidus* and *H. fraxineus* was comparable and low during the first year of monitoring. The noted level of *H. albidus* inoculum is consistent with the few records of its ascomata in Europe, the fungus being regarded as a relatively rare species (Baral and Bemmann, 2014). The lack of detection of *H. albidus* spores after 2013 suggests that the fungus was outcompeted by *H. fraxineus*, which showed

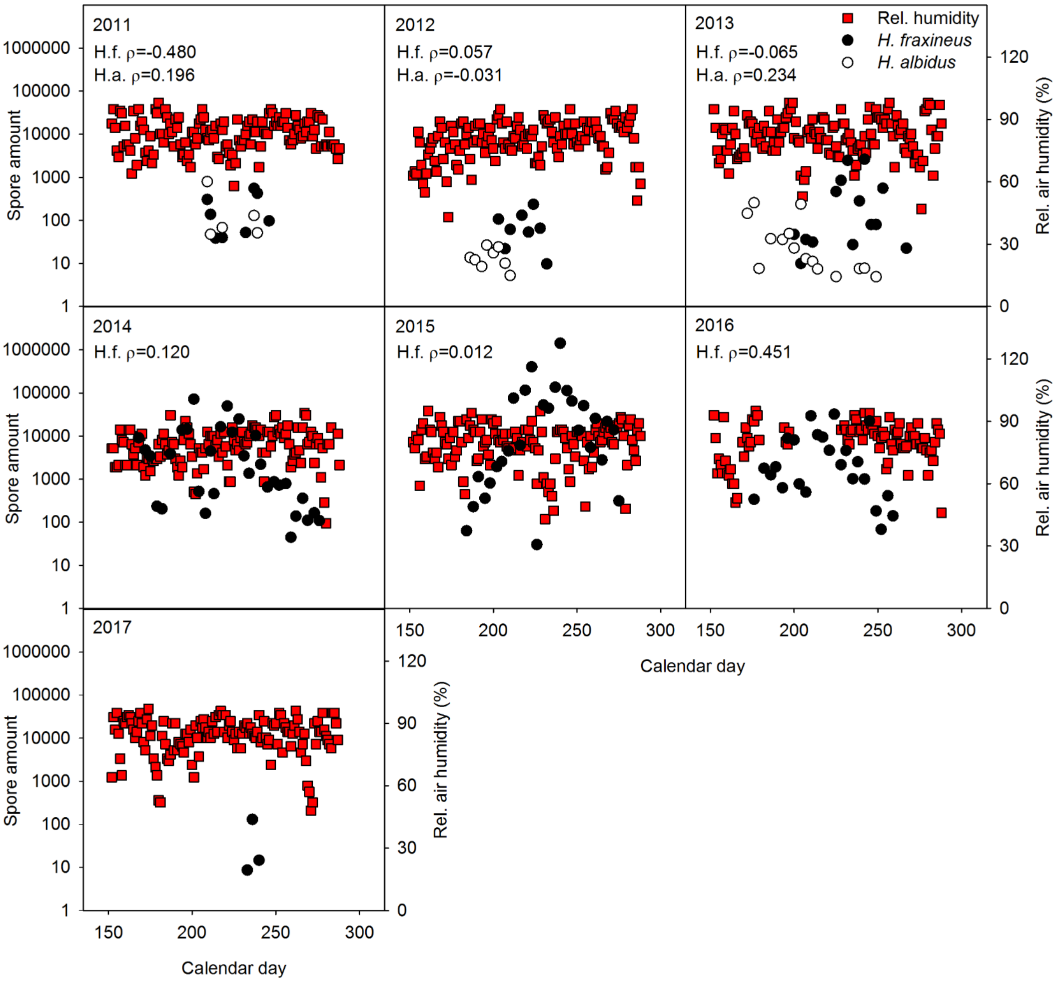

FIGURE 3 | The amount of *H. fraxineus* (H.f.) and *H. albidus* (H.a.) spores during 2011–2017 in relation to relative air humidity (%). The spore amount is calculated from amount DNA (pg/12 h) in the day period 12 midnight to 12 noon, each data-point constituting a single biological replicate. The spore data represent two randomly chosen days per each sampling week, whereas air humidity data are provided for each day. Spearman correlation coefficient (ρ) between the spore amount and relative air humidity data sets is enclosed, significant relationships being indicated by *.

an exponential increase in maximum spore level between 2012 and 2015. Studies from Denmark (Mckinney et al., 2012) and UK (King and Webber, 2016) also reported that *H. albidus* has become rare and probably even locally extinct due, whereas spore trapping studies from the Czech Republic, though based only on one year of sampling, imply that *H. albidus* could persist even after several years of competition by *H. fraxineus* (Dvorak et al., 2016; Koukol et al., 2016). For *H. albidus* detection, these Czech studies, like us, employed the primer/probe set designed by Husson et al. (2012). Whether the contrasting result could be influenced by study-specific differences in real-time PCR parameters (annealing temperature 65°C in the study of Husson et al. (2012) and the present work, and 60°C in the studies by Dvorak et al., 2016 and Koukol et al., 2016) is not clear to us. In Central Europe, first fruiting bodies of *H. fraxineus* appear already in May–June, distinctly earlier than *H. albidus*, and early initiation of sporulation has been proposed to provide an advantage to the invader (Baral and Bemmann, 2014). In the current stand sporulation of *H. fraxineus* initiated in 2012 and 2013 clearly later than that of *H. albidus*. This inconsistency between the present study and observations from central Europe relates presumably to different climatic conditions. Our ash stand represents oceanic climate with average daily temperatures normally between 13 and 14°C in summer, this being well below the optimum growth temperature that is close to 20–22°C for *H. fraxineus* (Kowalski and Bartnik, 2010; Pham et al., 2013) but also for *H. albidus* (Isabella Børja, pers. comm.). Taken together, the observations of fruiting body formation by *H. albidus* and *H. fraxineus* in central Europe and the sporulation data recorded now suggest that the order of sporulation initiation by *H. albidus* and *H. fraxineus* is not a decisive factor in their competition.

The exponential increase in maximum spore level of *H. fraxineus* between 2012 and 2015 is typical to invasive species, which may increase in population size according to their biotic potential in a new environment where access to resources is not a growth-limiting factor (e.g., Arim et al., 2006).

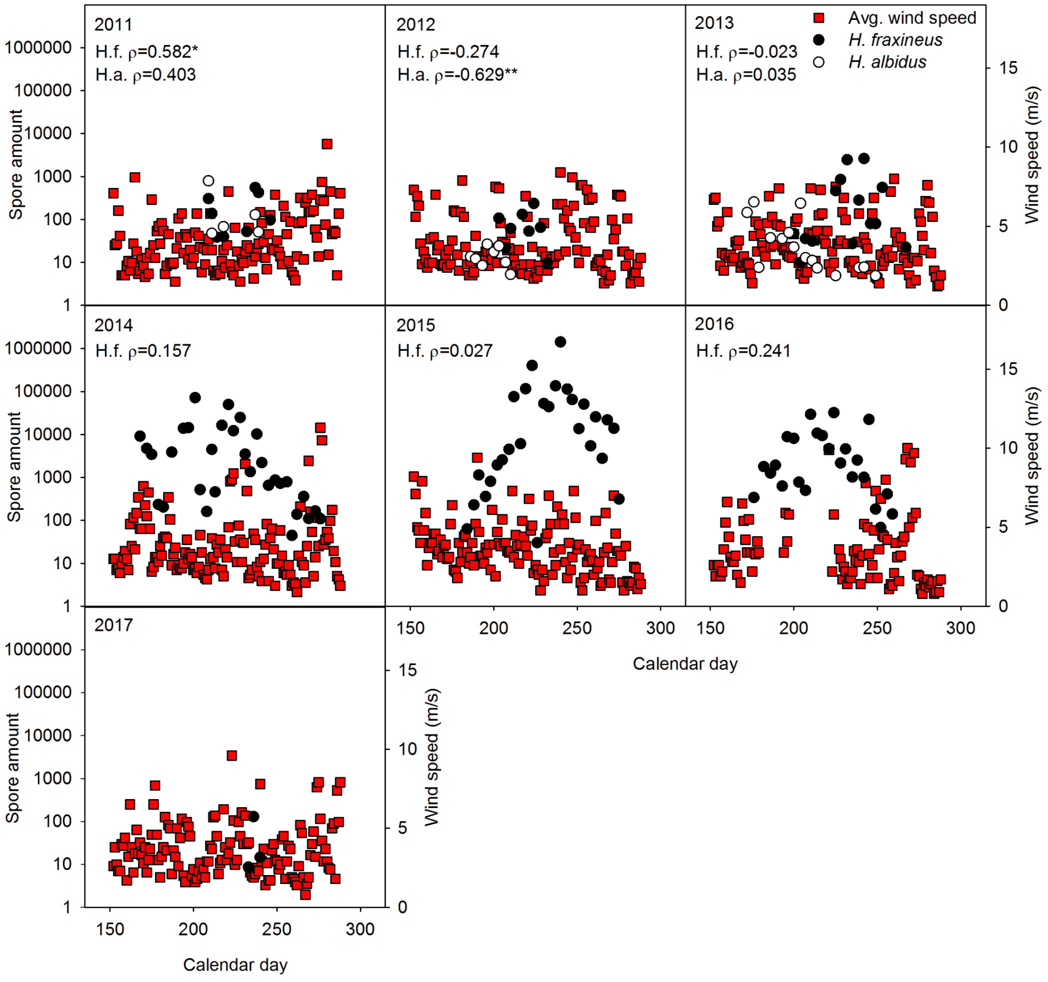

FIGURE 4 | The amount of *H. fraxineus* (H.f.) and *H. albidus* (H.a.) spores during 2011–2017 in relation to average wind speed (m/s). The spore amount is calculated from amount DNA (pg/12 h) in the day period 12 midnight to 12 noon, each data-point constituting a single biological replicate. The spore data represent two randomly chosen days per each sampling week, whereas wind data is provided for each day. Spearman correlation coefficient (ρ) between the spore amount and average wind speed data sets is enclosed, significant relationships being indicated by *.

Based on experiments under defined laboratory conditions, carpogenic germination of fungal pseudosclerotia to form fruiting bodies requires sufficient moisture, cumulative chill-hours and degree-days (e.g., Scherm et al., 2001). All these factors presumably influence also carpogenic germination of *H. fraxineus* pseudosclerotia but controlled experiments to determine the relations, to the best of our knowledge, have not yet been carried out. The region of the current stand is characterized by mild winters with average temperature around 1°C, high level of precipitation throughout the year and cool summers with average temperatures around 13–14°C in June, July and August. The significant correlation between annual maximum spore levels and degree-day sums suggest that in oceanic climate, cool summer temperatures are presumably the most limiting factor when it comes to carpogenic germination of *H. fraxineus* pseudosclerotia. An opposite scenario has been proposed for southern France, where high summer temperatures presumably restrict dissemination of *H. fraxineus* (Grosdidier et al., 2018). However, it is challenging to use field data to model the relationship between weather parameters and annual changes in species population size, especially during early stand infestation. At this phase invasive species may increase in population size according to their biotic potential (e.g., Arim et al., 2006), which makes distinguishing between inherent increase in population size and influence of weather on infection success and rate of carpogenic germination of pseudosclerotia difficult. For example, the maximum spore level of *H. fraxineus* was recorded in 2015, which was temperature-wise a typical summer in the region, with average temperature of 13.3°C in July which is usually the warmest month in the area. The previous summer was exceptionally warm, with average temperature of 17.8°C in July, and it seems plausible that besides the biotic potential of *H. fraxineus* to increase in population size, also the warm 2014 summer facilitated success of leaf infection and thereby contributed to the high level of spore production in 2015.

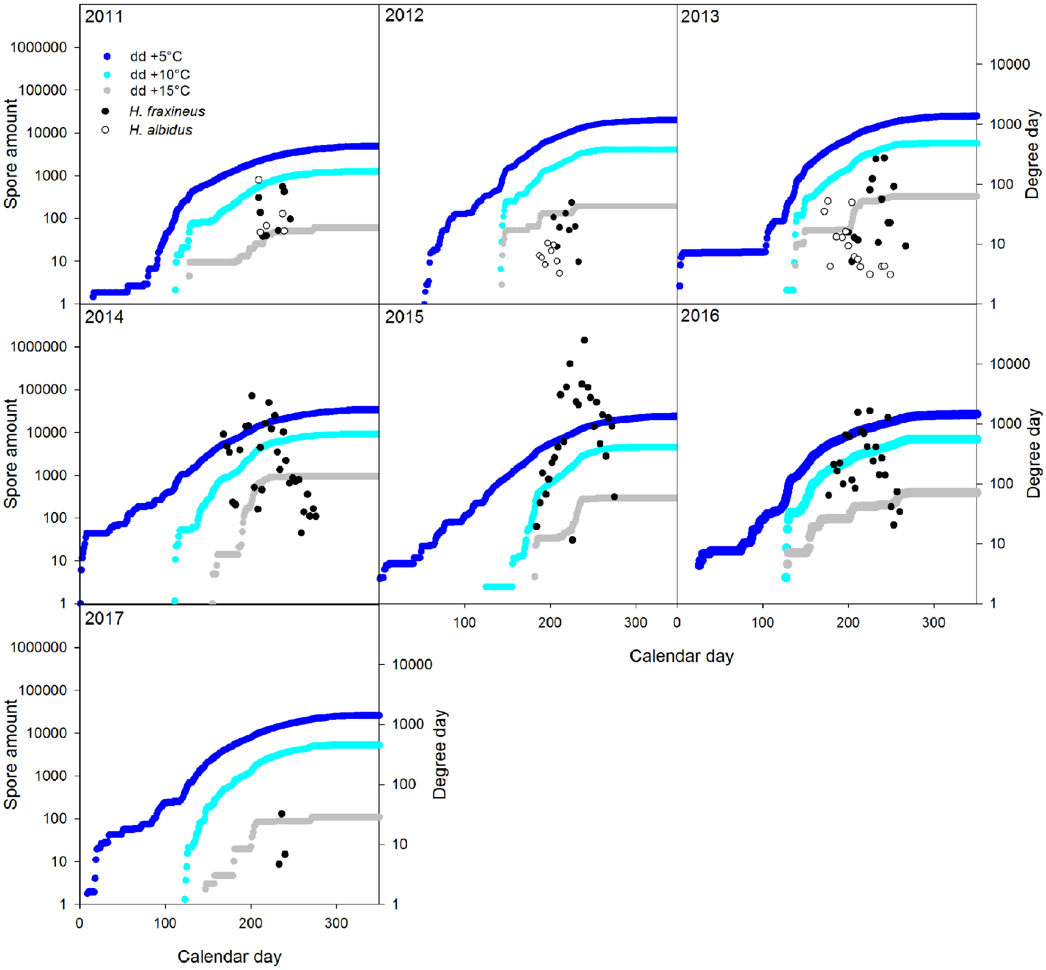

FIGURE 5 | Accumulated degree days and amount of *H. fraxineus* and *H. albidus* spores detected 2011–2017. 5°C, 10°C or 15°C were used as basal temperature in degree day calculation. For the spores, each data-point constitutes a single biological replicate.

The drastic decline in sporulation level of *H. fraxineus* after 2015 resembles a population crash that takes place when a population overshoots the carrying capacity of the environment. Considering the increased defoliation of ash trees during the experimental period, the carrying capacity for *H. fraxineus* might have been reduced in the stand along with the degree of defoliation. Results from two of our ash dieback monitoring plots at the Norwegian west coast with a time series from 2012 have shown rapidly increasing damage and mortality for young and intermediate ash trees, while disease development in the large, dominant trees has been progressing slowly (Timmermann et al., 2017). The monitoring plots are located 110 km south and 40 km north, respectively, and show currently a health condition comparable to that observed in the present stand (Supplementary Figure 1). Regarding precipitation, rainfall can facilitate the deposition of spores on the ground and thereby reduce the amount of spores captured by volumetric air sampling, this wet deposition of spores becoming more important as the distance from spore source increases (Aylor and Sutton, 1992). The record level of precipitation in summer 2016 could have somewhat reduced aerial dispersal distances of *H. fraxineus* ascospores at the stand. However, the drastic reduction in spore levels of *H. fraxineus* between 2016 and 2017 can hardly be explained by changes in the carrying capacity alone or by rainfall scavenging of spores – it is likely that the low degree-day sum in June and July in 2016 and especially in 2017 were less favorable to carpogenic germination of *H. fraxineus* pseudosclerotia. In this context it is highly significant that *H. fraxineus* can survive several months in air-dried petioles covered with a pseudosclerotial layer (Gross and Holdenrieder, 2013). Further, ascomata of *H. fraxineus* can be formed not only in the year after leaf fall but also on older petioles, at least up to five growing seasons after the leaves have been shed (Kirisits, 2015). This testifies for a strong competence in defending the saprobic niche against abiotic and biotic stress, an ability that may allow survival of *H. fraxineus* through unfavorable seasons for ascomata production. Should the summer 2018 turn out to be warm in southwestern Norway, we predict that the spore levels of *H. fraxineus* increase again in the subjected stand, via carpogenic germination of pseudosclerotia formed during previous years.

According to the stochastic niche theory of resource competition, invasion and community assembly (Tilman, 2004), the probability that an invader can survive, reach maturity and reproduce depends on its traits relative to the traits of the established species. This theory predicts that successful invaders should decrease the abundance mainly of species that are competitively similar to themselves. The genome sequence data indicate that *H. albidus* and *H. fraxineus* are very close to each other on the trade-off surface (Stenlid et al., 2017) but the species show some obvious differences in life history traits (e.g., review by Hietala et al., 2018). The largest ascomata recorded for *H. fraxineus* and *H. albidus* were 8.5 and 4 mm in diameter, respectively (Baral and Bemmann, 2014). Due to the larger hymenium, *H. fraxineus* has probably a higher ascospore production than *H. albidus*. Moreover, in laboratory experiments *H. fraxineus* shows a considerably larger ascocarp production capacity than *H. albidus* (Wey et al., 2016), and in forest *H. fraxineus* can form as many as 10000 ascocarps per m^2 (Hietala et al., 2013) or 13 million per hectare (Kowalski et al., 2013). As discussed by Wey et al. (2016), *H. albidus* may simply have inherently low fecundity, and thereby be at a disadvantage to *H. fraxineus*. When it comes to adaptation to changes in environmental conditions, a characteristic of invasive species, also in offspring quality *H. fraxineus* has a clear advantage over *H. albidus*; while *H. fraxineus* is an outcrossing heterothallic species with recombinant offspring, *H. albidus* is homothallic species reproducing via haploid selfing (Gross et al., 2012; Wey et al., 2016; Brasier et al., 2017).

Besides offspring quantity and quality, *H. fraxineus* differs from *H. albidus* owing to its extensive pseudosclerotial layers that may cover the entire vein system of the compound leaf, while the pseudosclerotial layer of *H. albidus* appears usually sharply isolated on the otherwise straw-colored rachis (Baral and Bemmann, 2014). The high investment in survival structures, high fecundity and production of recombinant offspring by *H. fraxineus* may represent an adaptation to harsh environmental conditions, the native hosts of the fungus spanning across multiple climate zones in Asia, these including regions with cold dry winters and either hot, warm or cold summers (Wei et al., 2004; Peel et al., 2007). To our knowledge, there are no other long-term data available about annual changes in *H. fraxineus* population size, either from Asia or from Europe. It is noteworthy that in European arboreta the Asian host trees of *H. fraxineus* support equally well pathogen ascomata formation as common ash (Nielsen et al., 2017). We hypothesize that under Asian climatic conditions *H. fraxineus* is not able to grow according to its biotic potential over several years and rarely reaches a critical population size when crowding effects become a selective force, whereas the more stable continental climate of Europe enables *H. fraxineus* to frequently increase in population size over several years, to the effect that the fungus overshoots the environment carrying capacity and causes ash decline. We predict that the boom and bust cycle now observed in *H. fraxineus* sporulation level is not specific to the subjected stand but a more general phenomenon, and that the length of the exponential increase in *H. fraxineus* population size depends on how stable the climatic conditions that favor ascocarp sporulation and leaf infection are locally across consequent years. Our results are coherent with the predictions of Goberville et al. (2016), who proposed that climate change will expand the range of *H. fraxineus*.

AUTHOR CONTRIBUTIONS

AH, IB, HS, NN, and VT contributed to the design of the project, analysis and interpretation of data, and to drafting and revising of the manuscript. Processing of qPCR data was done by AH. AH and NN conducted the statistical analysis.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2018.01087/full#supplementary-material

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Radial Growth and Wood Density Reflect the Impacts and Susceptibility to Defoliation by Gypsy Moth and Climate in Radiata Pine

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Drought stress causes a reduction in tree growth and forest productivity, which could be aggravated by climate warming and defoliation due to moth outbreaks. We investigate how European gypsy moth (*Lymantria dispar dispar* L., Lepidoptera: Erebidae) outbreak and related climate conditions affected growth and wood features in host and non-host tree species in north-western Spain. There, radiata pine (*Pinus radiata* D. Don) plantations and chestnut (*Castanea sativa* Mill.) stands were defoliated by the moth larvae, whereas Maritime pine (*Pinus pinaster* Ait.) was not defoliated. The gypsy moth outbreak peaked in 2012 and 2013, and it was preceded by very warm spring conditions in 2011 and a dry-warm 2011–2012 winter. Using dendrochronology we compared growth responses to climate and defoliation of host species (radiata pine, chestnut) with the non-host species (Maritime pine). We also analyzed wood density derived from X-ray densitometry in defoliated and non-defoliated trees of radiata pine. We aimed to: (i) disentangle the relative effects of defoliation and climate stress on radial growth, and (ii) characterize defoliated trees of radiata pine according to their wood features (ring-width, maximum and minimum density). Radial growth during the outbreak (2012–2013) decreased on average 74% in defoliated (>50% of leaf area removed) trees of radiata pine, 43% in defoliated trees of chestnut, and 4% in non-defoliated trees of Maritime pine. After applying a BACI (Before-After-Control-Impact) type analysis, we concluded that the difference in the pattern of radial growth before and during the defoliation event was more likely due to the differences in climate between these two periods. Radiata pines produced abundant latewood intra-annual density fluctuations in 2006 and 2009 in response to wet summer conditions, suggesting a high climatic responsiveness. Minimum wood density was lower in defoliated than in non-defoliated trees of radiata pine prior to the outbreak, but increased during the outbreak. The pre-outbreak difference in minimum wood density suggests that the trees most affected by the outbreak produced tracheids with wider lumen and were more susceptible to drought stress. Results of this study illustrate (i) that the pattern of radial growth alone may be not a good indicator for reconstructing past defoliation events and (ii) that wood variables are reliable indicators for assessing the susceptibility of radiata pine to defoliation by the gypsy moth.

Keywords: *Castanea sativa*, dendroecology, extreme climate event, defoliation, intra-annual density fluctuation, *Lymantria dispar dispar*, *Pinus radiata*, wood density

INTRODUCTION

In the Anthropocene, forests face unprecedented pest problems that are outside managers' range of experience (Liebhold, 2012). Outbreaks of herbivorous insects causing widespread disturbances may be favored by climate warming, land-use changes, increasing host abundance or susceptibility and range shifts of pests (Raffa et al., 2008). There may also be feedbacks between components of the changes at the global scale which may aggravate pest incidence (Ayres and Lombardero, 2018). Tools to quantify how susceptible trees are to pests and to monitor effects of pests on forest growth and health will help managers to develop strategies to face new challenges (Trumbore et al., 2015).

Rapid land-use changes during the twentieth century have resulted in millions of hectares of pine plantations, particularly in areas in the southern Hemisphere where pines are introduced species (FAO, 2010). These commercial plantations involve also biota alterations because they are monocultures of non-native tree species which may face new herbivores (Wingfield et al., 2015). In some cases, these plantations can reduce herbivory relative to their native environments via geographical escape from their natural herbivores. However, sometimes the native herbivores can become important pests of non-native tree species (Nuñez et al., 2008).

Outbreaks of defoliating insect herbivores may reduce productivity in commercial plantations, such as those of the widely planted radiata pine (*Pinus radiata* D. Don), because they reduce stand productivity. Radiata pine is native to California but has been extensively used for commercial forestry, becoming the most extensively planted softwood worldwide (Mead, 2013). More than four million ha of radiata pine plantations exist, and 90% of them are located in the southern Hemisphere (Manley and Maclaren, 2009). In this regard, we need an improved monitoring of the responses of radiata pine in plantations to insect defoliators which may make their management challenged by the changes at the global scale described above.

The European gypsy moth (*Lymantria dispar dispar* L., Lepidoptera: Erebidae) is a problematic defoliating pest of angiosperm and gymnosperm tree species in the northern Hemisphere because it is a polyphagous species (Tobin and Liebhold, 2011). In north-eastern USA the gypsy moth is invasive, and outbreaks have been observed throughout the twentieth century, differently affecting tree species indicating that tree species vary in their susceptibility to defoliation (Allstadt et al., 2013). Radiata pine has been conventionally considered resistant to defoliation, i.e., needles are consumed only by some larval stages or when susceptible hardwood species (consumed by all larval stages) are not present (Liebhold et al., 1995).

In the laboratory it was demonstrated that European gypsy moth could complete development to adult by feeding on needles of radiata pine (Miller and Hanson, 1989). Moreover, the outbreak in the present study showed the ability of this insect to complete development in the field and severely defoliated trees of radiata pine (Castedo-Dorado et al., 2016).

Here, we capitalize on the outbreak reported by Castedo-Dorado et al. (2016) which mainly affected plantations of radiata pine but also caused defoliation in the native chestnut (*Castanea sativa* Mill.) but not in planted Maritime pine (*Pinus pinaster* Ait.) trees in northern Spain (Lago-Parra et al., 2016). An increase in gypsy moth populations was first noticed in 2011, peaking in 2012 and 2013 (maximum defoliation severity) and then insect populations collapsed in 2014 (Castedo-Dorado et al., 2016). We used tree-ring data to evaluate the impacts of the outbreak and related climate anomalies (i.e., 2011 and 2012 were very dry and warm years over most northern Spain; cf. Camarero et al., 2015) on radial growth of susceptible (i.e., radiata pine, chestnut) and immune or non-host species (e.g., Maritime pine). Since wood density is a key characteristic for the timber industry, we also quantified changes in maximum and minimum wood density of defoliated and non-defoliated trees of radiata pine.

Dendroecology has been used to evaluate the effect of insect defoliations on radial growth by comparing tree-ring width series of host vs. non-host species (Swetnam et al., 1985). Narrow rings or morphological wood anomalies (e.g., xylem cells with poorly lignified walls) have been also associated with defoliations (Sutton and Tardif, 2005, 2007; Paritsis et al., 2009). Densitometry has also been used to characterize the reduction in maximum wood density caused by some outbreaks (Esper et al., 2007; Koprowski and Duncker, 2012).

In this study, (i) we assessed the relative effects of gypsy moth defoliation and climate on radial growth and wood density, in the case of radiata pine, and (ii) we compared the responses of susceptible or host (chestnut, radiata pine) vs. immune or non-host (Maritime pine) tree species to this outbreak. We expected that insect defoliation to result in a severe loss of radial growth and reduction in wood density in radiata pine trees, whereas warm-dry conditions leading to water scarcity to result in growth reduction in non-defoliated trees of radiata pine and in immune trees of Maritime pine.

MATERIALS AND METHODS

Study Area and Tree Species

The gypsy moth outbreak occurred in the Municipality of Cubillos del Sil, province of León, north-western Spain (42°

41’ N, 6° 36’ W). According to the records of the Office responsible for forest pest control in the region (Junta de Castilla y León), it was the first record of outbreak by the gypsy moth in the area since plantation establishment in the 1990s. The mean altitude of the study area is 570 m a.s.l. The stand is facing east and has a slight slope (5–10°). Climate in the study area is Mediterranean temperate with oceanic influence. According to climatic data for the period 1971–2015 taken from the Ponferrada climatic station (42° 33’ N, 6° 35’ W, 534 m a.s.l.), located at about 12 km from the study site, the mean annual temperature is 12.7°C, with a mean daily minimum of 0.9°C in January and a mean daily maximum of 29.1°C in July. The mean annual rainfall is around 672 mm (**Figure S1a**). The soils are acidic and low in nutrient concentrations. Vegetation in the area is dominated by radiata pine plantations, although stands of native broadleaved species are also present, including chestnut and oak species (*Quercus ilex* L., *Quercus pyrenaica* Willd). Maritime pine occurred in clumps within radiata pine stands, and originated from natural regeneration from seeds produced by trees in the previous rotation.

The outbreak mainly affected a pure stand of radiata pine planted in 1994 (trees were 18 years old at the beginning of the outbreak in 2012). The site index of the forest was around 20 m (according to the height growth curves of Diéguez-Aranda et al., 2005). Density at establishment was ∼1,600 trees ha^{-1}, which was reduced to ∼800 trees ha^{-1} by a thinning of under-performing trees in early 2012.

After the defoliation event of 2012–2013, *ca.* 6 ha were severely defoliated during two consecutive years (2012 and 2013), and *ca.* 40 ha were severely defoliated in 2012. Moderate defoliation was observed in surrounding stands of radiata pine affecting *ca.* 15 ha. Virtually all noticeable defoliation was limited to radiata pine (**Figure 1a**), except a small chestnut stand of *ca.* 1 ha, located within a radiata pine stand (**Figure 1b**). Isolated Maritime pines were not affected indicating a different susceptibility to the gypsy moth of the two pine species (**Figure 1c**).

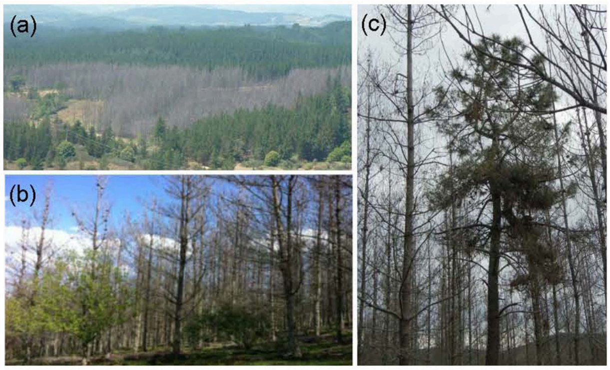

FIGURE 1 | Some illustrative views of the defoliation caused by the gypsy moth outbreak in the studied area (Cubillos del Sil, north-western Spain). **(a)** Landscape view of radiata pine affected plantations, **(b)** defoliated radiata pine trees and chestnut stands, and **(c)** defoliated radiata pines surrounding a Maritime pine not affected by defoliation.

Field Sampling and Dendrochronology

In April 2014, at the end of the outbreak, we selected and labeled 46 dominant *P. radiata* trees (18 defoliated and 28 non-defoliated trees; see **Figure S1b**). A threshold of 50% of loss in leaf area was used to classify trees as defoliated or non-defoliated individuals. Defoliation was noted in the field by visual assessment and, when necessary, using binoculars. The defoliation class assigned to each tree at the time of the defoliation assessment (April 2014) was assumed to be the same as during the defoliation period (2012–2013).

In January 2016, all the selected radiata pine trees, along with 21 chestnut trees, and 13 Maritime pine trees were sampled for dendrochronological analyses. The 13 Maritime pine trees and the 11 defoliated trees of chestnut were randomly selected in the vicinity of sampled radiata pine trees (**Figure S1b**). The remaining 10 chestnut trees were sampled in a nearby stand where defoliation did not occur. All the sampled trees of the three species were georeferenced using a sub-meter GPS receiver. Given that the study area is quite homogenous regarding topography and soil type, selected trees for each species and defoliation class were very similar in size, and therefore, we consider this number of trees sufficient to characterize the radial-growth patterns.

Tree diameter at breast height (dbh) was measured at 1.3 m for each tree using tapes. Sampling was performed following standard dendrochronological methods (Fritts, 2001). Two cores were taken at 1.3 m using a 5 mm Pressler increment borer from the 80 sampled trees. The wood samples were air-dried and polished with successively finer sand-paper grits until rings were clearly visible. Then, wood samples were visually cross-dated.

In radiata pine, we quantified (percent annual frequency) intra-annual density fluctuations (IADFs) in the latewood which reflect the tree responsiveness to intra-annual variability in precipitation (Pacheco et al., 2018). Latewood IADFs are characterized by the presence of earlywood-like cells, i.e., having wider lumen and thinner cell walls than latewood tracheids (Vieira et al., 2010). Latewood IADFs were visually identified in all cross-dated sampled under a binocular microscope.

Tree-ring width was measured to the nearest 0.001 mm with a stereomicroscope mounted above a LINTAB System (Rinntech, Heidelberg, Germany) device linked to a computer. Cross-dating of the tree rings was carried out using the program COFECHA (Holmes, 1983). The trend due to the geometrical constraint of adding a volume of wood to a stem of increasing radius was corrected by converting tree ring widths into basal area increment, hereafter abbreviated as BAI, which is a more biologically meaningful descriptor of growth trends than ring widths (Biondi and Qeadan, 2008). Annual basal area increment was calculated from tree-ring widths as the difference between consecutive basal areas using the following expression:

$$\text{BAI} = \pi \left(r_t^2 - r_{t-1}^2 \right) \quad (1)$$

where BAI is the basal area increment and r_t and r_{t-1} are the radii corresponding to rings formed in years t and t-1, respectively.

Densitometry Analyses

For the densitometry analyses, we additionally sampled 20 radiata pine trees, 10 defoliated and 10 non-defoliated. Trees were sampled at 1.3 m using a 12 mm Pressler increment borer. Wood samples were air dried and glued onto wooden mounts in the laboratory. For each core, thin laths of 1.2 mm thickness were cut by a twin bladed saw. The laths were then x-rayed in an Itrax Multiscanner (Cox Analytical Systems, Sweeden) at the CETEMAS laboratory (Asturias, Spain). The Multiscanner, equipped with a Cu-tube (Bergsten et al., 2001), was operated at 30KV and 50 mA, with an exposure of 25 s at each sample point and a step size of 20 μm (Moreno-Fernández et al., 2018).

The radiographic images obtained from the Itrax Multiscanner were analyzed in WinDENDRO (Regent Instruments, Canada) to determine wood density values for each point in the wood sample. This allowed us to define wood density profiles and to determine maximum and minimum wood density values for each annual ring. Cross-dating was again carried out by using the COFECHA software (Holmes, 1983).

Statistical Analyses

Comparisons of variables of interest (BAI and wood density) between defoliated and non-defoliated trees of the same species were assessed using the Mann-Whitney U-test.

To perform climate-growth correlations in non-defoliated trees, tree-ring width data were converted into ring-width indices to remove any size- or age-related growth trend (Cook et al., 1990). First, we removed the long–term trends of tree-ring width series by detrending them using negative exponential functions, which preserve high-frequency variability in the indices. Residuals were obtained by subtracting observed tree-ring width data from fitted values. Second, autoregressive models were fitted to each detrended series to remove most of the first-order autocorrelation. Lastly, a bi-weight robust mean was used to obtain chronologies for each species. A similar procedure was used for maximum and minimum wood density data of radiata pine but in this case indices were calculated as ratios from the fitted growth curves. Chronology development was done using the ARSTAN program (Cook and Krusic, 2005).

Using the ring-width indices obtained as described in the previous paragraph, the climate-growth correlations were calculated for the common and best-replicated period 2000–2015 considering only non-defoliated trees (Fritts, 2001). Tree radial growth was described by averaged annual ring-width indices whereas climatic variables (mean of the maximum and mean of the minimum temperatures, precipitation) were calculated annually from the Ponferrada station. Climatic variables were calculated for each month and season (winter, previous December to February; spring, March to May; summer, June to August; autumn, September to November). Mean of the maximum and mean of the minimum temperatures refers to the mean of the daily maximum and minimum temperature, respectively, for a given period. The window of analyses included from the previous October to current September based on previous studies (Downes et al., 1993; Ivković et al., 2013). The correlation analyses of the 16 climate-growth data pairs were carried out using the Pearson correlation coefficient. This correlation coefficient was also used for assessing the climate-wood density correlations for the period 2000–2015 for both defoliated and non-defoliated trees. We did not remove temporal autocorrelation of climate data since most of the considered variables it variables was not significant ($P > 0.05$) at 1–10 lags (**Table S1**).

For disentangling the effect of climate and defoliation on radial growth, we applied the BACI (Before-After-Control-Impact) type concept. As we have available information prior and subsequent to a stressor (defoliation), and information of stressed (defoliated) and control (non-defoliated) entities (trees), our data can be viewed as a case of a BACI design. The method for analysis this type of data involve comparison of stressed entities with control entities, and before and after they were affected by the stressor (e.g., Green, 1979; Stewart-Oaten et al., 1986).

Following this BACI-type concept we fitted a linear mixed-effects model of BAI of the current year (BAI_t) for a given tree as a function of climate data of the current growing season (or of the previous growing season), the defoliation class (defoliated or non-defoliated), the type of period (before defoliation or during the defoliation) and the interactions among them. The whole period 2000–2013 was considered because exploratory analysis of the data showed that the different length of the time series before the defoliation event (12 years) and that during the event (2 years) did not condition the results. Unlike in the correlation analysis described earlier, the BAI data used in the linear mixed model were not detrended.

For radiata pine and chestnut trees, the following full model was fitted:

$$y = b_0 + b_1x_1 + b_2x_2 + b_3x_3 + b_{12}x_{12} + b_{13}x_{13} + b_{23}x_{23} + b_{123}x_{123} + d\,z + \varepsilon \tag{2}$$

where y = response variable (annual BAI_t for a given tree), b_0 = intercept; x_1 = climate covariate, x_2 = an indicator variable distinguishing between defoliated and non-defoliated trees, x_3 = an indicator variable distinguishing between before defoliation and during the defoliation period, x_{12} = interaction between climate and defoliation, x_{13} = interaction between climate and period, x_{23} = interaction between defoliation and period, x_{123} = interaction among climate, defoliation and period, z = tree identity (the random effect); b_1, b_2, b_3, b_{12}, b_{13}, b_{23}, and b_{123} = coefficients for the above-mentioned covariate, fixed-effects factors, or interaction terms; d = coefficients for the random effect; and ε = error term, which was modeled using a first-order autoregressive correlation structure [AR(1)] for dealing with the temporal autocorrelation of the BAI_t data.

Nested reduced models considering a three two-way interactions (one model), two two-way interactions (three models) and only one two-way interaction (three models) were also tested for assessing the impact of subtracting predictors from the full model.

For Maritime pine trees, which did not suffer defoliation, the full mixed model fitted was the following:

$$y = b_0 + b_1x_1 + b_3x_3 + b_{13}x_{13} + d\,z + \varepsilon \tag{3}$$

The nested reduced model for this species did not considered the interaction term.

For all the models, BAI_t was log-transformed to meet the assumption of normality of the data.

Seasonal climate variables were used to avoid fitting too complex models based on monthly climate variables. It must be noted that the climate data may also be correlated at short to long time scales, but the possible autocorrelation in the climate data is not accounted in the models since previous analyses revealed that the climate series did not show significant temporal autocorrelation at 1–10 years scales (**Table S1**).

Comparisons between models were carried out through the log-likelihood ratio statistic (-2LL), which follows a chi-square distribution with degrees of freedom equal to the difference between the number of coefficients of the models (Zuur et al., 2009). The random effects and the covariance parameters were estimated using the maximum-likelihood method (Zuur et al., 2009) implemented in the lme function of the nlme library in R (R Core Team, 2017).

Taking into account that differences in tree radial growth can arise because of different microsite conditions (e.g., access to soil water), and the experimental design did no factor out microsite differences, we examined whether spatially autocorrelated factors, such as microsite differences, were present. Using ArcGIS® software (ESRI, 2011) we calculated Moran's I index for average BAI of both the non-defoliation and the defoliation periods. This index evaluates whether the pattern is spatially autocorrelated or not (random; Goodchild, 1986).

RESULTS

The 2011–2012 Climate Anomalies

The outbreak was preceded by very warm spring conditions in 2011 (+6.0° C anomaly in the mean of the maximum April temperatures; warmest on record since 1971) and a dry 2011–2012 winter (**Figures S2,S3**). The 2 years of the gypsy moth outbreak culmination (2012 and 2013) were characterized by very dry conditions, particularly in early 2012 (−50 mm anomaly in February precipitation; driest February on record), and were comparable in terms of water shortage with other years characterized by dry spells as 2005 (**Figure S3**).

TABLE 1 | Size (diameter at breast height, dbh) and radial-growth data (basal area increment, BAI) measured for the sampled tree species.

Species	Tree status	No. trees	dbh (cm)				BAI 2000–2011 (cm^2)				BAI 2012-2013 (cm^2)			
			Max	Min	Mean	SE	Max	Min	Mean	SE	Max	Min	Mean	SE
Pinus radiata	Defol	18	21.3	16.4	18.7	1.8	20.49	4.61	13.11a	1.20	10.43	1.26	2.95a	0.87
	Non-defol	28	24.6	18.2	21.0	2.1	21.64	5.48	16.89b	1.28	28.45	1.52	11.28b	2.32
Castanea sativa	Defol	11	20.5	16.7	18.4	0.4	14.09	6.11	9.71a	1.40	12.66	5.92	8.96a	0.43
	Non-defol	10	20.0	16.9	18.2	0.3	14.56	7.07	10.11a	1.44	25.06	7.63	15.66b	0.55
Pinus pinaster	Non-defol	13	32.2	24.9	28.5	2.6	42.41	7.99	20.21	1.21	38.51	1.84	19.39	4.29

Basal area increment was calculated for the period previous to defoliation (2000–2011) and for the outbreak period (2012–2013). Different letters indicate significant BAI differences (α = 0.05) between defoliation classes (defoliated –Defol–, and non-defoliated –Non-defol) within each species (radiata pine and chestnut) based on Mann-Whitney U-tests. Values are means, maximum (Max), and minimum (Min) values, and standard errors (SE).

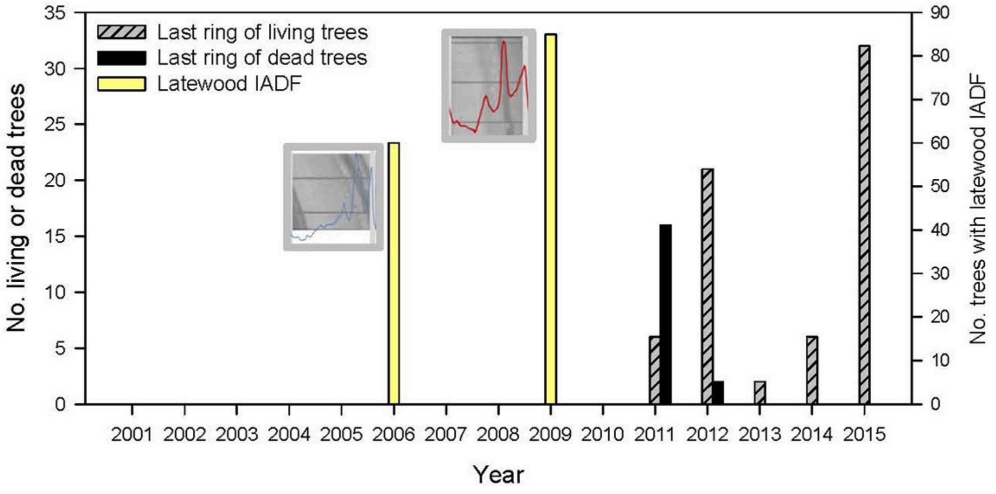

FIGURE 2 | Number of living or dead radiata pine trees according to their last formed tree-ring (left *y* axis) or according to the presence of intra-annual density fluctuations (IADF) in the latewood (right *y* axis). The insets show density profiles and images corresponding to the 2006 and 2009 rings when latewood IADFs were abundant. Dead pines died during the gypsy moth outbreak.

Tree Size, Radial Growth, IADFs, and Wood Density: Comparisons of Defoliated and Non-defoliated Trees Within Each Species

The dbh of defoliated and non-defoliated trees were similar in radiata pine and chestnut (**Table 1**). According to the cross-dated ring-width series of defoliated trees of radiata pine, most of them (89%) formed the last complete ring in 2011, whereas 33 and 47% of living trees formed it in 2012 and 2015, respectively (**Figure 2**). This means that many severely defoliated trees did not form a complete ring in 2012, when defoliation peaked, whilst other defoliated trees of radiata pine presented extreme growth reduction in 2012 and stopped growing. Two years (2006, 2009) presented abundant IADFs in the latewood of radiata pine trees (**Figure 2**). Using 2009 to illustrate the climate conditions linked to IADF formation, these corresponded to warm conditions but mainly elevated summer (June to July) and autumn-winter (November-December) precipitation (**Figure S3**).

Prior to the outbreak (period 2000–2011), we found no significant differences in BAI between defoliated and non-defoliated trees of chestnut, but defoliated trees of radiata pine showed a lower BAI than their non-defoliated counterparts ($U = 64$, $P < 0.001$; **Table 1** and **Figure 3**). Defoliated trees of radiata pine and chestnut showed significantly lower BAI values during the outbreak (2012–2013) than their non-defoliated conspecifics (**Table 1** and **Figure 3**). The mean BAI values of the defoliated trees of radiata pine continue to decrease during and after the defoliation, while that of the non-defoliated trees of radiata pine continue to increase during the same period (2012–2015; see **Figure 3**). The temporal pattern of changes in the mean BAI values of Maritime pine trees was similar to that of non-defoliated trees of radiata pine. In contrast to the radiata pine, the mean BAI of the defoliated trees of chestnut showed an increasing trend after the defoliation (2014–2015; see **Figure 3**). The mean reductions of BAI in defoliated trees during 2012–2013 compared with 2000–2011 were 74% and 43% in radiata pine and chestnut, respectively. For immune maritime pines (i.e., non-defoliated) BAI declined 4% during the outbreak period compared with the pre-outbreak period.

Prior to the outbreak, defoliated trees of radiata pine had lower mean maximum wood density (mean ± SE = $0.87 \pm 0.02\,\mathrm{g\ cm^{-3}}$) than non-defoliated ($0.96 \pm 0.02\,\mathrm{g\ cm^{-3}}$) pines ($U = 50$, $P = 0.003$). Similarly, defoliated trees presented lower mean minimum wood density ($0.44 \pm 0.01\,\mathrm{g\ cm^{-3}}$) than non-defoliated ($0.46 \pm 0.01\,\mathrm{g\ cm^{-3}}$) trees ($U = 63$, $P = 0.015$). However, in the year when outbreak peaked (2012), defoliated pines showed higher mean minimum wood density ($0.54 \pm 0.03\,\mathrm{g\ cm^{-3}}$) than non-defoliated ($0.48 \pm 0.02\,\mathrm{g\ cm^{-3}}$) pines ($U = 20$, $P = 0.003$) (**Figure 4**).

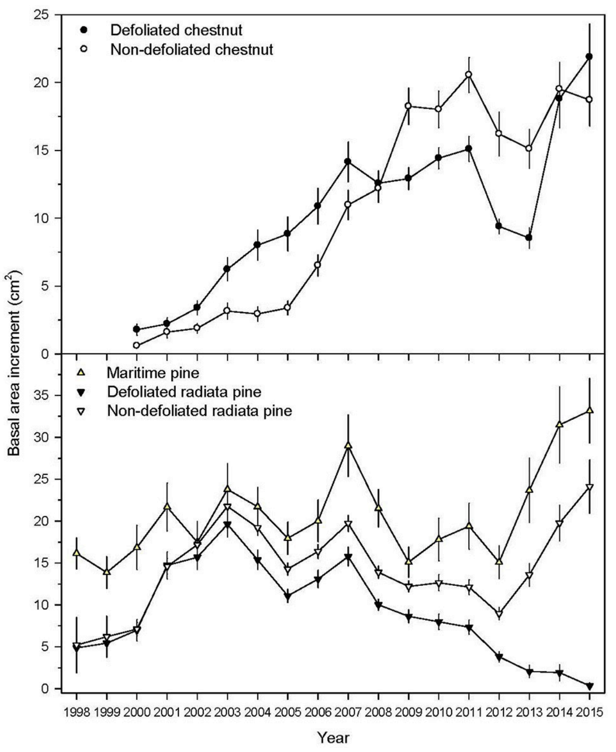

FIGURE 3 | Growth patterns (basal area increment) of the three studied tree species and considering defoliated and non-defoliated trees due to the gypsy moth outbreak in the case of chestnut and radiata pine. Values are means ± SE.

Climate and Defoliation Effects on Radial Growth

The mean BAI series of radiata pine and Maritine pine for the 2000–2011 period were highly correlated ($r = 0.68$, $P < 0.0001$), but the chestnut BAI series was neither related to the radiata ($r = -0.27$, $P = 0.39$) nor to the Maritime pine BAI series ($r = -0.007$, $P = 0.98$). Considering the ring-width indices (plotted in **Figure S4**), we found that high mean maximum temperatures during the previous December were positively related to Maritime pine growth, and high mean minimum temperatures during the previous autumn were associated with improved growth in the two pine species (**Figure 5**). However, high maximum February temperatures were negatively related to growth of both pine species. Warm spring temperature favored chestnut growth. Wet conditions during the previous autumn were positively related to growth of the three species, whereas wet February and July conditions favored Maritime pine and chestnut growth, respectively.

The climate variables selected in the linear mixed-effects models are in agreement with the climate-growth correlations observed in **Figure 5**. According to these results, annual BAI was affected by prior minimum autumn temperatures in the case of the two pine species and by maximum spring temperatures in the case of chestnut.

For radiata pine, the three-way interaction (defoliation × climate × period) was not significant ($P = 0.55$). Moreover, all the two-way interactions were not significant (P-values ranging from 0.44 to 0.66), except the climate × period ($P < 0.001$ in all cases; **Table S2** and **Figure S5**). The model containing this interaction was selected as the best according to the likelihood ratio test (**Table 2**). For Maritime pine, the full model (including the climate × period interaction) was also selected as the best (**Tables 2**, **S2**). For chestnut, none of the interaction terms was significant, and none of the models could be considered as the

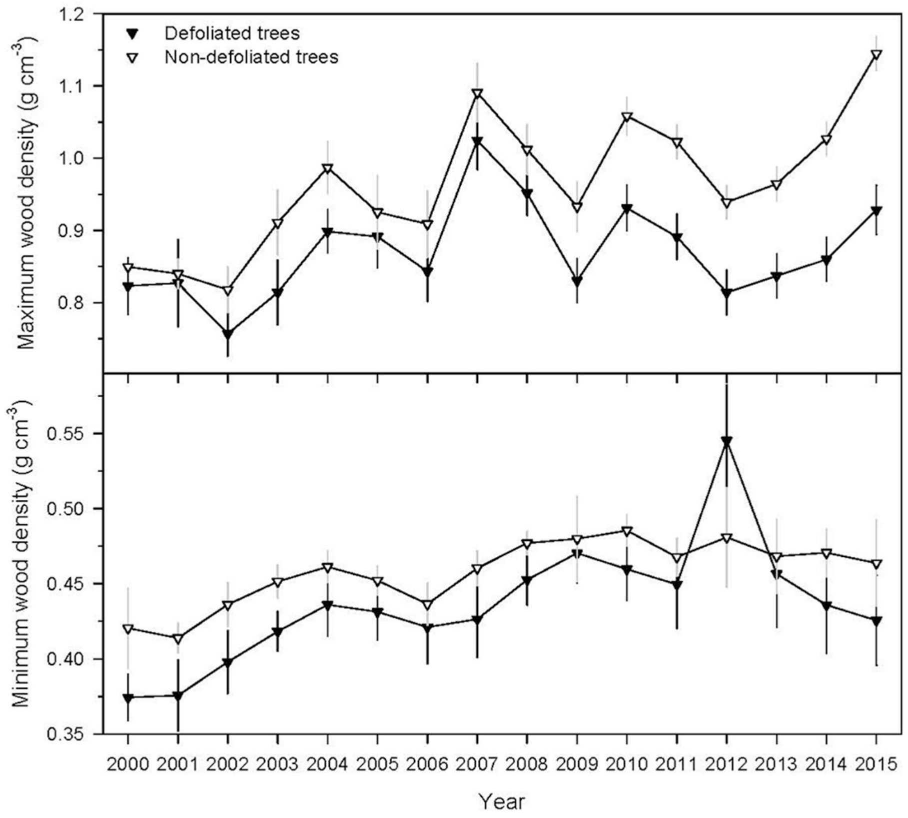

FIGURE 4 | Maximum and minimum wood density of defoliated and non-defoliated radiata pines. Values are means ± SE.

best according to the likelihood ratio test. Since none of the interaction terms was significant, we tested the significance of the main factors one at a time. The results indicate that both the climate variable (Tmax spring$_t$) and the period were significant (**Table 2**).

Concerning the influence of climate and defoliation in wood density, it was observed that in non-defoliated trees of radiata pine, minimum wood density was positively associated with June precipitation ($r = 0.61$, $P = 0.012$) but negatively associated with March minimum temperatures ($r = -0.64$, $P = 0.008$). In defoliated trees of radiata pine, minimum wood density decreased as minimum April temperatures increased ($r = -0.52$, $P = 0.040$). High September minimum temperatures were negatively associated with maximum wood density in defoliated ($r = -0.60$, $P = 0.014$) and non-defoliated trees ($r = -0.53$, $P = 0.038$).

Average BAI prior to the outbreak or during the outbreak was not spatially autocorrelated for any of the three tree species, since the *P*-values were not statistically significant ($P > 0.05$ in all cases; **Table 3**) associated with Moran's I index.

DISCUSSION

For radiata pine, the absence of significance for the three-way interaction (defoliation × climate × period) indicates that the interactive effects of climate and defoliation on growth did not differ before and during the defoliation event. The significance of climate × period interaction highlights that the effect of climate differ before and during the defoliation event, i.e., the climate affected radial growth in a different manner in the two different periods. The absence of significance for the climate × defoliation interaction states that the effect of climate on growth did not differ between the defoliated and non-defoliated trees, whereas in the case for the defoliation × period interaction points out that the effect of defoliation did not differ before and during the defoliation event.

The fact that the two way-interaction terms involving defoliation were not significant suggests that, for radiata pine, the difference in the pattern of radial growth before and during the defoliation event (74%) was more likely due to the difference in climate before and during the defoliation event than to the defoliation effect *per se*. These results are also consistent with the fact that the model including climate × period interaction was the best for Maritime pine: both pine species follow a similar radial growth pattern (**Figure 3**), led by climate characteristics.

The susceptible or host chestnut presented a moderate growth reduction (43%, **Figure 3**) which might be mainly attributed to the climatic characteristics, since the defoliation effect was found to be non-significant.

All these results illustrate that the differences between susceptible (radiata pine, chestnut) and resistant (Maritime pine)

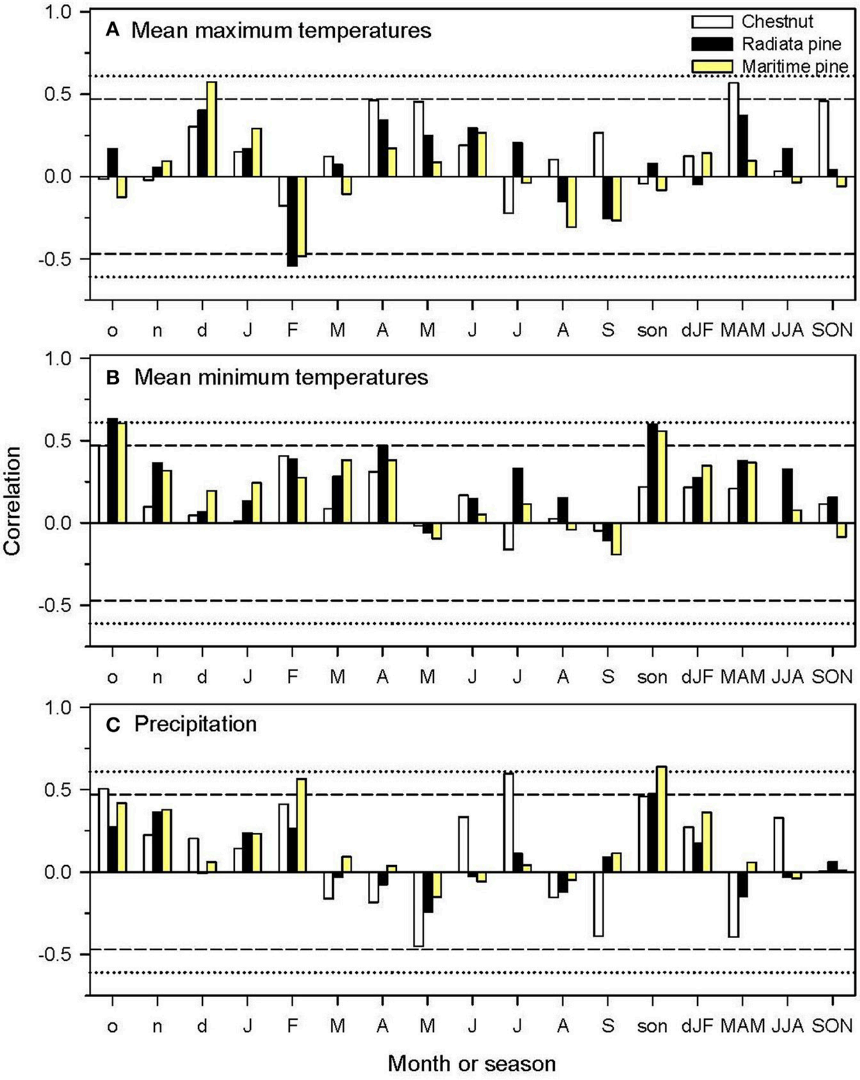

FIGURE 5 | Climate-growth relationships calculated by relating monthly or seasonal climatic variables [**(A)**, mean maximum temperatures; **(B)**, mean minimum temperatures; **(C)**, precipitation] with the mean series of ring-width indices of the three study species (chestnut, radiata pine, maritime pine). The dashed and dotted horizontal lines correspond to the 0.05 and 0.01 significance levels, respectively. Months or seasons abbreviated by lowercase or uppercase letters correspond to the previous and current years, respectively.

species to gypsy moth defoliation cannot be straight-forwardly explained in terms of differences in the pattern of radial growth as quantified by BAI, highlighting the difficulty of reconstructing past outbreak history solely by comparing tree-ring width series of coexisting host vs. non-host tree species. Outbreak tree-ring signals, as the sharp increase in minimum wood density observed in radiata pine, should be used as complementary information to tree-ring width data (Paritsis et al., 2009).

Several types of interactions between climate and insect defoliations can be discussed in this case study. First, it is possible that the particular warm and dry climatic conditions during 2011-2013 favored the gypsy moth outbreak. It is well known that spring temperature is an important factor influencing the temporal course of defoliator outbreaks, mainly due to its effects on herbivore survival and plant–herbivore synchrony (Fitzgerald, 1995). Specifically for gypsy moth, dry and warm weather in winter and spring seems to support outbreaks (Alalouni et al., 2013). However, variation in climate is not the only factor to consider since the population size of herbivorous insects must be increasing gradually prior to the outbreak, and host plants may show a progressive loss in vigor due to stressful climatic conditions (Ayres, 1993). Nevertheless, recent meta-analysis have shown that drought-stressed host plants show variable responses to herbivorous insects (Jactel et al., 2012), and therefore the subject remains under debate. Second, these particular climate conditions also contributed to reduce radial growth in non-defoliated species as Maritime pine, which growth is enhanced by wet-cool conditions during the prior

TABLE 2 | Parameter estimates and their corresponding *P*-values of the best linear mixed-effects models fitted to annual basal area increment (BAI_t) for *Pinus radiata*, *Pinus pinaster* and *Castanae sativa*.

Term	*Pinus radiata*		*Pinus pinaster*		*Castanea sativa*	
	Parameter est.	*P*-value	Parameter est.	*P*-value	Parameter est.	*P*-value
Intercept	34.69	< 0.0001	37.14	< 0.0001	–	–
Tmin $autumn_{t-1}$	−3.562	< 0.0001	−3.813	< 0.0001	–	–
Tmax $spring_t$	–	–	–	–	0.07918	< 0.0001
Defoliation	−0.3825	0.02	–	–	−0.06078	0.91
Period	−28.26	< 0.0001	−30.40	< 0.0001	0.3110	0.007
Tmin $autumn_{t-1}$ × Period	3.624	< 0.0001	3.885	< 0.0001	–	–
Phi	0.85		0.89		–	

For Castanea sativa the parameter estimates and P-values come from fitting a linear mixed-effects model using each covariate or factor one at a time. Phi is the autocorrelation parameter for the AR(1) error structure.

TABLE 3 | Values of the Moran's *I* index values used to test for the presence of spatial autocorrelation in growth data (BAI, basal area increment).

Species	BAI 2000-2011			BAI 2012-2013		
	Moran's *I*	*Z* score	*P*-value	Moran's *I*	*Z* score	*P*-value
Pinus radiata	0.0668	0.7112	0.48	0.1900	1.5792	0.11
Castanea sativa	−0.0984	−0.2910	0.77	0.1239	1.0792	0.28
Pinus pinaster	−0.2358	−1.4302	0.15	−0.2022	−1.1159	0.26

Z scores and P-values evaluate the significance of the index. Analysis was carried out for the period previous to defoliation (2000–2011) and for the outbreak period (2012–2013).

autumn and winter (e.g., February) seasons (Caminero et al., 2018). Maritime pine is a plastic tree species regarding its growth responses to Mediterranean climate (Sánchez-Salguero et al., 2018), characterized by a high year-to-year variability in precipitation, which is consistent with the growth reductions observed in 2009 and 2012 in response to adverse climatic conditions. Dry and warm conditions in previous autumn and winter may reduce carbohydrate reserves by increasing respiration before the onset of tree-ring formation in spring (Kagawa et al., 2006).

In radiata pine the growth reduction started in 2012, and growth decline was irreversible in defoliated trees. Indeed, the mortality rate of severely defoliated trees of radiata pine reached in some areas values of 87% (Castedo-Dorado et al., 2016), as confirmed the last rings observed (2011-2012) in many wood samples of defoliated trees.

The absence of defoliation effect in the radial growth of chestnut trees can be explained by rapid coppicing by defoliated trees of chestnut in summer. These trees used reserved photoassimilates to rebuild their foliage and recover growth levels after the outbreak (Palacio et al., 2012). The growth pattern observed in chestnut agrees with previous studies on gypsy moth outbreaks affecting hardwood forests in north-eastern USA. There, gypsy moth caused a loss in radial growth of host oak species proportional to the defoliation severity during the year of defoliation and, in a lower degree, during the following year, whilst plot-level defoliation positively influenced the radial increment of non-host species (e.g., ash) which benefitted from increased radiation and nutrients after the death of severely defoliated trees (Muzika and Liebhold, 1999). This potential benefit for non-host species could explain the rapid increase of growth observed in non-defoliated trees of Maritime pine in 2013, whilst defoliated trees of chestnut rapidly recovered growth rates and even surpassed non-defoliated tree of chestnut in 2015.

The abundant latewood IADFs observed in 2006 and 2009 confirmed the growth sensitivity of radiata pine to the intra-annual variability in climate of the study area. Probably, wet June to July conditions favored the radial expansion of latewood tracheids leading to an increase in hydraulic conductivity and the formation of cells with wide lumen and possibly thin walls (Vieira et al., 2010). In Aleppo pine (*Pinus halepensis* Mill.) and Stone pine (*Pinus pinea* L.) latewood IADFs were also formed in response to humid conditions in summer and autumn when cell-wall thickening and lignification are active phases of xylogenesis (Campelo et al., 2007; Pacheco et al., 2018). The IADFs did not translate into enhanced radial growth but the formation of earlywood-like tracheids within the latewood probably explained the reduction in maximum wood density in combination with warmer temperature in autumn.

One of the most striking results of this study was the increase of minimum wood density of radiata pine in response to the 2012 defoliation. Planted Maritime pine subjected to induced defoliation by *Thaumetopoea pityocampa* Den. & Schiff. showed a ca. 30% reduction in tree-ring width but no differences in maximum wood density when compared with non-defoliated trees (Polge and Garros, 1971). However, the minimum wood density of the defoliated trees decreased the defoliation year, but increased two years later. In planted Norway spruce (*Picea abies* L.), outbreaks of nun moth (*Lymantria monacha* L.) were associated with the production of narrow rings with higher minimum wood density (Koprowski and Duncker, 2012). Most studies using tree-ring data to reconstruct or to quantify the impacts of population outbreaks of forest Lepidoptera have documented reductions of radial growth associated with severe defoliations (Swetnam and Lynch, 1993; Krause, 1997; Weber, 1997; Rolland et al., 2001; Speer et al., 2001; Camarero et al., 2003; Vejpustková and Holuša, 2006;

Sutton and Tardif, 2007; Sangüesa-Barreda et al., 2014), and sometimes a decrease in maximum wood density reflecting a reduction in the thickening and lignification rates of latewood tracheids (Esper et al., 2007). The finding that gypsy moth defoliation increased minimum wood density may be explained considering xylogenesis processes and its climate drivers. In planted radiata pine stands, wet conditions are associated with improved growth, whereas warm conditions during the growing season are associated with a reduction in wood density (Ivković et al., 2013). Minimum wood density is positively associated with spring temperatures, prior winter precipitation and drought severity in plantations in Australia and New Zealand (Harris, 1965; Downes et al., 1993). This indicates that warmer spring conditions favor photosynthesis and increase wood density by increasing wall thickness, whereas drought stress will increase density by decreasing the radial diameter of tracheids. In fact, drought was associated with increases in minimum wood density of planted radiata pines (Downes et al., 1993). In this case defoliation probably altered the water use within trees, reduced the turgour of cambial derivatives and induced the formation of earlywood tracheids with narrow lumen (Skene, 1969) leading to an increase in minimum wood density as has been observed in other conifers under natural conditions (Camarero et al., 2014). To disentangle the defoliation from the climate effects on wood formation, further studies could quantify density changes in non-host species as Maritime pine as compared with host species as radiata pine. Regarding maximum wood density in radiata pine, it has been found to be negatively associated with severe stress due to summer drought (Downes et al., 1993), in our case this could correspond to warm September conditions amplifying late-summer drought.

Interestingly, defoliated trees of radiata pine showed lower growth and maximum and minimum wood density values than non-defoliated trees during the 2000–2011 period, i.e., prior to the 2012–2013 outbreak. According to the growth-defense trade-off hypothesis (Herms and Mattson, 1992), we would expect that trees showing low growth rates can invest more on defenses and therefore are less susceptible to defoliation, whilst fast-growing trees would be more susceptible to defoliation. In this case slow-growing radiata pines were also more defoliated, not supporting that trade-off at the within-species level. Differences in radial growth cannot be attributed either to microsite differences since BAI previous to defoliation and during the defoliation was not found to be spatially autocorrelated; i.e., the spatial distribution of BAI is likely to be the result of random spatial processes.

In chestnut, BAI of defoliated trees was also lower than that of non-defoliated trees from 2009 to 2011. Remarkably, chestnut trees show lower grow rates and may be genetically predisposed to invest more in defense than fast-growing radiata trees according to the growth-defense trade-off hypothesis applied at the between-species level. Probably as a consequence of greater amounts of defense in chestnut, the impact of defoliation has been negligible. Nevertheless, we note that growth-defense trade-offs are complex to disentangle within and between species and may be attenuated under favorable climate conditions (Züst and Agrawal, 2017).

All these results confirm that the most defoliated trees, particularly in the case of radiata pine, were previously growing less and forming less dense wood than trees less affected by defoliation. This could mean that they were predisposed to show more pronounced gypsy-moth defoliation and they were more prone to die than fast-growing trees as has been observed in drought-induced forest dieback studies in natural pine populations (Camarero et al., 2015). Regrettably, the radiata pines used for obtaining BAI and density were not the same. Therefore, future studies could concentrate on evaluating the relationships between BAI and wood density in defoliated trees. The 2012 increase in minimum wood density, possibly reflecting a reduction in the transversal area of earlywood tracheids, was coherent with the reduction in growth since earlywood accounts for most of the hydraulic conductivity within the ring (Domec et al., 2009).

CONCLUSIONS

Abnormal warm and dry conditions in the winter prior to a gypsy moth outbreak reduced radial growth in both host (radiata pine and chestnut) and non-host (Maritime pine) species and might have resulted in the defoliation event occurred in 2012–2013.

Defoliation induced an increase in minimum wood density of radiata pine, probably reflecting a reduction in earlywood hydraulic conductivity and radial-growth rates. Therefore, this complementary information to tree-ring width data must be used in reconstructing past outbreak history of coexisting host vs. non-host tree species.

Radiata pine plantations could show relevant losses in productivity and changes in wood density due to potential interactions between climate warming and gypsy-moth outbreaks.

AUTHOR CONTRIBUTIONS

JC, FA-T, and FC-D conceived the idea. AH performed and interpreted the densitometry analyses. All authors contributed to the field work and the interpretation of the results. The dataset was analyzed by JC, FC-D, and FA-T. JC and FC-D wrote the first draft of the manuscript; thereafter all authors revised the first draft by rewriting, discussing and commenting. All authors read and approved the final draft.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2018.01582/full#supplementary-material

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Drought and *Phytophthora* Are Associated With the Decline of Oak Species in Southern Italy

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Forest decline induced by climate change is a global phenomenon that affects many tree species, mainly in drought-prone areas as the Mediterranean region. In southern Italy, several oak species have shown decline symptoms and elevated mortality since the 2000s due to drought stress. However, it remains to be answered whether decline occurred alone or whether a pathogen was also involved. To this aim, we compared two coexisting oak species in a forest located in southern Italy which are assumed to be less (*Quercus cerris*) and more tolerant to drought (*Quercus pubescens*). We sampled fifteen couples of neighboring declining (D) and non-declining (ND) trees of both species. Wood cores were taken from all trees to perform dendrochronological analyses to detect the decline onset and link it to potential climatic drivers. Carbon isotope ratios ($d^{13}C$) were analyzed in wood of the two vigor classes to compare their water-use efficiency. *Phytophthora* presence was also assessed in soil samples from ten D-ND couples of trees per species. The oak species most affected by drought-induced decline in terms of leaf shedding and mortality was *Q. cerris*, i.e., the least tolerant to drought. In both species, the D trees showed a reduced growth rate compared with ND trees from 2000 onward when drought and warming intensified. *Q. pubescens* showed higher growth sensitivity to precipitation, temperature and drought than *Q. cerris*. This sensitivity to climate was magnified in D trees whose growth decreased in response to warm and dry conditions during the prior winter and the late summer. The *Q. pubescens* D trees were more efficient in their water use than ND trees before the growth divergence between D and ND trees amplified. In the studied area, *Phytophthora quercina* was isolated from 40% of the sampled trees, and tended to be more frequent amongst ND than amongst D trees. Our data suggests that droughts and warm summer conditions triggered oak decline. The high prevalence of *P. quercina* in the studied area warrants further study as a potential predisposing factor.

Keywords: carbon isotopes, dendroecology, drought stress, forest dieback, *Phytophthora* species, *Quercus cerris*, *Quercus pubescens*, water-use efficiency

INTRODUCTION

Forest decline is a complex phenomenon characterized by a loss in tree vigor and increased mortality, and it is usually triggered by extreme climate events (e.g., droughts) but also involves pathogens and pests (Manion, 1991; Sangüesa-Barreda et al., 2015).

In oaks, climate warming and altered precipitation regimes may negatively affect the carbon and water use of trees and indirectly benefit root pathogens (e.g., *Phytophthora* spp.) causing the dead of fine roots and making trees more susceptible to water shortage (Wargo, 1996; Führer, 1998; Thomas et al., 2002; Haavik et al., 2015). However, it remains to be answered to what degree severe climatic stressors (drought or heat waves) trigger forest decline making trees more vulnerable to biotic stressors (root pathogens).

Drought and high evapotranspiration rates linked to warmer conditions have been found to reduce radial growth, increase stress and trigger forest decline in many oak species worldwide (Tainter et al., 1990; Jenkins and Pallardy, 1995; Pedersen, 1998; Andersson et al., 2011; Corcuera et al., 2004a,b; Sohar et al., 2014). Comparisons of tree-ring width series through time has revealed that declining trees showed lower radial-growth rates than non-declining trees about 5–25 years prior to the decline onset or before tree death (Haavik et al., 2011; Levaniè et al., 2011; Helama et al., 2016; Cailleret et al., 2017; Gentilesca et al., 2017; Colangelo et al., 2017a, 2018). Declining trees are usually characterized by recent low growth rates but also by leaf shedding and architectural anomalies such as abundant epicormic and dead shoots (Drobyshev et al., 2007).

The most likely mechanism causing drought-induced decline in anisohydric species as many ring-porous oak species is hydraulic failure (McDowell et al., 2008). Such prolific use of water during drought leads to a reduction of stem water potential and an elevated loss in hydraulic conductivity of declining individuals showing intense crown desiccation and leaf shedding (Nardini et al., 2013). If unusually severe and sustained droughts modify C supply (photosynthesis, leaf area) and demand (growth, root production) in oaks, and alter aboveground and belowground C reserves and translocation, trees could become more susceptible to necrotrophic root-rot pathogens (Oliva et al., 2014) such as several *Phytophthora* species (Brasier, 1996; Jung et al., 1999, 2000). Drought and soil pathogens can synergistically amplify stress and cause the decline and death of oaks (Haavik et al., 2015).

In Europe, *Phytophthora quercina* has been commonly associated with oak root rot and decline predominantly in wet sites (Jung et al., 1999, 2000; Vettraino et al., 2002; Balci and Halmschlager, 2003; Jönsson, 2004). However, it remains unclear whether *Phytophthora* is the main trigger for oak decline episodes, because the relationships between environmental conditions, root damage caused by the pathogen and the loss in tree vigor are poorly understood (Jönsson et al., 2005; Jönsson, 2006). Although associations between oak crown transparency and the presence of *Phytophthora* species in the rhizosphere have been reported (Jung et al., 2000; Vettraino et al., 2002), information on the growth rates of coexisting non-declining and declining trees which show disease development is still missing (Jönsson et al., 2005).

Drought stress has been considered among the main triggering factors of oak decline, but it remains to be answered if both drought and pathogens synergistically contribute to the long-term loss in growth and tree vigor. Furthermore, to our best knowledge there are not experiments which investigated the effect of both stressors under controlled conditions on our studied species, except for other *Quercus* species (see for instance Sànchez et al., 2002 on *Q. ilex* where *P. cinnamomi* was investigated).

Here we use tree-ring width data to reconstruct growth and pinpoint the onset of decline in two affected oak species coexisting in the same forest located in southern Italy (*Quercus pubescens*, *Quercus cerris*). This comparison of the two species allows testing if the species more abundant in xeric sites (*Q. pubescens*) was less affected by drought-induced decline than the species from mesic sites which is assumed to be less tolerant to water shortage (*Q. cerris*). Both *Q. pubescens* and *Q. cerris* seem to be susceptible to *P. quercina*, because incidence of the pathogen has been previously reported in stands of these oak species (Vettraino et al., 2002; Balci and Halmschlager, 2003). Although differences in drought tolerance among oak species are widely reported in literature, there are only few comparative studies on Mediterranean oaks (e.g., Dreyer et al., 1992; Valentini et al., 1992; Tognetti et al., 1999). Based on the distribution of the main oak species in southern Italy, the following ranking in drought tolerance can be suggested: *Q. robur* (least tolerant) < *Q. cerris* < *Q. frainetto* < *Q. pubescens* < *Q. ilex* < *Q. suber* (most tolerant).

We hypothesize that the severe 2000s droughts triggered oak decline, and that *Phytophthora quercina* was involved in the decline syndrome either as predisposing or as a contributing factor. To test this idea, we reconstructed the tree-ring growth, isotopic signature of declining oaks and we assessed the association between the presence of symptoms of decline and *Phytophthora* infestation.

MATERIALS AND METHODS

Study Area, Tree Species and Forest Decline

The study site (Gorgoglione forest, 40° 21′ 51″ N, 16° 10′ 34″ E, 800–850 m a.s.l.) is located in the mountainous Basilicata region, southern Italy. It has a mean slope of 25%. The study area is a mixed high forest with a mean density of 600 stems ha^{-1} dominated by *Quercus cerris* L. (71%) followed by *Quercus pubescens* L. (25%) and by other broadleaf species (4%). No recent disturbance has been reported for the study sites (e.g., insect outbreaks or fires) and no silvicultural treatment has been applied in the last five decades. In the past, these oak forests were traditionally managed as coppices for firewood and timber production in combination with livestock grazing. Soils are a mixture of sand, silt, and clay textures.

Climate in the study area is Mediterranean (cool dry-summer climate according to the Köppen classification), characterized by dry and warm summers (total precipitation from June to August is 93 mm) and wet and mild winters (total precipitation from December to February is 230 mm) with mean annual temperature of 11.6°C and annual precipitation of 722 mm (data from Gorgoglione station, 40° 24′ N, 16° 09′ E, 796 m). The warmest and coldest months are August (mean temperature of 20.5°C) and January (mean temperature of 3.7°C), respectively, whereas the driest and wettest months are July (22 mm) and November (96 mm). Drought occurs from June to August.

The two studied tree species showed recent drought-induced decline symptoms since the early 2000s (shoot dieback, leaf loss and withering, growth decline, and high mortality). Regarding the incidence of the decline syndrome according to local reports, annual oak mortality in the study area affected ca. 450 ha and shifted from 5 to 10% from 2002 to 2004. In the most affected stands, more than 50% of mature specimens showed decline symptoms and 15% were dead, while the remaining individuals (35%) were non-declining trees.

Long-Term Climate and Drought Data

Due to the shortness and heterogeneity of local climate data we used gridded (0.25° resolution) climate data from the E-OBS dataset version 13.0 (Haylock et al., 2008) to quantify climate trends and climate-growth associations for the period 1950–2016. Climate was extracted from the 0.25° grid with coordinates 40.00–40.25° N, 16.00–16.25° E. To evaluate drought-growth associations we downloaded the Standardized Precipitation Evapotranspiration Index (SPEI) for the 0.5° grid where the study sites are located and considering 1–24 months long scales using the Global SPEI database webpage[1]. The SPEI is a multiscalar drought index, which considers the effects of temperature and evapotranspiration on drought severity and indicates wet (positive SPEI values) and dry (negative SPEI values) conditions (Vicente-Serrano et al., 2010).

Field Sampling and Tree-Ring Data

In the field, first we randomly located seven circular plots (radius of 15 m) to estimate the density of trees with different vigor. Then, we sampled dominant trees of the two oak species. We selected pairs of neighboring (located at less than 20 m apart) declining or symptomatic (D trees) and non-declining or asymptomatic (ND trees). Declining oaks (hereafter D trees) were considered those with crown transparency higher than 50%, whereas non-declining oaks (hereafter ND trees) were considered those with transparency lower than 50%. Crown transparency was estimated by a visual assessment performed by two independent observations on the same tree using binoculars (Colangelo et al., 2017a). Using other crown-transparency thresholds (40 and 60%) the main results presented here did not change (cf. Colangelo et al., 2017a). Note, however, that recently dead trees could have been logged by people from nearby villages so our data may underestimate the actual density of D and recently dead trees.

The diameter at breast height (dbh), i.e., at 1.3 m, and height of each tree were measured using tapes and a laser rangefinder, respectively. To study radial growth we extracted one increment core per tree at breast height (1.3 m) using 5-mm Pressler increment borers. We avoided taking two cores per tree due to restrictions imposed by forest managers for preserving the study stands.

To quantify radial-growth changes in trees of contrasting vigor we used dendrochronology. In total we sampled 42 D and 32 ND *Quercus cerris* trees and 17 D and 21 ND *Quercus pubescens* trees. Within each ND-D couple, trees were located 10–15 m apart at maximum. Wood samples were air–dried and the surface of the cores was cut using a sledge core microtome (Gärtner and Nievergelt, 2010). Tree rings were visually cross-dated and measured with precision of 0.01 mm using a binocular microscope coupled to a computer with the LINTAB package (RINNTECH, Heidelberg, Germany). The COFECHA program (Holmes, 1983) was used to evaluate the visual cross-dating of tree-ring series. To estimate age at 1.3 m we counted the number of annual rings. In cores without pith, the missing distance was estimated by fitting a template of concentric circles with known radii to the curve of the innermost rings, which allowed the estimation of the missing radius length and transforming it into the number of missing rings (Camarero et al., 2015).

To quantify climate- and drought-growth relationships, first we removed the long–term trends of tree-ring width series by detrending them through the Friedman super smoother, which preserves high-frequency (annual) variability in the resulting ring-width indices. In addition, an autoregressive model was applied to each detrended series to remove most of the first-order autocorrelation related to the previous-year of growth. We obtained series at the tree level of dimensionless ring-width indices. Lastly, a biweight robust mean was used to obtain mean chronologies of ND and D trees at each site. Chronology development and standardization were carried out using the ARSTAN program (Cook and Krusic, 2005).

Climate-growth associations were calculated for the common and best-replicated period 1950–2016, considering monthly climatic variables (mean maximum and minimum temperatures, precipitation). The window of analyses included form the previous September to current September based on previous studies (Tessier et al., 1994). Drought-growth associations were calculated for the same time period using 1- to 24-month long SPEI values obtained from January to December.

Finally, we calculated dendrochronological statistics to compare the ring-width series of ND and D trees. Specifically, we calculated the first-order autocorrelation of ring-width data (AR1) and the mean sensitivity of ring-width indices (MS) which assesses the relative variability in width between consecutive rings (Briffa and Jones, 1990).

Carbon Isotopes in Wood

We compared carbon isotope ratios ($^{13}C/^{12}C$, $\delta^{13}C$) in wood between non-declining (n = 5 trees) and declining (n = 5 trees) individuals of each species. We considered three different 5-year periods including different phased of the decline event (2002–2006, 2007–2011, and 2012–2016). The wood samples were dried in the oven at 70°C for 48 h and ground to a fine powder. Wood aliquots (0.001 g) were weighed on a microbalance (AX205 Mettler Toledo, OH, United States) into

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[1] http://sac.csic.es/spei/database.html

tin foil capsules and combusted to CO_2 using a Flash EA-1112 elemental analyzer interfaced with a Finnigan MAT Delta C isotope ratio mass spectrometer (Thermo Fisher Scientific Inc., MA, United States). Isotope analyses were conducted at the Isotope Lab of the "Istituto di Geoscienze e Georisorse" (CNR; Udine, Italy). Stable isotope ratios were expressed as per mil deviations using the δ notation relative to Vienna Pee Dee Belemnite (VPDB). The standard deviation for repeated analyses was better than 0.1‰.

To account for the $\delta^{13}C$ depletion of atmospheric CO_2 ($\delta^{13}Ca$) due to the combustion of fossil fuels, we calculated C isotope discrimination in wood ($\Delta^{13}C$) from $\delta^{13}Ca$ and plant $\delta^{13}Cp$ following Farquhar et al. (1989):

$$\Delta^{13}\mathrm{C} = \left(\delta^{13}Ca - \delta^{13}Cp\right)/(1 + \delta^{13}Cp/1000) \qquad (1)$$

$\delta^{13}Ca$ was obtained from Graven et al. (2017).

Soil Characteristics

Soils were sampled at ten points separated by 10 m and located within the sampled stands. Soils were taken at a depth of 15–30 cm. Soils were characterized them by measuring the following variables: texture, C and N concentrations, pH and electrical conductivity. Soil texture was determined with laser diffraction method in Coulter Mastersizer (2000) and clay content was corrected (Taubner et al., 2009). Soil C and N concentrations and the C/N ratio were determined with an elemental analyzer (Elementar Vario MAX N/CM, Hanau, Germany).

Detection of Phytophthora Presence

We focused on the detection of *Phytophthora* since this pathogen has been often associated with decline and mortality of oak stands across Europe (Jung et al., 1999, 2000). To evaluate whether dieback was associated with soilborne *Phytophthora* infection we sampled the rhizosphere of twenty trees (five defoliated and five healthy trees ×2 tree species). Approximately 400 gr of shallow soil were collected after removing the organic layer from three points situated below the crown of each oak taken at a mean 25 cm depth in the A horizon. Samples were pooled for each tree. Soil baiting was carried out following Jung's (2009) using fresh leaves of Valencian oak (*Quercus faginea*) as baits. Baits were inspected regularly during 10 days. Necrotic spots were plated on CMA-PARPBH medium (Jeffers and Martin, 1986) and then incubated at 20°C in darkness. Within 2–4 days, *Phytophthora*-like colonies were transferred onto V8 agar media (Erwin and Ribeiro, 1996), and stored at 20°C. DNA from 14 *Phytophthora*-like isolates was extracted following the NaOH extraction procedure described by Wang et al. (1993). The ITS region was amplified using the ITS6f and ITS4 primers (White et al., 1990; Cooke and Duncan, 1997), following the PCR settings specified in Samils et al. (2011). PCR product was sequenced by Macrogen (South Korea), and a BLAST search was later performed on the Phytophthora ID curated database[2], establishing a minimum identity threshold for species identification of 98%.

Statistical Analyses

Tree characteristics of the two tree vigor classes were compared between species and between vigor classes using *t* or Mann-Whitney *U* tests in the case of variables that followed or did not follow normal distributions, respectively. To assess trends in monthly climate data (mean maximum and minimum temperatures, precipitation) we used the non-parametric tau statistic (τ) considering climate data for the 1950–2016 period. We used the Wilcoxon rank-sum test to check if mean tree-ring width differed between ND and D trees because this non-parametric statistic is robust against deviations from normal distributions and the presence of temporal autocorrelation (Gibbons and Chakraborti, 2011).

RESULTS

Climate Trends and Drought Variability

Significant ($P < 0.05$) and positive trends in mean maximum temperatures of January ($\tau = 0.26$), July ($\tau = 0.17$), October ($\tau = 0.20$), and November ($\tau = 0.21$) were found for the period 1950–2016 (**Figure 1**). All months excepting February showed significant and positive trends in mean minimum temperatures, being particularly strong in July–August ($\tau = 0.40$). Only December showed a significant negative trend in precipitation ($\tau = -0.18$), followed by May ($\tau = -0.14$). Lastly, the May SPEI calculated at 11-month long scales showed dry conditions (SPEI < -1.75) in 1957, 1995, 2000, 2002, and 2013, whereas wet conditions (SPEI > 1.75) occurred in 1980, 2006, and 2009.

Density and Size of Declining and Non-declining Trees

The densities of D trees were 262 trees ha^{-1} (64% of trees) and 43 trees ha^{-1} (30% of trees) in *Q. cerris* and *Q. pubescens*, respectively (**Table 1**). ND trees had thicker stems (greater dbh) and were taller than D trees in both species, but age did not differ between the two vigor classes (**Table 1**).

Growth Patterns and Comparisons of the Two Vigor Classes

Radial growth increased in the 1901s, from the 1930s to 1940s, in the late 1950s and 1960s, in the and early 1970s, and in the 2000s and 2010s; but it decreased in the 1920s, early 1950s and early 1960s, 1980s, and 1990s (**Figure 2A**). Tree-ring width was higher in ND than in D trees for the periods 2014–2016 and 2010–2016 in *Q. pubescens* and *Q. cerris*, respectively (**Figure 2A**). Considering the common period (1950–2016) ND trees had wider rings than D trees (*Q. pubescens*, 0.94 vs. 0.73 mm; *Q. cerris*, 1.22 vs. 1.07 mm), whilst the rest of dendrochronological statistics did not differ between the two vigor classes (**Table 2**). It is also remarkable the higher correlations of the individual series with the mean series of each vigor class observed in *Q. cerris* as compared with *Q. pubescens*, indicating a higher coherence and a higher responsiveness to climate of the former species as compared with the latter.

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[2] http://phytophthora-id.org

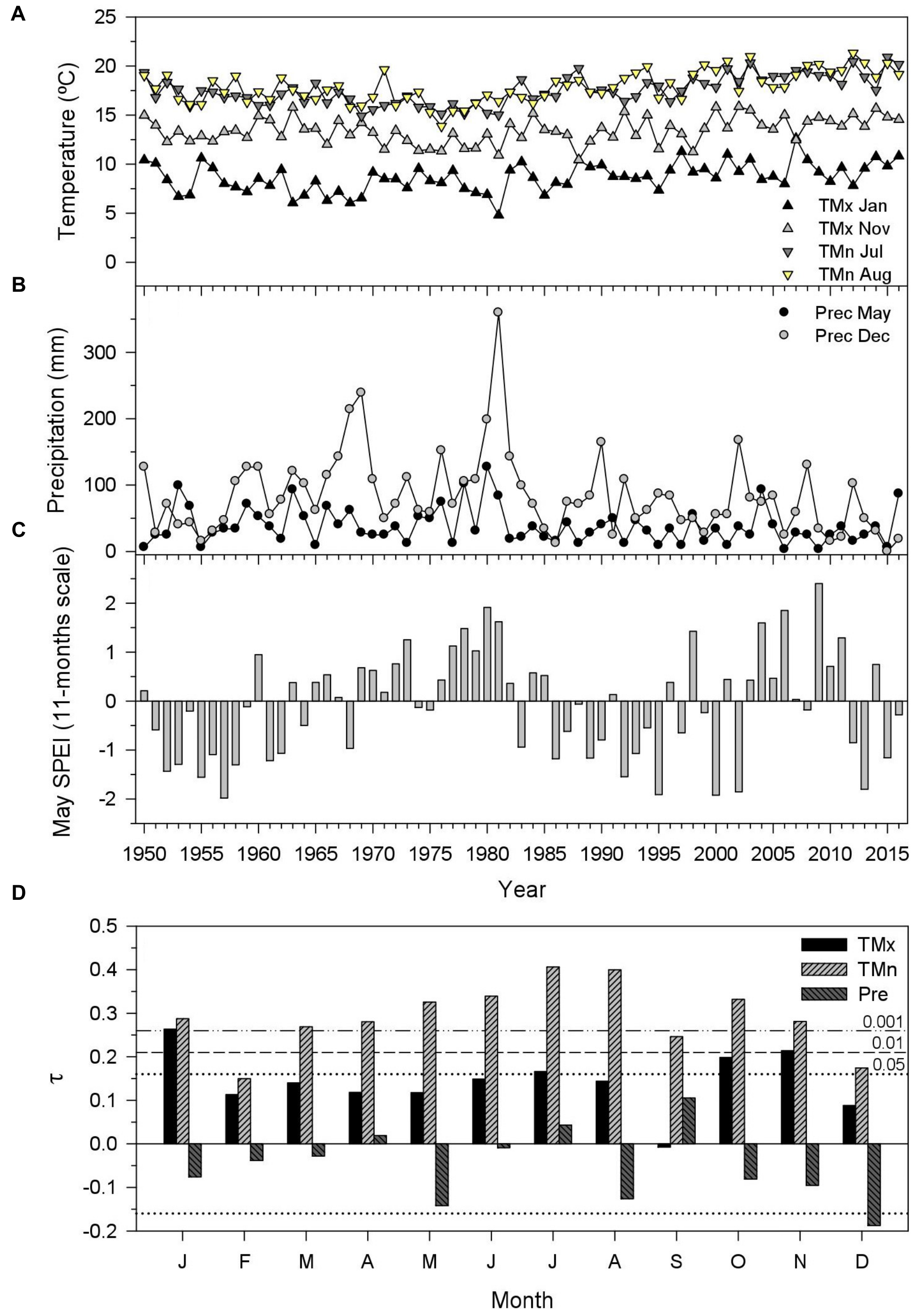

FIGURE 1 | Climate trends and drought patterns in the study area. The selected climate variables correspond to monthly mean maximum (TMx) and minimum (TMn) temperatures **(A)** and precipitation [**(B)** Prec] showing the most pronounced trends for the 1950–2016 period as assessed through the tau (τ) statistic **(D)**. The drought severity was assessed using the May Standardized Precipitation Evapotranspiration Index (SPEI) for 11-months long scales **(C)**, which showed high associations to tree growth (see **Figure 4**).

During the common period 1950–2016, sharp growth reductions in both species and vigor classes (e.g., 1957, 1962, 1983, 1986, 2002, and 2012–2013) usually corresponded to dry conditions, whereas growth increases corresponded to wet conditions (1960, 1972–1973, 1977, 1980, 1985, 1998, 2004, and 2009; compare **Figures 1C**, **2B**). An abrupt increase of the ring-width index was observed in ND trees of both species in 2014. The four ring-width chronologies were highly and significantly correlated during 1950–2016 suggesting a high coherence and similar responses to climate (**Figure 2B**), but the highest and lowest correlations were observed for *Q. cerris* ND and D trees (r = 0.90, P < 0.001) and for

TABLE 1 | Main structural characteristics of the non-declining (ND) and declining (D) oak trees sampled in Gorgoglione forest.

Tree species	Vigor class	Density (trees ha^{-1})	Diameter at 1.3 m (cm)	Height (m)	Age at 1.3 m (years)
Quercus pubescens	ND	100 ± 11b	26.5 ± 1.3b	11.3 ± 0.3b	115 ± 4
	D	43 ± 6a	22.5 ± 0.8a	8.6 ± 0.3a	111 ± 3
Quercus cerris	ND	148 ± 13a	33.0 ± 0.9b	15.0 ± 0.6b	102 ± 4
	D	262 ± 16b	28.3 ± 0.7a	12.2 ± 0.4a	107 ± 2

Values are means ± SE. Different letters indicate significant differences at the 0.05 level based on t-tests.

TABLE 2 | Tree-ring width statistics of the non-declining (ND) and declining (D) oak trees sampled in Gorgoglione forest and calculated for the common and best-replicated period 1950–2016.

Tree species	Vigor class	Time span	Tree-ring width (mm)	AR1	MS	Correlation with mean series
Quercus pubescens	ND	1881–2016	0.94 ± 0.10b	0.66 ± 0.03	0.32 ± 0.01	0.45 ± 0.03
	D	1883–2016	0.73 ± 0.04a	0.63 ± 0.04	0.33 ± 0.01	0.50 ± 0.04
Quercus cerris	ND	1890–2016	1.22 ± 0.04b	0.69 ± 0.02	0.34 ± 0.01	0.67 ± 0.02
	D	1879–2016	1.07 ± 0.04a	0.72 ± 0.01	0.34 ± 0.01	0.66 ± 0.02

Values are means ± SE. Different letters indicate significant differences at the 0.05 level based on t-tests. Variables' abbreviations: AR1, first-order autocorrelation of ring-width data; MS, mean sensitivity of ring-width indices.

Q. cerris ND and *Q. pubescens* D trees (r = 0.68, P < 0.001), respectively.

Growth Responses to Climate and Drought

Both *Q. pubescens* and *Q. cerris* growth responded negatively to warm previous September conditions, regardless the tree vigor (**Figure 3**). However, the growth of *Q. pubescens* D trees was sensitive to high maximum and minimum temperatures in January, April and from current July to September. In *Q. cerris*, D trees were more sensitive to warm April conditions, whilst warm conditions in current September were negatively associated to growth of both vigor classes. In *Q. cerris*, high minimum temperatures in the prior late autumn and early winter (November and December) enhanced growth of ND trees. Wet January conditions benefitted growth of both species, particularly of D trees, whereas wet current September conditions (and also in the prior September for *Q. pubescens*) improved growth of ND trees.

In all species and vigor classes, the maximum positive correlations between ring-width indices and SPEI (r = 0.37–0.42) were found for May and considering 11-months long scales, i.e., from the previous July up to May (**Figure 4**) The highest correlations with May SPEI calculated at 11-months scales were found for *Q. pubescens* D trees (r = 0.42). In *Q. cerris* ND trees, very high correlations (r = 0.38) were also observed with January SPEI and considering a 2-month scale.

Soil Features and Phytophthora Presence

Soils were of silt loam texture with mean percentages of 55, 32, and 13% for silt, sand and clay. The mean (±SE) soil C and N concentrations were 3.21 ± 0.24 and 0.17 ± 0.01%, respectively (mean C/N ratio is 20.5 ± 4.0). The soil pH was 7.5 ± 0.1 and the soil electrical conductivity was 184.0 ± 8.7 ms cm^{-1} indicating slight salinity.

We isolated *Phytophthora quercina* in 40% of the sampled trees. Phytophthora tended (p = 0.068, Chi-square test without Yates correction) to be more frequent on ND than on D trees (same proportion was observed for *Q. pubescens* and *Q. cerris*). The low number of infected trees per defoliation class did not allow the comparison of tree ring width between D and ND trees. Nevertheless, comparison of tree-ring width data between *Phytophthora*-infected and -uninfected trees (regardless of the defoliation class) within each tree species for the periods 2014–2016 and 2010–2016 did not reveal significant differences (*Q. pubescens*, Mann-Whitney U = 81–149, P = 0.41–0.62; *Q. cerris*, Mann-Whitney U = 271–319, P = 0.43–0.69; see Supporting Information, **Supplementary Figure S1**).

Wood C Isotopes

Regardless the vigor class, *Q. pubescens* showed higher $d^{13}C$ values (mean ± SE = −27.33 ± 0.22‰) than *Q. cerris* (−27.45 ± 0.19‰), but differences between the two species were not significant (t = 0.42, P = 0.67). Considering the two vigor classes, D trees (−26.85 ± 0.27‰) showed significantly higher $d^{13}C$ values than ND trees (−27.79 ± 0.29‰) in *Q. pubescens* (t = 2.35, P = 0.026), but differences were not significant in the case of *Q. cerris* (D trees, −27.39 ± 0.30‰; ND trees, −27.53 ± 0.25‰; t = 0.36, P = 0.72; **Table 3**). Considering different 5-year periods, $d^{13}C$ and $\Delta^{13}C$ were higher in D than in ND trees of *Q. pubescens* for the period 2007–2011.

DISCUSSION

The study area has experienced a rise in minimum temperatures since 1950 and this warming trend coincided with the occurrence of severe droughts in the early 2000s and early 2010s. As expected, these warm and dry conditions probably triggered the

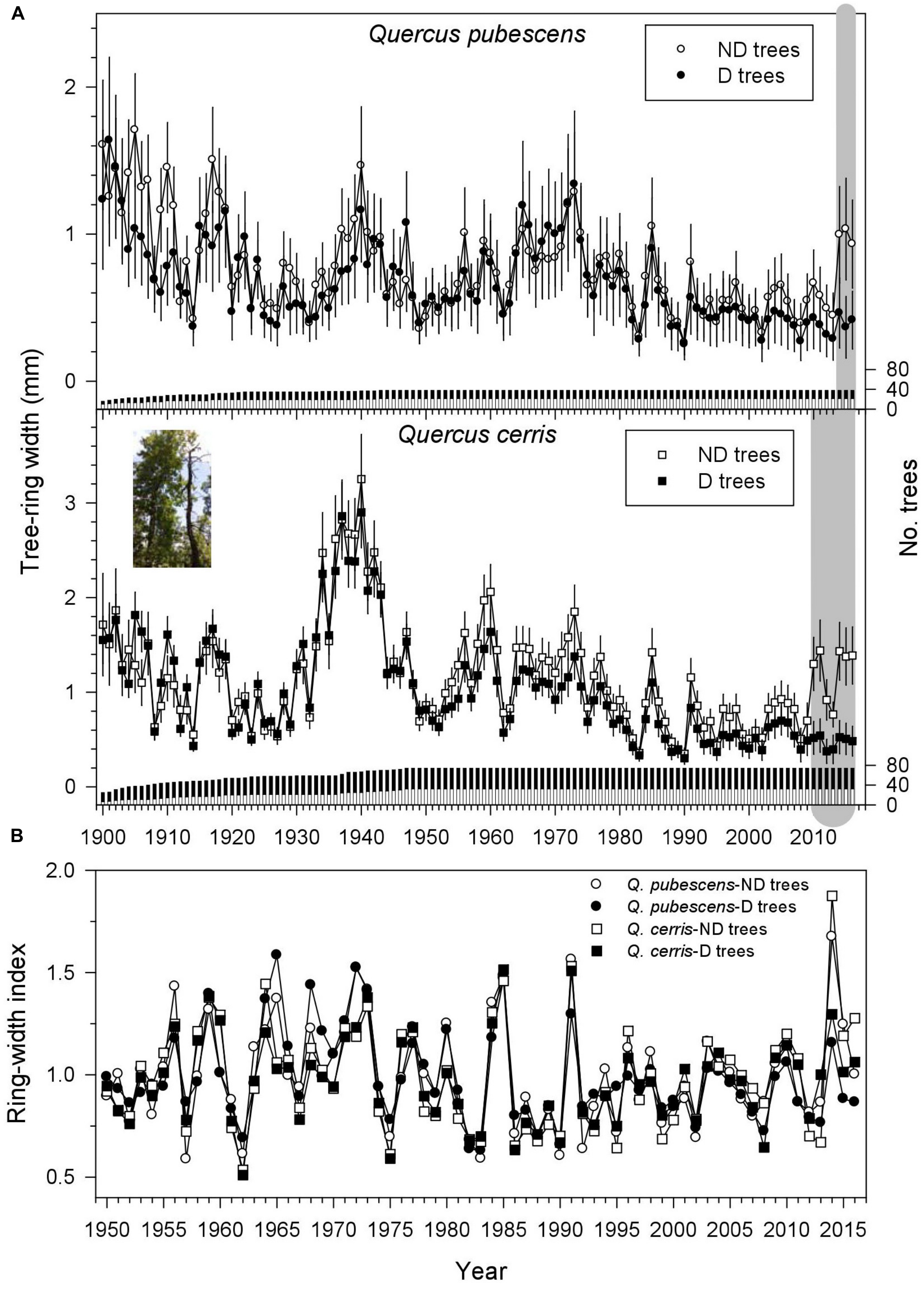

FIGURE 2 | Radial-growth patterns from 1900 to 2016 of non-declining (ND) and declining (D) trees in **(A)** *Q. pubescens* and *Q. cerris* and **(B)** residual ring-width chronologies for the best-replicated period (1950–2016). The right *y* axes show the sample depth. In the plot **(A)** the gray areas indicate periods when tree-ring width of ND trees was significantly ($P < 0.05$) higher than in D trees according to Wilcoxon rank-sum tests. Values are mean tree-ring widths ± SE. The inset shows one of the sampled couples of ND and D *Q. cerris* trees.

oak decline phenomena affecting more *Q. cerris* (less tolerant to drought) than *Q. pubescens* (more tolerant to drought). Similarly, Gentilesca et al. (2017), in a recent review, reported drought as the main driver triggering oak decline within the Mediterranean area, although other causes (i.e., increasing temperature, low temperature, tree nutritional status, overaging,

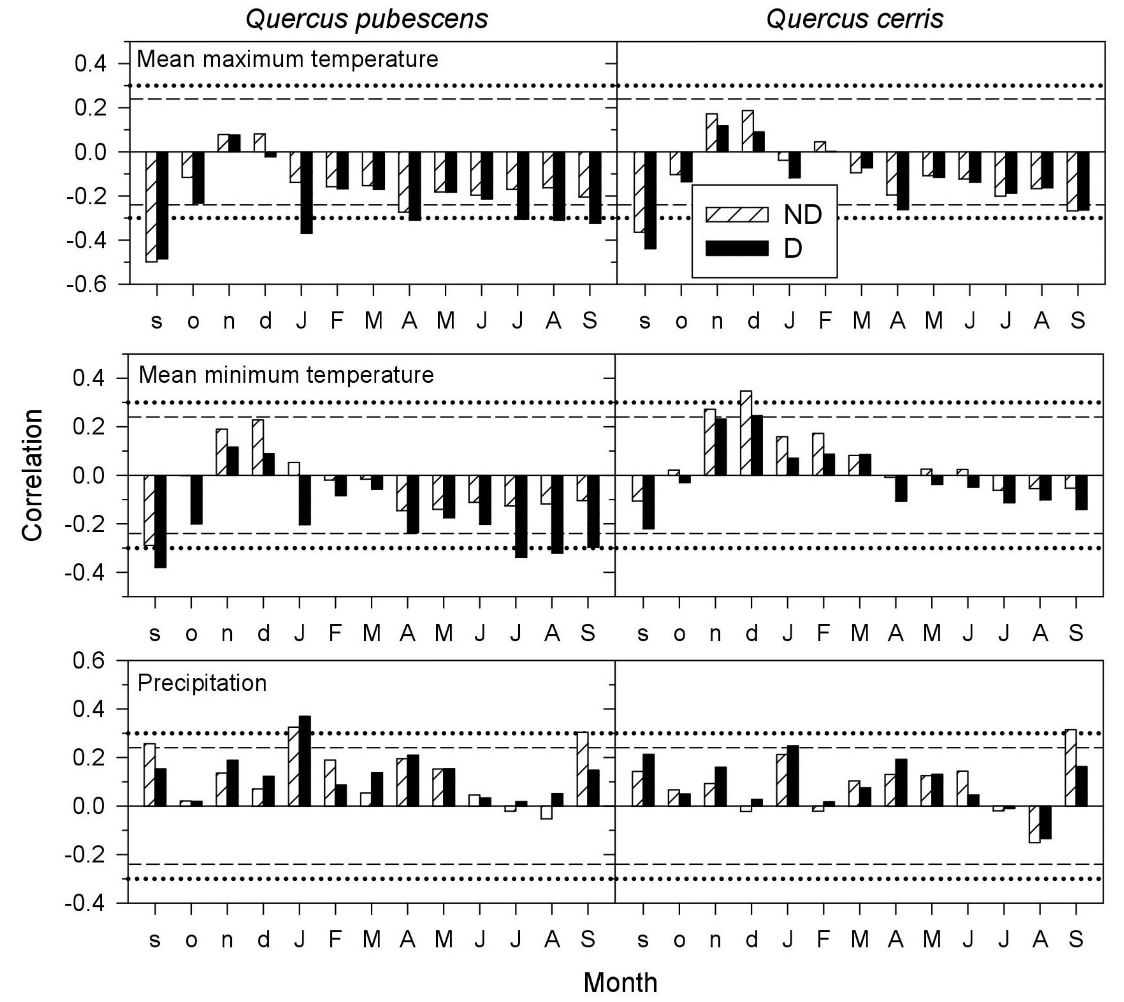

FIGURE 3 | Climate-growth relationships (Pearson correlations) in declining (D, filled bars) and non-declining (ND, hatched bars) trees of *Quercus pubescens* and *Quercus cerris*. The dashed and dotted horizontal lines indicate the 0.05 and 0.01 significance levels.

excessive stand density or pathogens attack) could have also exacerbated decline.

In this study, the oak decline was characterized by conspicuous leaf shedding and high mortality rates which were preceded by recent divergences in radial growth between ND and D trees (2010–2016 in *Q. cerris*, 2014–2016 in *Q. pubescens*).

Our findings agree with previous studies in the region which found that ND trees were larger (thicker diameter, longer stems) than D trees (Colangelo et al., 2017a,b). However, they do not support global reviews suggesting that the tallest trees are more vulnerable to drought-induced hydraulic failure (Bennett et al., 2015). This discrepancy may be explained by several factors making smaller, but dominant, trees more vulnerable to drought damage. First, it is possible that oaks forming short stems develop more shallow roots or do not have access to deep soil-water pools, but this aspect remains understudied. Second, tree height is related to the size of the stem sapwood water reserves and may determine hydraulic capacitance and vulnerability to drought (Zhang et al., 2009).

Considering the period 1950–2016, ND trees presented slightly higher growth rates than D trees in both oak species (29 and 14% higher in *Q. pubescens* and *Q. cerris*, respectively). The divergence in growth between both vigor classes was recent, thus indicating that the growth reduction started about 3–7 years in *Q. pubescens* and *Q. cerris*, respectively, before the decline intensified and trees started to die. This pattern indicates that slow-growing trees were prone to show decline and does not concur with other studies showing that trees growing vigorously produce vessels of wider lumen and are more prone to xylem embolism and decline (Levaniè et al., 2011; Voltas et al., 2013). In *Quercus rubra* currently D trees were growing at slower rates than ND trees but D trees grew more in the past than ND trees suggesting that rapid growth early in development led to later decline and mortality (Haavik et al., 2011). However, we did not observe this pattern characterized by D oaks showing rapid growth early in life. The growth increase in the 1930s, which was more evident in *Q. cerris* than in *Q. pubescens*, suggests past management in the study forest, probably logging

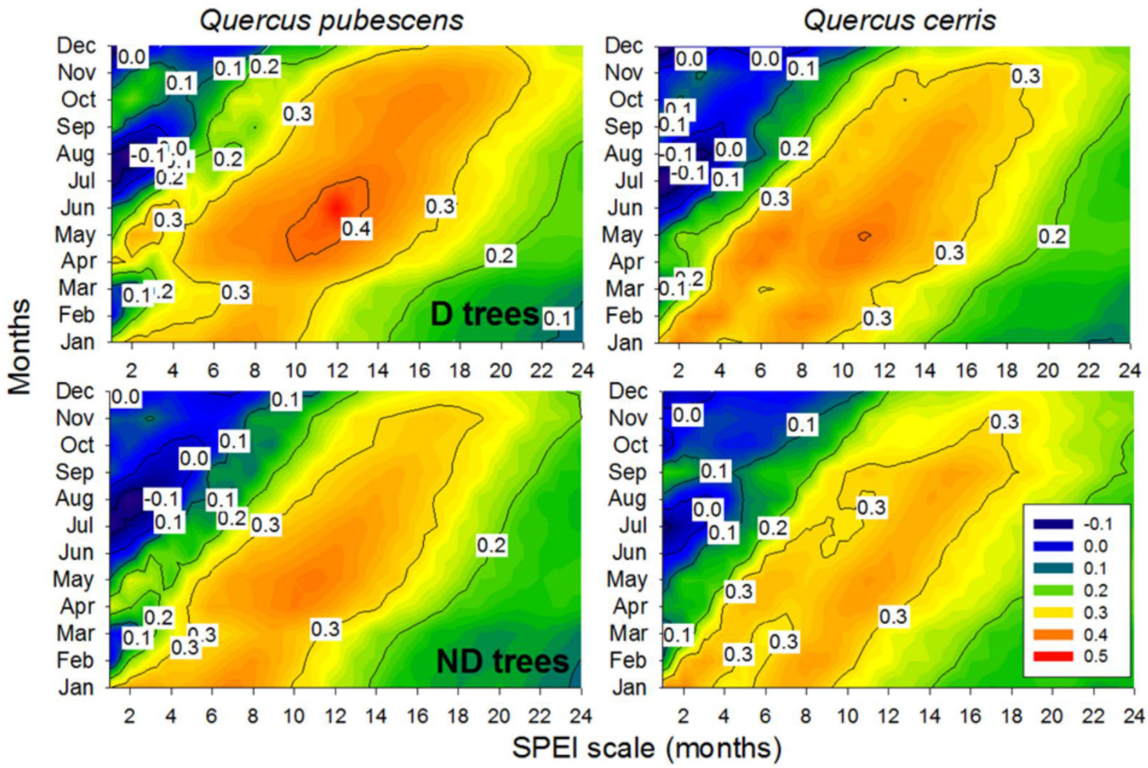

FIGURE 4 | Drought-growth relationships (Pearson correlations) in declining (D) and non-declining (ND) trees of *Quercus pubescens* and *Quercus cerris*. The color scale shows the correlations between ring-width indices and the Standardized Precipitation Evapotranspiration Index (SPEI) calculated at 1–24 months long scales (*x* axes) from January to December (*y* axes). Significance levels are as in **Figure 2**.

TABLE 3 | Wood C isotope radios (d^{13}C) and discrimination (Δ^{13}C) in non-declining (ND) and declining (D) *Q. cerris* and *Q. pubescens* individuals considering three 5-year periods.

Tree species	**Period**	**d^{13}C (‰)**		**D^{13}C (‰)**	
		ND trees	**D trees**	**ND trees**	**D trees**
Quercus cerris	2002–2006	−26.92 ± 0.45	−26.76 ± 0.39	19.17 ± 0.41	19.33 ± 0.47
	2007–2011	−27.77 ± 0.41	−27.57 ± 0.48	19.90 ± 0.50	20.11 ± 0.43
	2012–2016	−27.99 ± 0.32	−27.83 ± 0.64	20.01 ± 0.67	20.32 ± 0.30
Quercus pubescens	2002–2006	−27.14 ± 0.51	−26.08 ± 0.36	18.45 ± 0.37	19.56 ± 0.53
	2007–2011	−27.85 ± 0.42a	−26.73 ± 0.28b	19.02 ± 0.30a	20.19 ± 0.45b
	2012–2016	−28.38 ± 0.52	−27.94 ± 0.37	19.99 ± 0.33	20.54 ± 0.54

Different letters indicate significant ($P < 0.05$) differences between ND and D trees within each period based on t-tests.

of trees for timber or firewood. This past legacy effects could interact with recent climate warming if formerly logged trees were growing better and were also less vulnerable to drought damage (Camarero et al., 2011), but this potential interaction merits further research.

As previously observed, we also found that radial growth of Mediterranean deciduous oaks is enhanced by mild and wet spring conditions and that the decline was triggered by severe droughts and warm conditions during the growing season (Tessier et al., 1994; Corona et al., 1995; Amorini et al., 1996; Haavik et al., 2011; Alla and Camarero, 2012). Climate-growth associations were usually stronger in non-declining than in declining trees, particularly in the sensitive *Q. pubescens*, which agrees with Romagnoli et al. (2018). In oaks growth responses to drought are often lagged 1 year and they are characterized by leaf shedding, shoot dieback and the lack of latewood production leading to altered patterns of hydraulic conductivity (Corcuera et al., 2004a,b; Di Filippo et al., 2010). We also observed those patterns but working with mature and dominant individuals in a high forest, whereas other studies on the decline of Mediterranean oak species using dendroecology were developed on coppice stands (e.g., Amorini et al., 1996; Di Filippo et al., 2010). Spring drought was the main triggering factor of dieback in these studies where declining trees often showed lower growth rates than non-declining trees prior (about 20 years before in *Q. cerris*) to the onset of the decline episode (Amorini et al., 1996). In recently dead *Q. robur* trees, growth of dying trees was also lower than in surviving trees 1–25 years prior to death (Andersson et al., 2011). By contrast, here the ND-D divergence in growth was recent and lasted from 3 (*Q. pubescens*) to 7 (*Q. cerris*) years. These different periods indicate distinct periods of stress and complex interactions between climate stress (drought) and perhaps opportunistic pathogens causing dieback and tree death (Marcais and Bréda, 2006).

The higher responsiveness to year-to-year climate variability of *Q. pubescens* compared to *Q. cerris* corresponds to the highest vulnerability to drought damage of *Q. cerris*. These results agree with previous dendroecological research (Garfi, 2000; Cherubini et al., 2003). It is noticeable that *Q. pubescens* growth mainly responded to January precipitation whereas *Q cerris* growth responded more to warm November-December mean minimum temperatures suggesting a higher sensitivity of *Q. cerris* to warm winter conditions. This could explain the vulnerability to drought of *Q. cerris* since we detected the most marked decrease of precipitation and increase in maximum temperatures during winter. Interestingly, the growth responsiveness to climate was more marked in D than in ND trees of *Q. pubescens* trees and the growth loss in response to warm night conditions from July to August observed in D confirms their highest sensitivity to summer drought as confirmed the SPEI-growth analyses.

Q. pubescens shows higher and more variable radial-growth rates than *Q. cerris* and also a better post-drought resilience or recovery capacity (Tognetti et al., 2007). According to these authors both oak species are able tolerate some drought stress but *Q. pubescens* better tolerates water shortage than *Q. cerris* by uptaking deep soil water and using it more efficiently. Overall, this suggests that *Q. pubescens* can better tolerate drought stress than *Q. cerris* in terms of wood production and associated processes (carbon uptake and storage, hydraulic conductivity, fine root production, etc.).

We isolated *Phytophthora quercina* from the rhizosphere of 40% of the trees that we sampled. Whether the observed prevalence may pose a threat for these stands is unknown, but the fact that our isolation frequency of *P. quercina* was four times higher than the one reported in the north of Italy (Vettraino et al., 2002), may suggest that further monitoring could be carried out. The fact that soil sampling was carried out in winter, when the pathogen is less active, may have underestimated its true incidence, and could indicate that *P. quercina* is even more widespread than reported. *P. quercina* is a primary and aggressive pathogen, which causes root necrosis and dieback of fine roots leading to tree decline and death (Jung et al., 1999). In this area, *P. quercina* was observed more frequently in healthy than in declining trees, indicating that the pathogen did not seem to play a crucial role in the observed decline as a contributing factor. However, the tendency to isolate the pathogen from healthy trees suggests that *P. quercina* could instead have played a role in the onset of decline, as a predisposing factor (*sensu* Manion), although the lower number of samples collected in this survey requires caution about the possible role played by the pathogen. In other ecosystems in which *Phytophthora* spp. also act as primary pathogens, a similar pattern has been observed. For instance, *P. alni* was more frequently isolated from cankers in relatively symptomless alder trees rather than from highly symptomatic trees (Redondo et al., 2015). Further studies comparing the frequency of decline in areas with and without *P. quercina* should be carried out in order to confirm the role of *P. quercina* as predisposing factor. Considering not only the presence, but other more quantitative measures of inoculum could also help elucidating the role of this pathogen in the decline of this area. In parallel, managers could improve forest resilience against *Phytophthora* attacks by improving soil drainage and restricting further spread or by selecting genotypes resistant to this soil-borne pathogen (Sena et al., 2018).

The carbon isotope discrimination ($\Delta^{13}C$) was used as surrogate of intrinsic water-use efficiency (*WUEi*), i.e., the ratio between the photosynthesis (*A*) and the stomatal conductance (*g*) rates (cf. Ripullone et al., 2004). The D trees of *Q. pubescens* present a higher *WUEi* than conspecific ND trees for the period 2007–2011 when the growth divergence between D and ND trees increased. This could be explained because those D trees presented a persistent growth reduction in response to warm and dry conditions during the growing season but at the same time reduced *g* by forcing stomatal closure, and probably showed a reduction in hydraulic conductivity as is common in anisohydric oak species (Granda et al., 2017). It is unclear why the difference in *WUEi* between D and ND *Q. pubescens* trees diminished in the most recent period but it could be a common response to severe dry conditions regardless the vigor class. By contrast, D trees showed lower *WUEi* than ND trees after the dieback onset in *Quercus frainetto*, which was associated to higher *g* and water loss through transpiration (Colangelo et al., 2017a). In *Q. robur*, dying trees had also lower *WUEi* than surviving trees (Levanič et al., 2011), which was interpreted as lower *A* or to higher *g* (Farquhar et al., 1989). The difference in *WUEi* between ND and D trees was found in *Q. pubescens* but not in *Q. cerris* which can be attributed to their different ecophysiological behaviors. When these species coexist, *Q. pubescens* shows a more conservative water-use strategy than *Q. cerris*, since *Q. pubescens* presents higher *WUEi*, and lower photosynthesis rates, stomatal conductance rates and negative midday water potential than *Q. cerris* (Tognetti et al., 2007). These complex responses related to *WUEi* indicate the need to increase the samples collection and more studies considering different decline episodes, using several functioning (growth, wood anatomy) and ecophysiological proxies (C and O isotope discrimination) and involving tree species with diverse susceptibility to drought and pathogen damage (Gessler et al., 2018).

CONCLUSION

Drought and warmer minimum temperatures triggered oak decline in the 2000s and 2010s. Such drier conditions affected more negatively *Q. cerris* than *Q. pubescens*. The more tolerant *Q. pubescens* was also the most responsive to climate and drought in terms of wood production, particularly declining trees.

The sensitive *Q. pubescens* declining trees showed higher water-use efficiency than non-declining trees of the same species before the growth divergence between these two vigor classes started in 2014. *Phytophthora quercina* was isolated from both oak species, and its incidence tended to be higher in non-declining than in declining trees, suggesting a possible role as predisposing factor. However, considering the reduced number of individual surveyed in this field experiment further studies are needed to support this hypothesis. These findings illustrate complex responses to drought and soil-borne pathogens of coexisting oak species suggesting different tolerances to the combined effects of climate and biotic stressors under the forecasted warmer and drier conditions.

AUTHOR CONTRIBUTIONS

MC, JC, MB, TG, and FR conceived the idea and contributed to writing the manuscript. MC, TG, and FR carried out the field work. The dataset was analyzed by JC, MC, and JO. *Phytophthora* analyses were carried out by JO and M-AR. All authors contributed to the interpretation of the results and the revision, discussion, and approval of the final draft.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2018.01595/full#supplementary-material

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Importance of Ecological Variables in Explaining Population Dynamics of Three Important Pine Pest Insects

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Climate change challenges forest vitality both directly by increasing drought and heat periods and indirectly, e.g., by creating favorable conditions for mass outbreaks of phyllophagous insects. The large forests dominated by Scots pine (*Pinus sylvestris* L.) that cover the lowland regions in northeast Germany have already been affected regularly by cyclic mass propagations of defoliating insect species in the past with climate projections implying an even more advantageous environment for devastating outbreaks in the future. To improve predictive and responsive capacities we have investigated a wide range of ecological parameters to identify those most strongly related to past outbreak waves of three central species. In total, we analyzed 3,748 variables covering stand and neighborhood properties, site quality, and climatic conditions for an area of roughly 750,000 ha of pine forests in the period 2002–2016. To reflect sensitivity against varying climate, we computed "floating windows" in relation to critical phenological phases of the respective insects. The parameters with the highest explanatory power resulted from the variable importance measures of the Random Forest (RF) methodology and have been evaluated by a 10-fold cross-validation process. Our findings closely reflect the known specific gradation patterns and show that relative variable importance varies with species. While *Lymantria monacha* L. feeding was mainly dependent on the surroundings of the respective stand, *Diprion pini* L. proved to be almost exclusively susceptible to climatic effects in its population dynamics. *Dendrolimus pini* L. exhibited a mixed pattern of variable importance involving both climatic and forest structure parameters. In many cases the obtained statistical results support well-known ecological cause-effect relations and long-term population change dynamics. The RF delivered very high levels of sensitivity and specificity in the developed classifications and proved to be an excellent tool to handle the large amounts of data utilized for this study. While the presented classification approach may already support a better prognosis of the amplitude during the outbreak culmination, the obtained (most important) variables are proposed as preferable covariates for modeling population dynamics of the investigated insect species.

Keywords: ecological modeling, Random Forest, mass outbreak, *Pinus sylvestris*, *Lymantria monacha*, *Dendrolimus pini*, *Diprion pini*

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INTRODUCTION

Climate change is challenging forest management strategies around the globe (Kirilenko and Sedjo, 2007; Lindner et al., 2014). In addition to the expected detrimental effects of increasing heat and drought on major physiological processes of forest trees (Allen et al., 2015), the potential stimulation of mass outbreaks of thermophilic forest pest insects is threatening sustainable forest management (De Lucia et al., 2008; Jactel et al., 2012).

The federal state of Brandenburg, Germany, has been prone to severe damages in Scots pine (*Pinus sylvestris* L.) stands by defoliating forest pest insects for a long time (**Figure 1**). Around 75% of the forest area in Brandenburg (ca. 1,000,000 ha) is dominated by Scots pine which, particularly in even-aged pure stands, provides favorable feeding conditions for phytophagous pine pest insects (PPIs). Due to the frequent occurrence of mass outbreaks (Gräber et al., 2012), the forest protection service responsible for the Brandenburg area has been operating a comprehensive monitoring program for individual pest insects tracking their population dynamics since the beginning of the 1930s. Experience has shown that mortality sharply increases if needle losses due to feeding by PPI exceed a threshold of 90% (Wenk and Möller, 2013). In cases of defoliation prognosis of this dimension, selective insecticides are applied to prevent the total loss of the affected forests and their functions. Massive feeding damage causes major disruptions of ecosystem relations, which in turn may lead to long-term changes of forest structure (Otto, 1994).

Three of the most important PPI more or less regularly infesting large forest areas in northeast Germany are nun moth (*Lymantria monacha* L., "*Lym*"), pine-tree lappet moth (*Dendrolimus pini* L., "*Den*"), and pine saw fly (*Diprion pini* L. "*Dip*"). Specific monitoring and counteractive algorithms have been set up in reaction to their different biology and outbreak behavior (Böhme and Haffelder, 1999; Hielscher and Engelmann, 2012; Möller et al., 2017). While *Den* and *Dip* are feeding exclusively on *Pinus sylvestris*, *Lym* is a polyphagous species. Its larvae may develop, in addition to most conifer species, on many deciduous trees and shrubs. In the northeast German lowlands, however, destructive mass attacks tend to be limited to pine stands (Häußler et al., 2000; Hielscher and Engelmann, 2012).

Comparing the historical outbreaks of pine pests in the northeast German lowlands, Gräber et al. (2012) describe a relatively uniform frequency (8–12 years) of *Lym* gradations, while mass outbreaks of *Den* have increased in frequency with climate change. *Dip* shows a great irregularity in the timing of gradations over the observed period 1920–2013. Ray et al. (2016) and Schafellner and Möller (2018) described the preference of *Den* for warm and dry late summer months and classified this species as a climate-change winner. For *Dip* the observation of a new unexpected and strong mass outbreak in northeast Germany in 2016 was the reason to investigate the causal relations between weather and population development in detail (Möller et al., 2017).

In Brandenburg, 11 mass propagations have been documented for *Lym* between 1922 and 2010 (Gräber et al., 2012). The life cycle of the species in the region starts with the larvae developing in their eggs already in early fall. They hibernate in this form at the base of the tree trunks and hatch after warm periods in April or May (Schwenke, 1978). Significant needle feeding starts with the L2/3 stage (= second and third larval development stage) and lasts until pupation in June. A few weeks later the imagines appear and begin to mate; the females deposit their eggs immediately afterward, usually in coarse bark fissures in the lower part of the tree trunks. Warm and dry conditions, for example during the mating period, promote individual and population development (Zwölfer, 1935; Häußler et al., 2000).

The pine lappet moth has also been a potential threat to Scots pine forests in the region for a long time (Schwenke, 1978). The species is more dependent on climatic triggers in the timing of mass outbreaks than *Lym*, their frequency has increased throughout the past decades (Gräber et al., 2012; Schafellner and Möller, 2018). The first instar larvae hatch in late summer from eggs usually deposited at needles and twigs in the pine crowns and start to feed until the first frost events force them to climb down and enter hibernation in the upper layers of the forest soil. In early spring the L3/4 instars return to the crowns and resume feeding. Pupation occurs from June to July; the adults emerge shortly afterward and mate with each female laying up to 300 eggs (Schwenke, 1978; Möller et al., 2007). High population densities during mass outbreaks lead to extreme defoliation covering the complete age range of the host trees with heavy feeding even of juvenile plants. Additionally, caterpillars tend to consume all green parts of the host including buds and needle sheaths (Weckwerth, 1952; Möller and Engelmann, 2008; Schafellner and Möller, 2018).

The life cycle of *Dip* is very complex and may change from univoltine to bivoltine patterns in Brandenburg. Large-scale defoliation events are usually coupled with bivoltine years with the second generation feeding in fall. Massive damage and widespread tree mortality may occur in these years because the first generation larvae feed on needles from the previous year and the second generation on current year needles. A detailed description of the biology and of the frequency and consequences of mass outbreaks is provided by Möller et al. (2017). Among the three selected PPI, *Dip* seems to show the largest dependency on climatic factors with warm and dry summer periods contributing significantly to mass outbreak probability (Geri, 1988; Möller et al., 2017).

Scientific analyses and practical experience have shown that population dynamics of the PPI are partly characterized by cyclic patterns leading to mass gradations of a more or less stable frequency (Schwenke, 1978; Altenkirch et al., 2002). On the other hand, the exact timing, the extent, and the consecutive damage of outbreaks are shaped by abiotic factors, mainly weather conditions (Möller et al., 2017). The latter influence population dynamics directly (as favorable or disadvantageous environments) and indirectly by affecting the host plant (Sierpiñska, 1998; Breda et al., 2006). These indirect effects – together with the gradation history of the respective forest stands – shape the predisposition of trees and forests toward mass outbreaks. The complexity of these relationships is further aggravated by climate change processes which affect both the physiological composition and predisposition of the

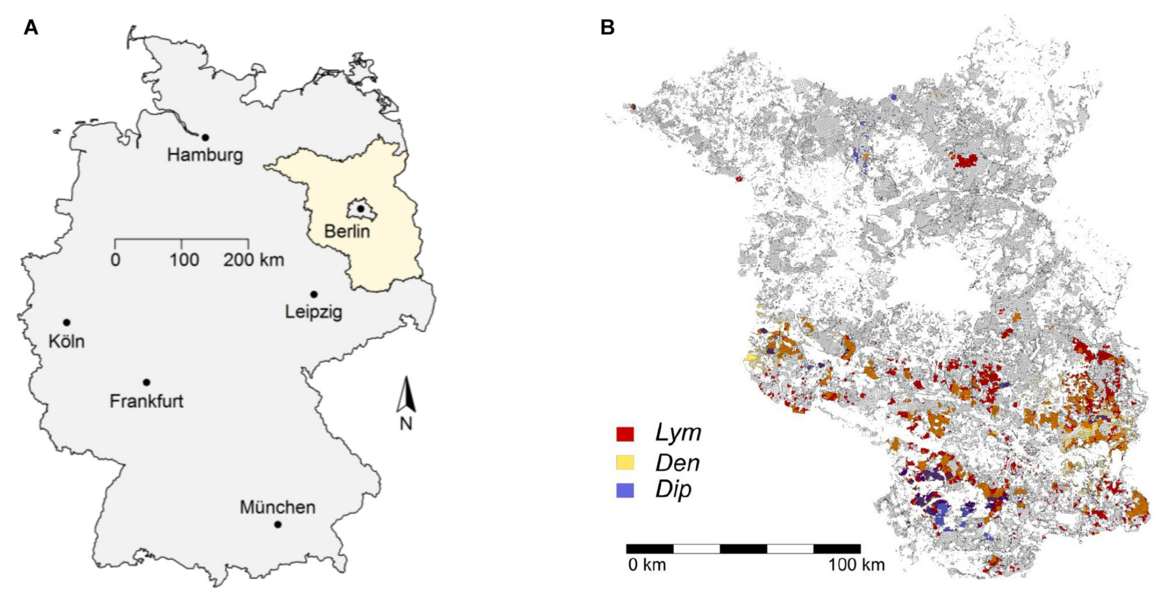

FIGURE 1 | The federal state of Brandenburg is located in the Northeast of Germany **(A)**. The 40,005 forest compartments (FC) in the state are shown in gray shading **(B)**. FC affected by PPI defoliation are filled with the color code of nun moth (*Lym* = red), pine-tree lappet (*Den* = yellow), and pine saw fly (*Dip* = blue). Outbreaks of more than one species in identical FC are indicated by divergent shading (e.g., orange colors show FC affected by *Lym* and *Den*).

forests and the population dynamics of the PPI (Jactel et al., 2012).

Against this background, there is an increasing need to explore the causal relationships of forest pest insects' population dynamics to environmental drivers in order to enhance the existing monitoring programs, to reduce insecticide treatments to a minimum and to develop forest management strategies accounting for biotic risk prognosis. Furthermore, the climate sensitivity of individual pest species needs to be contrasted to the forest development at landscape level which in turn affects the forests' predisposition and their quality as source of feeding and breeding ground for these insect species and their antagonists.

This study aims to determine the most important influencing factors controlling mass outbreaks of nun moth, pine-tree lappet moth, and pine saw fly in Scots pine forest ecosystems. For this purpose, we applied the "Random Forest" approach (RF; Breiman, 2001) to an extensive database covering the forests in the federal state of Brandenburg. Focusing on the variable importance measures of the RF methodology, we performed a variable selection procedure highlighting the most explanatory variables representing climatic conditions (clim_), site properties (site_), forest stand structure (stand_), and forest landscape description (forest_). The variable selection process, hence, provides a set of covariates most suited for modeling future feeding hot spots under consideration of stand development and climate change.

We hypothesize that (a) the PPI are sensitive to independent climatic triggers and temporal periods within the year relating to their particular biology. Furthermore, we propose that (b) the preferences to particular stand structures vary for the three PPI due to their specific adaptation to ecological (sub-) niches of pine forest ecosystems. Based on the relevant literature and practical experiences we assume that (c) PPI share a common preference for warm climate and that (d) a higher tree species diversity of the habitat is counteracting severe mass outbreaks due to the limiting effects exerted by the higher abundance of predators and parasitoids in such conditions.

MATERIALS AND METHODS

The database of this study was built as a comprehensive set of ecological variables available for almost the total forest area in Brandenburg for the years 2002–2016. This area is subdivided into "forest compartments" (FCs), which is the basic administrative unit of forest services in Germany. In Brandenburg, the forest area is organized into a total number of 40,005 FC (reference year 2014). The ecological variables of these plots were computed for different statistical metrics, weighting factors and temporal aggregates. We generated a total of 3,748 variables in a first step and condensed our analysis to a few variables of largely independent information at the end of the study. All analysis steps have been performed in "R" (R Core Team, 2014) and we used the 'randomForest' package (Liaw and Wiener, 2002) stressing the most important variables of PPI mass outbreaks.

Defoliation Assessment

Based on forest damage assessments of the years 2002–2016, the forest protection service of Brandenburg provided feeding statistics of the PPI for all FC affected by defoliation during this period. The spatial distribution of the historical defoliation areas is shown in **Figure 1B**. The forest area of Brandenburg is displayed by gray shading. Around 75% of all FC feature Scots

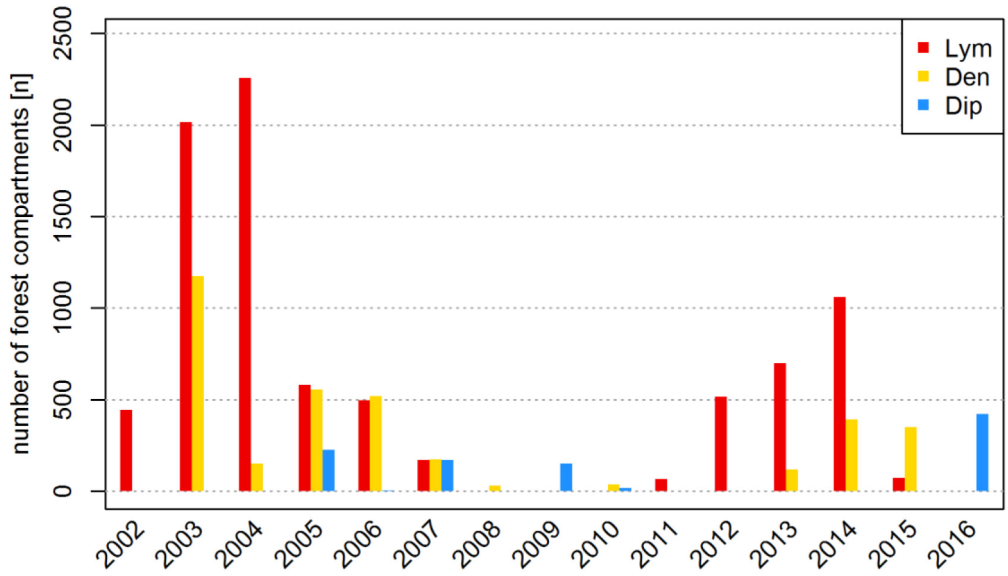

FIGURE 2 | Number of forest compartments (FC) affected by defoliation by *Lym*, *Den*, and *Dip* in Brandenburg from 2002 to 2016.

pine as the leading tree species. This amounted to an area of approximately 750,000 ha of potential feeding ground for the PPI. Defoliation damages were concentrated in the South of Brandenburg except for feeding events of *Lym* in 2003 and of *Dip* in 2005.

The terrestrial forest damage assessment in Brandenburg includes the area and the relative intensity of defoliation and the involved species of PPI. Feeding activities are documented by the local foresters who are in charge of 15,000 ha forest area on average, so defoliation events of low feeding intensity might have been missed. Another source of inaccuracy is introduced by airborne pesticide applications (PAs). Since PAs are restricted to FC predicted by the state forest protection service to become completely defoliated based on the related monitoring data and the specific regional population dynamics, we considered those FC as representing total defoliation. In **Figure 2**, the population dynamics of the three studied PPI is illustrated by the number of FC affected by defoliation over time.

Influencing Factors

We gathered an extensive and diverse set of potential influencing factors partitioned into climatic conditions (clim_), site properties (site_), stand description (stand_), and the forest structure within a circular buffer zone of 1,000 ha (forest_). In respect to these variable groups we computed various temporal compositions of climatic parameters and different aggregates of the forest characteristics based on their spatial abundance. We thus obtained a total number of 3,748 variables. A tabular description including an encoding scheme for the different variable groups is presented in the **Supplementary Tables 1–4**. The variable coding always starts with the acronym of the variable group and the accessed parameter, followed by further specification of computation routine. Important variables, however, have been expressed verbally within this article.

Climatic Conditions (clim_)

The German Meteorological Service (DWD) provides daily measurements of various climate parameters across a comprehensive network of weather stations in Germany. Köhler et al. (2015) made use of these data in a regionalization approach providing 100 m × 100 m grids of relevant climate data used in this study (**Supplementary Table 1**). Information on potential evapotranspiration was modeled according to Penman–Monteith (see Allen et al., 1998). Except for the precipitation data, all parameters were simulated using a generalized additive model (GAM; Wood and Augustin, 2002). Precipitation data were regionalized using an ordinary Kriging approach (Bivand et al., 2013).

According to the respective grid built by the center points of all FC, daily climate data were summarized to temporal aggregates of all available climatic parameters (**Supplementary Table 1**, second position). Furthermore, our investigation includes intervals attached to varying phenological dates rather than being fixed to a specific day of the year (DOY). In consequence, we estimated the beginning and end of crucial life stages of the respective populations. In addition, we tested conventional climatic windows such as annual and monthly aggregates and averages.

Based on the close temporal synchrony between host development and insect phenology we used the modeled day of bud burst (BBT) for *P. sylvestris* according to the phenological models developed by Menzel (2003) as an indicator of the specific climatic conditions in a given year. Annual dates of BBT usually vary between DOY 118 and 128 which translates to April 28 and May 8, respectively. In addition, we included periods related to the annual dates of BBT plus 90 days (BBT+90 = FLY), which translates to a range between July 27 and August 6, as physiologically important time windows for pupation. This period is particularly relevant for *Dip* because the species

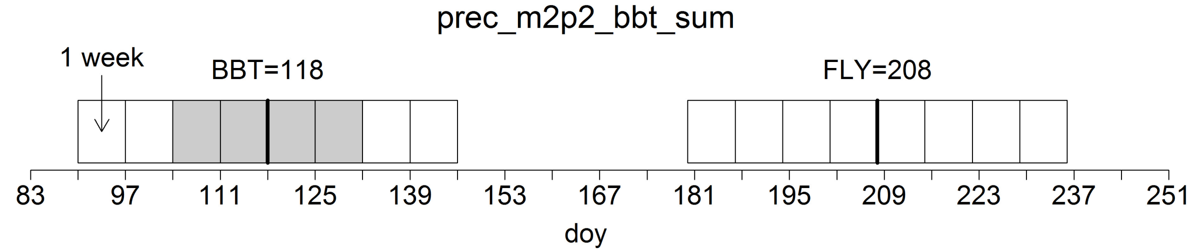

FIGURE 3 | Construction of temporal variables of the selected climatic parameters. In this exemplary case, the variable with the acronym "tmax_fly_m3p3_sum" represents the sum of daily maximum temperatures in a period beginning 3 weeks prior to and terminating 3 weeks after the day of year (DOY) 90 days after modeled bud burst (BBT) of *P. sylvestris*. The potential temporal range of variables is shown as weekly rectangles; the chosen time window is shaded in gray.

may switch into a bivoltine lifecycle with wasp hatching and swarming occurring in the weeks around FLY (Möller et al., 2017).

In relation to the phenological dates of BBT and FLY, we created factors for periods of at least 1 week covering all possible combinations of weeks before and after the respective dates, starting 28 days before and ending 28 days after BBT and FLY (see **Figure 3** for a visual example).

Site Properties (site_)

Similarly to the climate data, the "DSS-RiskMan" project provided regionalized raster data of basic site properties in 100 m × 100 m grids. Covariates for the regionalization process originated from geological and edaphic maps, a digital elevation model, long-term climate data, and information about the present forest types (Riek and Russ, 2014; Russ, 2015). Digital soil mapping (e.g., McBratney et al., 2003) was applied providing soil types and soil properties for the usage of appropriate pedotransfer functions (Renger et al., 2009).

Basic soil characteristics were introduced into the analyses as nutrient supply (nv_), actual field capacity (afc_), and plant available soil water (aws_). The latter parameter represents for the present forest type and the specific rooting depth. Furthermore, the long-term evapotranspiration rate (pet_) of the site has been computed according to Penman–Monteith model (see Allen et al., 1998) and a 30-year average of the respective climatic input variables. Considering the present forest type and the degree of stocking resulted in the long-term actual evapotranspiration rate (aet_) (**Supplementary Table 2**).

Stand Description (stand_)

Based on the state forestry inventory, each forest stand in Brandenburg should be gauged in a regular 5–10 years rotation by the angle-count sampling method. Basic stand information about, e.g., basal area and timber stock separated by tree species and stand layer are gathered in the Brandenburg forest database "DSW^2" which is updated annually with data covering forest growth, forest management activities, and calamity events.

Unfortunately, the support of the "DSW^2" database has been suspended by non-governmental forest owners since 2007. Hence, as of 2008, for around 73% of the forest stands in Brandenburg (i.e., the share of non-state owned forests) data are available only for mean age, diameter, height, and site index of the present tree species and layers. For these stands, we used yield-table references for pure forest stands in Brandenburg to estimate basal area (m^2 ha^{-1}) and standing timber volume (m^3 ha^{-1}). If information about stand density was missing, estimates of the basic stand parameters had to be assigned to fully stocked stands (stand density index = 1.0) introducing a not-quantifiable bias about the actual basal areas and forest stocks. This data completion was nonetheless necessary to target mass outbreak events of PPI on landscape level in consideration of the spatial correlation between feeding events and forest properties.

An additional step of data homogenization was necessary because this study focused on the level of the FC and not on the individual forest stands. Since the FC may contain up to 20 smaller management units and forest stands (**Figure 4A**), we had to aggregate individual stand data into a unique "average" description of the FC (stand_). All considered stand parameters have been weighted by the relative contribution of all forest stands within the FC. Stand parameters and weighting factors are probably biased due to differences in coverage and quality of the forest inventory data. We nevertheless suggest that the general distribution and abundance of forest characteristics within the FC can be preserved and that important influencing factors of PPI rather depend on the forest area properties on a larger scale rather than on detailed single stand properties.

In order to reduce the loss of information within the aggregation process, all stand_ variables were calculated for different tree types (angiosperms and gymnosperms) and tree species (pine and oak) additionally. We also computed the relative proportion of the forest area stocked by different mixture types, tree layers, and age classes. Two additional diversity indices (Shannon and Weaver, 1949; Simpson, 1949) were included which focus on the relative abundance of the respective tree species.

The aggregation of the forest inventory data resulted in 910 variables comprising the arithmetic means and standard deviations of classical stand parameters as well as forest area proportions and forest mixture types at the FC level. The encoding of the considered stand_ variables can be found in **Supplementary Tables 3, 4**.

Forest Structure (forest_)

In order to provide additional information about the neighboring forest stands and the surrounding of the individual FC, the

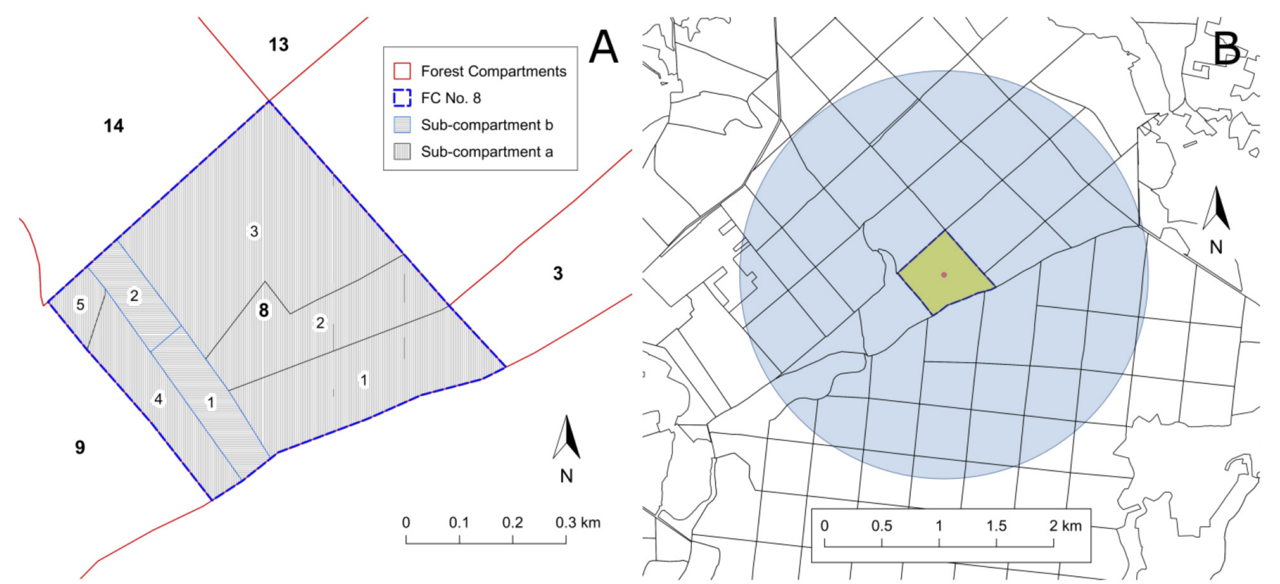

FIGURE 4 | Example of a FC (No. 8) subdivided into sub-compartments (a, b), sub-areas (1–5, 1–2) and management units which may contain several forest stands **(A)**. Individual stand properties are averaged to describe the "stand_" properties of each FC. **(B)** Forest compartment (yellow) with buffer circle of 1,000 ha (blue) analyzed to derive the forest_ properties.

computation routines developed for stand_ variables were adapted for circular buffer zone covering approximately 1,000 ha (**Figure 4B**). Each FC intersecting a circle with 1,784 m radius around the center of the focus FC was included in the calculation of respective forest_ variables. Except for the separation for pine and oak stands, **Supplementary Tables 3, 4** have been applied accordingly. The pre-processing resulted in 560 forest_ variables summarizing the forest inventory data of several hundred stands for one buffer zone.

Variable Importance

In order to detect the most explanatory variables on the presence of a defoliation event, we applied the RF approach for classification problems introduced by Breiman (2001). Its integrated variable importance measure provides a ranking of the variables according to their explanatory power (see Calle and Urrea, 2011). In fact, the RF has been stated to be the best classifier in comparison to a total number of 179 classifiers tested for "Average Accuracy" and "Friedman Ranking" (Fernández-Delgado et al., 2014).

We used the 'tuneRF' function implemented in the 'randomForest' package (Liaw and Wiener, 2002) for determination of the optimal number of predictors used for the random variable selection in the construction of the individual decision trees. Our default setting comprised of 1,000 decision trees. All RF were trained by a balanced training data set (see Chen et al., 2004). The validation on the classification performance, however, has been carried out by a 10-fold cross-validation (see Rodríguez et al., 2010) for classification of the entire data set (40,005 observations per year).

In a first step, we studied the variable importance of the individual variable groups and to eliminate non-important and highly correlated variables from the groups. Therefore we constructed three RF according to the three variable groups clim_, stand_, and forest_ (RF_{clim}, RF_{stand}, RF_{forest}). The site_ variables were considered in a later step. After computation of the average "mean decrease Gini" (MDG) based on the 10-fold cross-validation process (RF trained with 90% of the database and tested against the remaining 10%), we excluded variables of small importance as detected by falling below the standard deviation of the average MDG. We also excluded highly correlated variables (Spearman's rho > 0.9) in an iterative way keeping only the top-ranked variable. This was done although (as shown by Genuer et al., 2010) the relative importance between two variable groups could be preserved and important variables could be distinguished reliably from noise even for a high number of correlated variables. In our case, the applied pre-selection of important and "poorly" correlated variables should preserve the most important variables regardless of redundant data as introduced by variable generation.

In a second step, we analyzed the variable importance for mixed variable groups by means of an additional RF (RF_{all}) based on the reduced variable sets and the site_ variables. All previous steps of cross-validation, ranking, and variable elimination were repeated accordingly. This step was performed in order to further distinguish between the variable importance of different variable groups.

As an alternative approach we further eliminated highly correlated variables and condensed the available information to a small set of influencing factors of PPI. This was done in order to obtain a reduced variable set for both interpretation and prediction issues. Therefore, we applied the variable elimination process described by Genuer et al. (2015) on the same data as for RF_{all}. In contrast to the former approach, we here included the geographic coordinates (x = easting, y = northing; UTM ETRS89) of the FC in order to account for the spatial distribution of the feeding plots. Ordered by rank according to "mean decrease accuracy" (MDA), the algorithm inserted the variables step-wise into a nested model for classification. Variables are thus kept or eliminated from the nested model according to a threshold of the minimum error gain relating to the "out-of-bag" error (OOB). The threshold was calculated by the mean of the first-order differentiated OOB errors (see Genuer et al., 2015). Using the 'VSURF' package (Genuer et al., 2015), we provided an additional variable selection suggested for prediction (RF$_{VSURF}$).

RESULTS

The figures documenting the importance of the analyzed parameters use different colors for each of the studied pest insects (*Lym* = red, *Den* = yellow, *Dip* = blue). The encoding of the variable names can be found in the supplementary material and is also verbally expressed throughout the following passages. We compared the performance of the RF approaches on different variable selections and obtained the most important variables classifying non-defoliated ($y = 0$) and defoliated ($y = 1$) FC.

The five RF approaches strongly differed in the number of variables used (n_{var}) but showed a generally high hit ratio of the defoliation observations (TNR) with at least 70% and an even higher hit ratio of the non-defoliation observations (TPR) with at least 86% (**Table 1**). RF$_{VSURF}$ applied only a small number of variables and obtained a very high TNR of at least 94%. This is partly due to the slightly different methodology behind RF$_{VSURF}$ which uses MDA as importance measure minimizing the loss of accuracy in the variable elimination process.

In the case of RF$_{clim}$, observations of non-defoliated FC ($n_{y=0}$) were limited to FC that experienced defoliation by the particular PPI at least once within the observation period, whereas the other RF approaches considered observations of all FC. Thus, RF$_{clim}$ referred to a different data set focusing on the temporal occurrence of defoliation in relation to the respective climatic conditions rather than on the spatial distribution of the defoliation events. The statistical results were not directly comparable with the other RF approaches. Furthermore, RF$_{clim}$ used by far the highest number of variables. This first step of analysis has been performed to (a) preselect the most important time windows and climatic drivers (**Supplementary Table 1**), and (b) to identify the best explaining structural variables based on different weightings and spatial aggregations of forest inventory data (**Supplementary Tables 3, 4**).

The 10-fold cross-validation process obtained a generally high performance of the applied RF classification. The last column of **Table 1** shows the OOB calculated within the training procedure of the RF, whereas TPR and TNR of the classification resulted from the prediction on the entire forest area in Brandenburg. The poorest results were obtained for classifications based on stand variables exceeding an OOB of 10%. Except for RF$_{clim}$ focusing on the temporal occurrence of defoliation events and a reduced set of observations (see above), the lowest error rates were observed for the combined variable set excluding highly correlated variables within one group (RF$_{all}$). Both TPR and TNR showed the best results for *Dip* with 99% and 98% and the poorest results for *Lym* with 95% and 97%, respectively.

TABLE 1 | Input data and statistical output according to the five different RF approaches investigating the variable importance toward the feeding events of three PPI.

	PPI	$n_{y=0}$	$n_{y=1}$	n_{var}	TPR	TNR	OOB
RF$_{clim}$	*Lym*	70269	8443	2264	98%	80%	3.80%
	Den	49501	4428	2264	99%	89%	1.60%
	Dip	8527	797	2264	96%	98%	3.60%
RF$_{stand}$	*Lym*	515854	7642	910	86%	79%	14.00%
	Den	517636	2800	910	87%	82%	13.10%
	Dip	519664	772	910	89%	70%	11.50%
RF$_{forest}$	*Lym*	515854	7642	560	92%	93%	8.20%
	Den	520647	2849	560	93%	94%	7.20%
	Dip	522690	806	560	96%	92%	3.60%
RF$_{all}$	*Lym*	502663	7896	195	95%	97%	5.00%
	Den	506484	4075	182	97%	98%	3.00%
	Dip	510727	768	192	99%	98%	1.40%
RF$_{VSURF}$	*Lym*	502663	7896	29	97%	94%	5.60%
	Den	506484	4075	14	99%	96%	3.60%
	Dip	510727	768	8	99%	96%	3.60%

The number of observations of non-defoliated FC ($n_y = 0$) and defoliated FC ($n_y = 1$) for the particular PPI may vary due to the occurrence (and exclusion) of missing values depending on the variable selection. The number of variables processed (n_{var}) varied between RF approaches. The particular model accuracy is shown by the true positive rate (TPR), the true negative rate (TNR), and the out-of-bag error (OOB).

The variable importance of the first four RF approaches was determined by the arithmetic mean of the variable ranks based on the MDG measures of the 10-fold cross-validation process. To better illustrate the most important variables, we present only the top-30 ranked variables of RF$_{all}$ (**Figure 5**). Similar illustrations showing the results for the different variable groups including highly correlated variables can be found in the **Supplementary Figures 1–3**.

The most important variables of the three PPI originated from the variable groups clim_ and forest_ (**Figure 5**). For *Dip*, 29 of 30 variables originated from clim_. In contrast, the top ranked variables for *Lym* and *Den* originated from the forest_ group followed by clim_ variables. The stand_ variables never occurred within the top 30. One site_ variable, the mean long-term potential evapotranspiration per FC (site_pet_mean), appeared at rank 30 in the results for *Lym*. For all three PPI the standard deviation of the ranking increased with lower ranks.

According to **Figure 5**, the top three variables of *Lym* were expressed by the mean diameter and height of all forest stands within 1,000 ha exclusively calculated for the upper layer of the forest stands, and the Shannon index calculated by the relative tree species composition. For *Den*, the top three were the Shannon index, the relative site factor of gymnosperms, and the standardized Shannon index within 1,000 ha. In the case of *Dip* the three most important factors were the standard deviation of the sun duration in August, the average minimum temperature of the first week of the flight period, and the standard

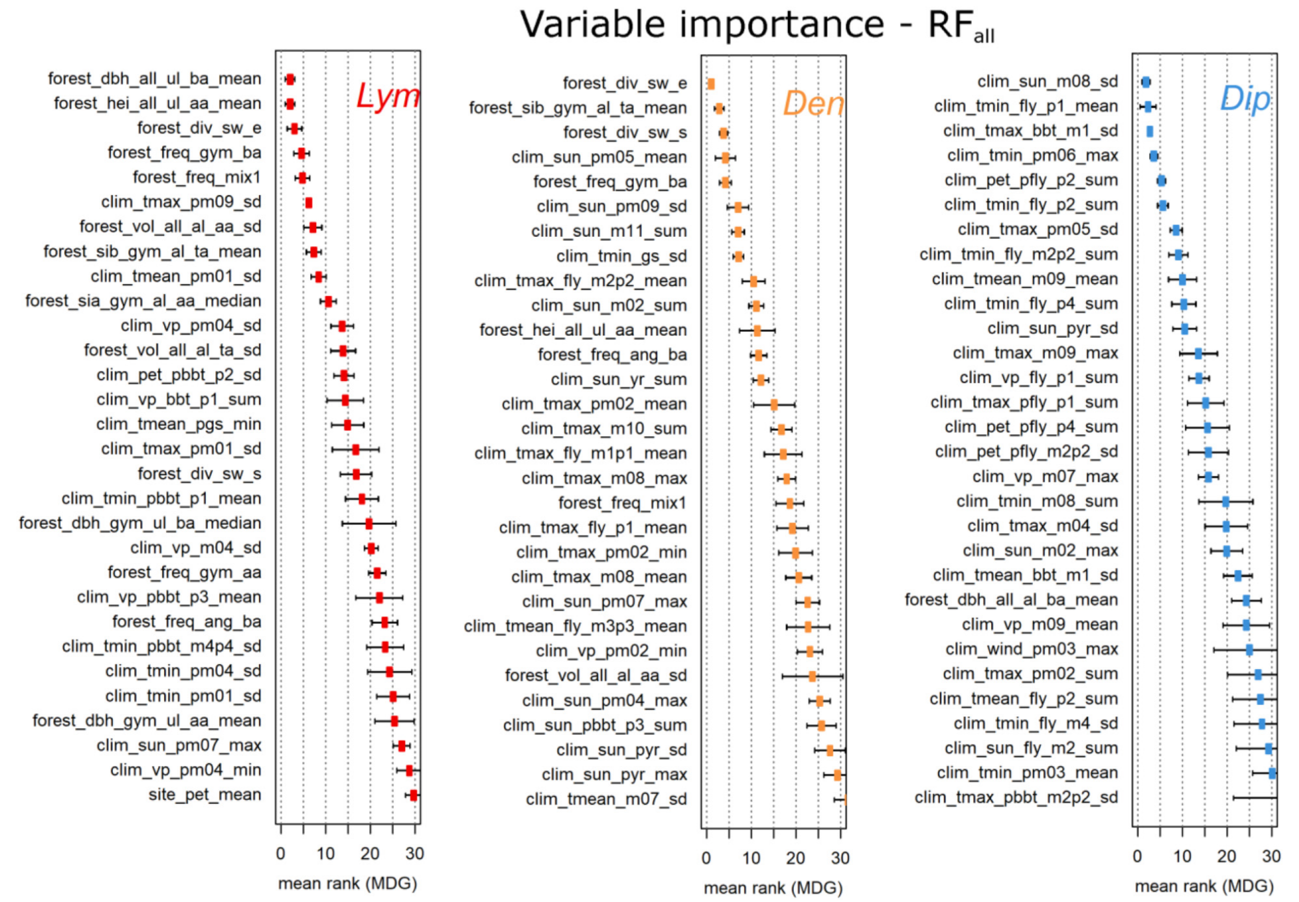

FIGURE 5 | Top 30 ranks of the RF$_{all}$ variables based on MDG. The three figures show the arithmetic mean and the standard deviation over the individual rankings within the 10-fold cross-validation process. Note that the data set of RF$_{all}$ was cleansed of variables of low importance or high correlation (see the Section "Variable Importance").

deviation of daily maximum temperatures 1 week before BBT of pine.

For a fully automatic variable selection, we additionally applied the VSURF algorithm (see above, Genuer et al., 2015). The final models of this approach (RF$_{VSURF}$) contained 29 variables suitable for predictions of *Lym* classification, 14 variables for *Den*, and 8 variables for *Dip* (**Table 2**).

The inclusion of the *x*–*y*-coordinates of the individual FC into our analyses exhibited a high predictive power of the latitude (see **Figure 1B**) and, respectively, a high variable importance (**Table 2**). Thus, the coordinates seem to be important variables for prediction purposes and, in turn, for the variable selection process of RF$_{VSURF}$. In contrast to variable importance based on MDG (**Figure 5**), the VSURF algorithms predominantly selected variables from clim_ for all PPI. Furthermore, there is an obvious shift in the ranking of (VSURF-) MDA variable importance compared to the MDG measure of RF$_{all}$ (e.g., for *Dip*, clim_tmax_pm05_sd switched from seventh to first rank; see **Figure 5** and **Table 2**).

The analyses produced the simplest RF$_{VSURF}$ model for *Dip*. The selected climatic variables are predominantly expressed by standard deviation (_sd) or maximum measures (_max). Almost twice the number of variables entered the RF$_{VSURF}$ model for *Den*. The Shannon index was the sole variable of the forest_ variable group. The sum of the maximum temperature 3 weeks before and 3 weeks after flight date appeared as second-ranked variable for *Den*. All other variables originated from the group derived from the number of sun hours. Standard deviations of sun_ within the current year appeared at third rank, whereas the following ranks were taken up by climate aggregates of the previous year. Similar to *Lym*, the *y*-coordinate was the most important variable predicting defoliation of *Den*. The next important variables for *Lym* were the arithmetic mean and the median of the height of the upper layer of all forest stands within 1,000 ha. The first three climate variables of *Lym* expressed the standard deviation of previous-year climate data. The climate conditions of the current year were represented by the sum of the vapor pressure of the first week after BBT. Another important forest_ variable was the frequency of pure (monospecific) stands within 1,000 ha.

In order to highlight the most influential climate variables for the three PPI we compared the top ranked variables

TABLE 2 | Variable selection of the three PPI based on the VSURF algorithm (RF$_{VSURF}$) and variable ranks according to the MDA importance measure.

Rank	*Lym*	*Den*	*Dip*
1	site_ycoo	site_ycoo	clim_tmax_pm05_sd
2	forest_hei_all_ul_ba_median	clim_tmax_fly_m3p3_sum	clim_sun_m08_sd
3	forest_hei_all_ul_ba_mean	clim_sun_yr_sd	clim_sun_m02_max
4	clim_tmax_pm09_sd	clim_sun_pm05_mean	site_ycoo
5	clim_pet_pbbt_p3_sd	clim_sun_pm07_max	clim_vp_m07_max
6	clim_vp_pm04_sd	clim_sun_pm04_max	clim_tmin_fly_m3p3_mean
7	clim_vp_bbt_p1_sum	clim_sun_pyr_sd	clim_tmin_m09_sum
8	forest_freq_mix1	forest_div_sw_i	clim_tmin_pm06_max
9	clim_sun_pm07_max	clim_sun_pm04_sum	
10	clim_tmax_pm01_sd	clim_sun_pm04_mean	
11	clim_sun_m04_max	clim_sun_pgs_max	
12	clim_vp_m04_sd	clim_sun_m06_max	
13	clim_tmean_pm01_sd	site_xcoo	
14	clim_vp_pm02_sd	clim_sun_m02_mean	
15	clim_tmean_pgs_min		
16	clim_sun_gs_sd		
17	clim_rain_bbt_m1_mean		
18	forest_dbh_all_al_aa_mean		
19	clim_rain_m10_sd		
20	clim_sun_pgs_sd		
21	clim_tmin_pm04_sd		
22	clim_vp_pfly_m1p1_sd		
23	clim_sun_pyr_sd		
24	clim_sun_m04_sd		
25	clim_sun_m09_mean		
26	site_pet_mean		
27	clim_sun_m09_sd		
28	clim_vp_pbbt_p2_mean		
29	clim_rain_m02_max		

achieved for prediction (RF$_{VSURF}$) and interpretation (RF$_{clim}$). The latter approach considered only climate data of FC that had experienced at least one defoliation event within the last 15 years. We thus suppose that the RF$_{clim}$ variable importance was predominantly driven by variables contrasting the individual years rather than reflecting the spatial distribution of the FC.

As an example for this approach, **Figure 6** shows an annual boxplot representation of the three most important climate variables of *Lym* (see **Table 2** and **Supplementary Figure 1**). Similar figures for *Den* and *Dip* can be found in the **Supplementary Figures 4, 5**. Note that the supplementary figures of variable ranking contain highly correlated variables, which were not considered for the selection of the top-ranked variables (see the Section "Variable Importance").

Except for the first ranked RF$_{clim}$ variable, the most important climate variables of *Lym* referred to previous-year climatic conditions (**Supplementary Figure 1**). RF$_{VSURF}$ variables were expressed by the standard deviation (**Table 2**), whereas RF$_{clim}$ showed measures of the arithmetic mean in the first and third ranked variables. In mass-outbreak years (gray bars), the selected variables frequently showed higher values compared to the mean level of all years (**Figure 6**). The outbreak years are marked by high mean sun duration in the first 2 weeks of the flight period of the actual year (RF$_{clim}$ first variable) and high mean vapor pressure in the first 3 weeks after BBT in the previous year (RF$_{clim}$ third variable). The second variable represents a high standard deviation of the maximum temperature of previous January. In RF$_{VSURF}$, the top three variables of *Lym* outbreak years are characterized by a high standard deviation of maximum temperature of previous September, potential evapotranspiration 3 weeks after BBT of the previous year, and vapor pressure in previous April.

Summarizing the results of *Den* and *Dip* (**Supplementary Figures 4, 5**), we obtained a much clearer separation of mass outbreak years from the other years for the top three climate variables for *Dip* than for *Den*. In RF$_{VSURF}$, the top three variables of *Den* outbreak years were related to a high (maximum) temperature sum 3 weeks before and after FLY, a low deviation of the sun duration in the previous year, and a low mean of the sun duration in May of the previous year. In RF$_{clim}$, the top three variables are characterized by high means of the maximum temperature in the first week of the flight period and of the vapor pressure 3 weeks before and 3 weeks after previous FLY, and by a low maximum of the daily sun duration in previous July. In RF$_{VSURF}$, the top three variables of *Dip* outbreak years showed a low standard deviation of the maximum temperature of previous May, a high standard deviation of the sun duration in August, and a low maximum of the sun duration in February. In RF$_{clim}$, the top three variables are related to a high sum of the potential evapotranspiration in the 2 weeks after previous FLY, a low maximum of the minimum temperature of previous June, and a low sum of the minimum temperature in the first week after FLY.

The top ranked forest_ variables allowed a clear differentiation of defoliated FC. In the example of *Den* (**Figure 7**), we observed a maximum value of the Shannon evenness of 0.6 for the defoliated FC (**Figure 7A**). The highest density of defoliated FC was reached for values from 0.16 to 0.18, whereas the distribution of Brandenburg showed highest densities of FC between 0.26 and 0.64. In accordance with a comparably low diversity and evenness, defoliated FC also showed a high proportion of gymnosperm forest stands with a minimum of 0.6. The most frequent mean-dbh classes in the defoliated FC were significantly lower than in the total of all FC (**Figure 7B**).

Similar relations could be found for *Lym* and *Dip* showing clearly different peaks for the respective variable classes of RF$_{forest}$ (**Supplementary Figures 6, 7**). We obtained a comparable value range of the respective forest_ variables, e.g., for basal-area weighted mean diameter of the upper layer within 1,000 ha (**Supplementary Figures 6A, 7A**). For all PPI, defoliated FC were characterized by rather small tree dimensions associated with a

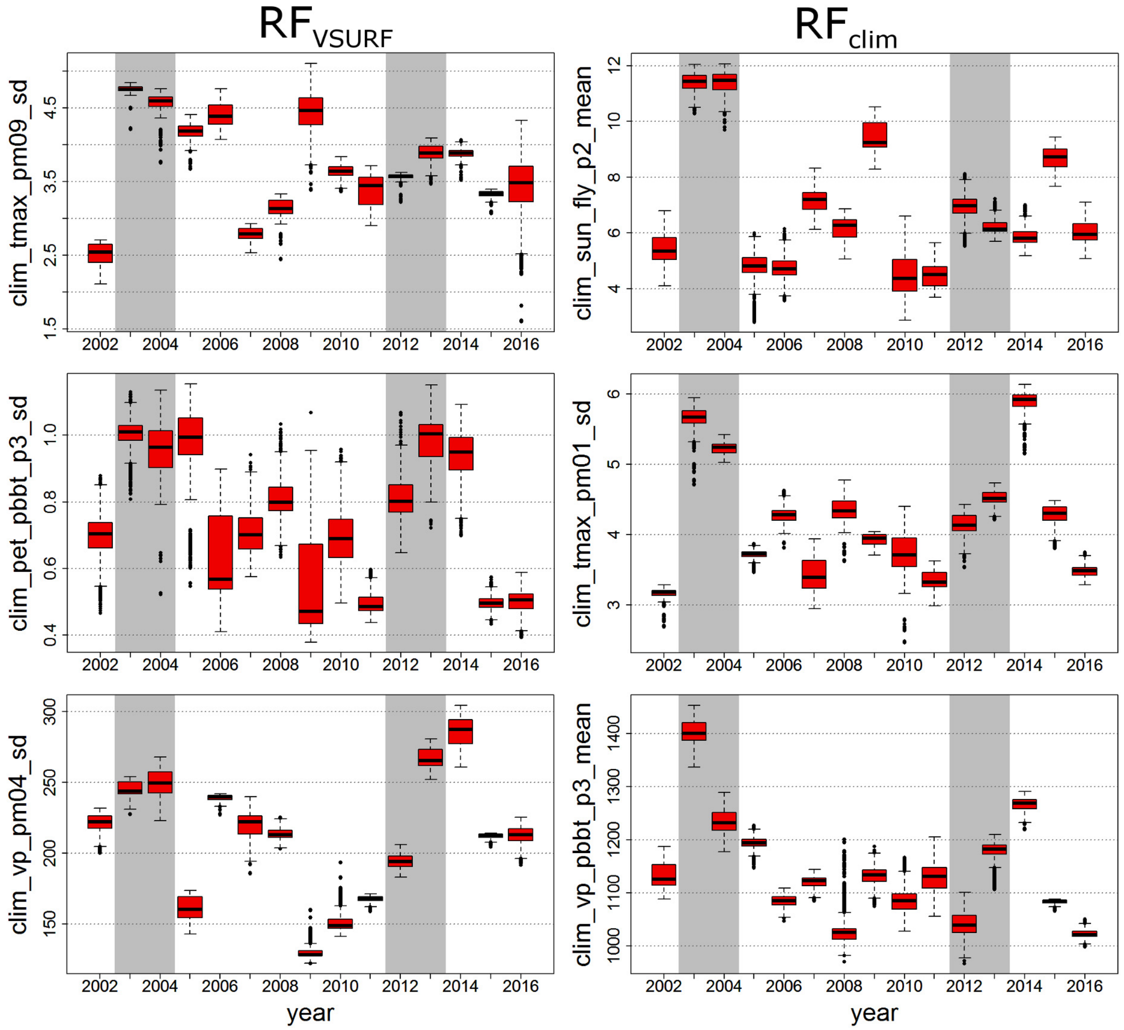

FIGURE 6 | Boxplot representation of the annual distribution of the top three climate variables for *Lym* as suggested by RF_{VSURF} **(Left)** and RF_{clim} **(Right)**. Mass outbreak years are indicated by gray background coloring.

stand age between 40 and 70 years, a low diversity as indicated by a low Shannon index or Shannon evenness, and a high proportion of gymnosperms, i.e., Scots pine stands.

Although no stand_ variables were observed among the most important variables for classification of PPI mass outbreaks (**Figure 5**), a comparable differentiation of the defoliated FC from the distribution of all FC in Brandenburg was registered. As an example the mean number of tree species per hectare was lower (**Figure 8A**) and mean relative site index of pine stands was higher (**Figure 8B**) for PPI defoliated FC compared to the total of all FC in the state.

DISCUSSION

Random Forest Variable Selection

We obtained a great performance of the RF algorithms handling huge databases of different PPIs. The settings for the RF and the applied methods proved to have a high accuracy in classification (**Table 1**). Even the sharply reduced sets of predictor variables selected by the VSURF algorithm (RF_{VSURF}) obtained remarkably high rates of correct prediction (TNR) for more than 90% of the forest compartments defoliated by the three investigated PPI species. TNR results comparable to RF_{VSURF} were found for RF_{forest} and RF_{all} using around 500 and 200 variables, respectively, whereas classification by RF_{stand} was somewhat poorer.

The achieved high model accuracy is a result of large amounts of information representing manifold causal relations, but also of the constitution of the analyzed databases. Due to the characteristic spatial distribution of the defoliation events in Brandenburg, the RF algorithms search for variables that optimally separate defoliation hotspots from other regions. We started our analysis with a set of climatic variables that consider varying time windows from two subsequent years, so various data patterns are provided to describe both the observation period

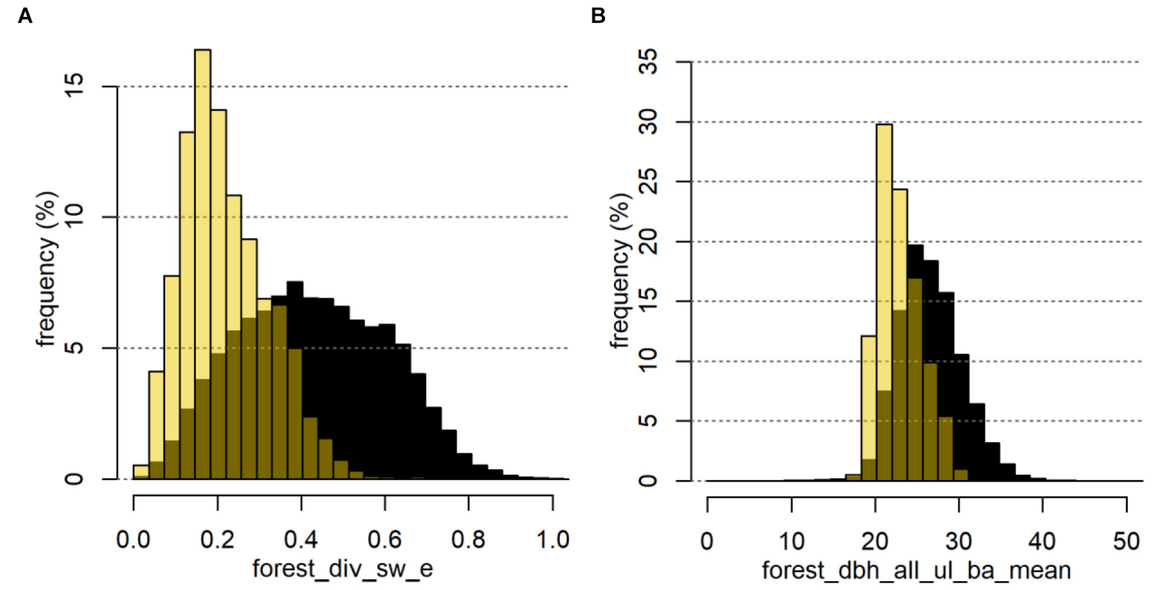

FIGURE 7 | Example of two important forest_ variables for *Den* represented by the density distribution of the mean Shannon evenness (A) and the mean basal-area weighted stand diameter at breast height of all upper forest layers within the 1,000 ha buffer zone (B). Black bars show the distributions for all FC in Brandenburg, yellow bars show the distributions for defoliated FC only.

of 15 years (**Figure 2**) and the spatial distribution (**Figure 1B**). Therefore, variable importance measures may identify a random data pattern rather than causal relationships to produce the most appropriate classification of the analyzed information.

Nevertheless, the extension of the analysis to a huge number of variables may firstly help to characterize past defoliation of mass outbreaks and is secondly essential for providing promising variable sets which describe reliable ecological relationships. Floating time windows, in particular, may provide hints on critical periods of the year that impact the development of mass outbreaks and feeding intensity (Möller et al., 2017). Furthermore, the identified climatic factors may impact the population dynamic on pest insects either directly (Netherer and Schopf, 2010) or via the physiological response of the host plant (Lindner et al., 2014). In addition, important climatic variables may represent both critical phases in the population dynamics of the species' natural antagonists and physiologically important periods according to particular biological needs (Chaves et al., 2003). Archaux and Wolters (2006) described the general impact of summer drought on forest biodiversity with decreased ecosystem productivity and increased mortality. The response to these extreme conditions depends on mobility, reproductive rate, or resistance mechanisms of the respective species. The consequences of climate change for the mostly host-specific natural antagonists of *Den*, *Lym*, and *Dip* are still largely unknown.

A critical remark has to be made on data availability: During the analyzed time covering 15 years of spatially referenced data we observed only two or three mass outbreaks, respectively (**Figure 2**). Depending on the pest species, mass outbreaks covered 1–3 years of defoliation in up to 2,000 forest compartments per year (**Figure 2**). The population dynamics of most forest pest insects follows a more or less fixed pattern for mass propagation with wave-shaped climax phases covering several years (Schwenke, 1978; Baltensweiler and Rubli, 1999; Hlásny et al., 2016). For example, mass outbreak periods of *Lym* occurred about every 10 years in Brandenburg. In the later years of these gradation cycles, the effect of the specific climatic triggers might become blurred by the effects of the initial population size in the previous year, even more so if feeding intensity is neglected as an influencing factor. In our analysis, we considered all defoliation classes as dichotomous "on-off" cases and thus accessed the maximal number of observations available. An exclusive focus on total defoliation (needle loss > 90%) events or the observation of specific phases of the population dynamic might further promote the identification of factors that trigger the onset of an outbreak cycle. In fact, the highest impact of the climatic conditions should be found before or during the first ("progradation") phase of mass outbreaks directly preceding a steep increase in population size. This narrowed approach, however, would be constrained by a severe reduction in data availability.

Data quality heavily depends on the origin of the particular parameters. The reliability of the regionalized environmental data (clim_ and site_) is limited by the uncertainties of the particular model application (Russ and Riek, 2011; Köhler et al., 2015). The core data used for stand_ and forest_ characterization, in contrast, originated from forest inventory data with a re-sampling rotation of 10 years that are corrected by annual harvest and mortality data provided by the local forest managers. Information from this background introduces an additional

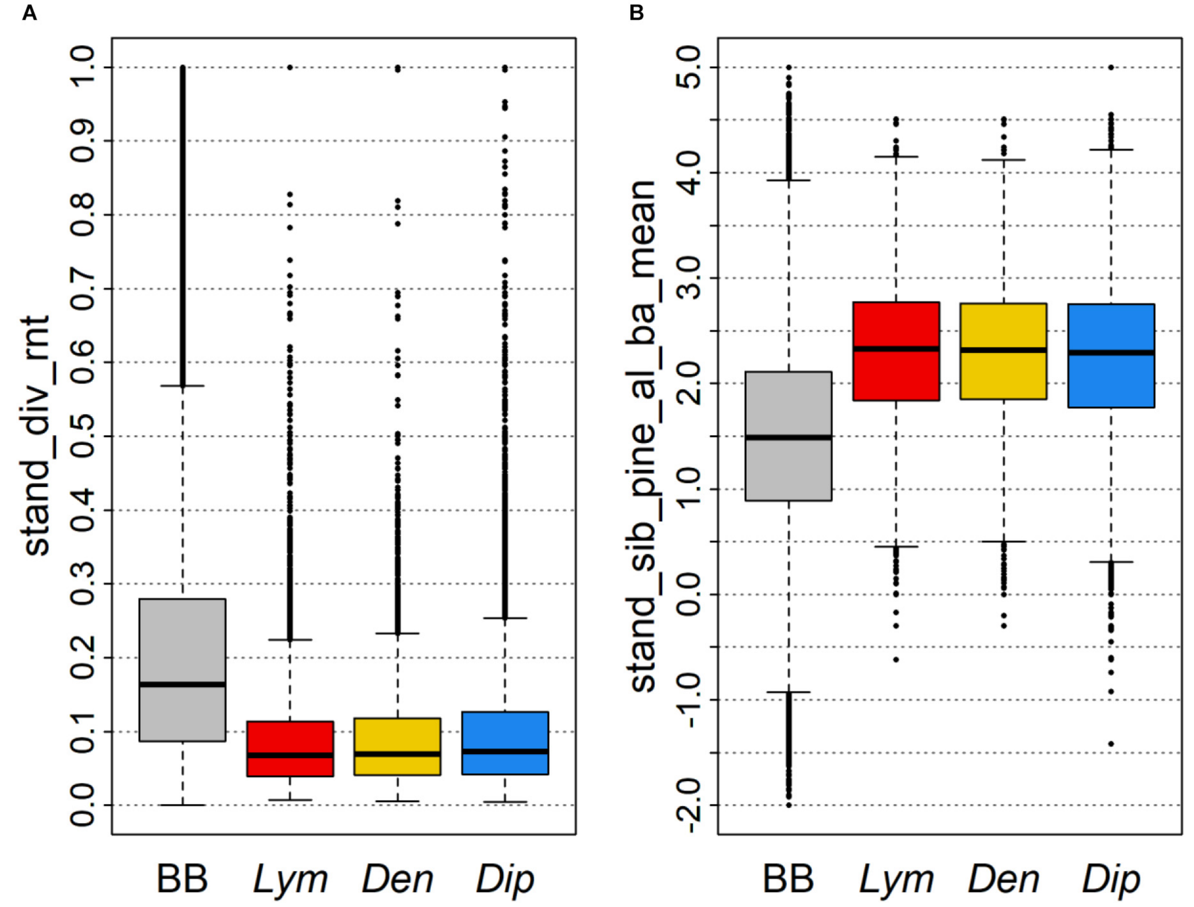

FIGURE 8 | Distributions of two important stand_ variables shown as boxplots of **(A)** the relative number of tree species per hectare and **(B)** the mean relative site index of pine stands within a FC. The baseline shows all data (BB) whereas PPI boxplots were calculated for FC defoliated by *Lym*, *Den*, and *Dip*, respectively.

level of uncertainty due to the potential subjective errors and temporally incoherent observation standards.

Since this study aimed to provide the most important explanatory variables for PPI defoliation inherent in the database, we computed different metrics of spatial and temporal data subsets for each set of parameters. While clim_ variables primarily explain the temporal appearance of PPI mass outbreaks, stand_ and forest_ variables should have a high impact on the spatial distribution of defoliation hotspots. Top variables of both variable groups clearly differentiated the continuum of forests attacked by PPI and the forest population of the whole study area (**Figures 7**, 8). On the landscape level, however, forest_ variables have proved to be most influential for classification and substituted stand_ variables in both variable selection approaches RF_{all} and RF_{VSURF} (**Figure 5** and **Table 2**).

We suggest that both compared variable importance measures (MDG and MDA) reliably select the variables that best represent the spatial distribution of PPI feeding events. The particular realizations of these variables deliver detailed information on the insects' ecological niche and, in turn, on the most likely feeding hotspots. However, the importance of clim_ variables largely depends on the particular setting of the RF, and the MDA measure might be misleading in some cases. In fact, the selection of standard deviation and maximum or minimum values (**Table 2**) may lead to the best representation of the pattern within the examined database without plausible biological links to the population dynamics of PPI. We also suppose that the consideration of climate data from all forest stands in the RF setting (RF_{all} and RF_{VSURF}) introduces additional noise to the temporal trend due to the spatial pattern of the climate data from specific years. Therefore, we suggest the application of RF_{clim} to interpret climatic triggers and crucial periods of the year. Despite of manifold uncertainties and shortcomings in the observation data and methodological details we are nevertheless confident about the quality of the primary sets of influencing factors for the three PPI. The selection of the climate variables, however, has to be evaluated by the years to come which will test the robustness of the respective parameters.

Finally, we were able to analyze a huge data set by different approaches using the RF algorithms implemented in "R" and obtained a reliable variable selection. We suggest that the quality of the variable importance measure might be restricted rather by the sample size and the timeframe available than by technical issues. Nevertheless, some methodological issues resulting from a high number of correlated variables (see Genuer et al., 2010) and strongly unbalanced data (see Chen et al., 2004) have to be considered. We recommend to spend more effort on database management providing continuous information on the predictive variables and to use the RF algorithms to detect changes in the data patterns as well as in the importance of ecological variables.

Ecological Relevance of Identified Classification Variables

The results achieved by the RF analyses are closely consistent with the known species-specific gradation patterns and their changes in the recent past. If a species exhibits a strong temporal regularity in the frequency of mass outbreaks, a tight dependence between population dynamics and the stand conditions (which remain more or less constant over a long time) has to be assumed. Consequently, the irregularity of mass outbreak patterns increases with a rising importance of climatic variables. Our findings indeed revealed a strong dependency of *Dip* mass outbreaks on climatic drivers (**Figure 5** and **Table 2**) which complies with the importance of the bivoltine lifecycle of *Dip* in Brandenburg and its close coupling to the climatic conditions of the current and the previous year (Möller et al., 2017). In contrast, *Lym* showed a higher variable importance for parameters of the forest_ variable group and a rather strong temporal regularity of outbreak events (Gräber et al., 2012).

Outbreak patterns and practical experiences have influenced the monitoring methods in forestry for a long time (e.g., Schwerdtfeger, 1934, 1981; Wellenstein, 1942; Sierpiñska, 1998; Häußler et al., 2000). The results of our investigations can help to further improve the forecast of timing and intensity of mass outbreaks and to adapt the respective methods to a changing environment. A reliable prognosis of the amplitude during the outbreak culmination, i.e., the infestation area and the intensity of needle loss to be expected, in dependence on the floating climatic windows could provide important essentials for evaluating and enhancing the current monitoring methods.

The obtained results on critical climatic factors correspond closely both with findings published by forest entomologists more than 100 years ago (e.g., Altum, 1881; Zwölfer, 1935) and with observations made by forest practitioners. A comparison of the found important climatic windows with the individual development of the species shows that there are a number of biologically plausible explanations for the statistical outcome. The three investigated pine pest species share a common preference for middle-aged, poorly structured pine forests. The successful development of mass gradations, however, depends on different critical climatic phases of the year relating to their specific life cycles.

Lymantria monacha

In accordance with other forest moths, it is widely accepted that *Lym* is highly sensitive toward temperature and prefers warm conditions. Vanhanen et al. (2007) suggested a northward shift of the range of *Lym* in the future triggered by warming climate conditions. Our study revealed some important climatic windows which strongly influence the development of this species.

The amplitude of an outbreak's culmination in terms of feeding damage depends heavily on the conditions in the initial phase of the outbreak. Increased sunshine hours during September of the previous year (clim_tmax_pm09_sd) have a positive effect on the severity of feeding. Zwölfer (1935), for example, observed high mortality rates in *Lym* eggs if they cannot finish their embryonal development in autumn due to detrimental weather. The egg stage shows three phases. The first phase is the highly temperature-dependent embryonic development of 2 up to 6 weeks following oviposition. The second phase is a hereditary development dormancy lasting for another 10 weeks; the third and last phase is winter rest due to low temperatures (Schwenke, 1978). Our findings emphasize the importance of the second phase for the survival of the young larva hibernating inside the egg.

The dominant influence of temperature on egg and larval development is further confirmed by the high rank of variation in average temperatures in January of the previous year (clim_tmean_pm01_sd). Winter conditions determine an insect's energy balance in a fundamental way. Temperature is an essential factor for egg and larvae metabolism even if other factors such as ambient moisture, nutrition status, or biotic opponents may also heavily influence dormancy regulation (Müller, 1992). A low mortality pressure on the parent generation caused by conditions favorable for the development during winter dormancy, for example, increases population density which in turn could lead to a mass outbreak in the next year (Wellenstein, 1942). The importance of vapor pressure in April of the previous year (clim_vp_pm04_sd) is related to the fact that intense drought periods can lead to raised egg mortality caused by desiccation (Wellenstein, 1942). This is one reason why egg vitality in winter should be investigated to further specify the forecast of feeding damage by *Lym*.

The effects of forest structure on the occurrence of mass outbreaks and defoliation were most significant for *Lym* as compared to *Den* and *Dip*. The largest and most severe *Lym* gradations commonly occur in pure conifer stands (Altum, 1881; Schwenke, 1978) with the highest proportion of defoliated pine stands located in forests with a dbh range of 20–24 cm for the dominant trees (**Supplementary Figure 6**). Similar results were found at the level of the individual forest compartments (stand_ variables) but are not shown here due to a lower explanatory power of this variable group compared to the forest_ variables. The high rank of the variable forest_div_sw_e, the standardized Shannon-Weaver index averaged over the 1,000-ha buffer area, proves that increasing tree species diversity reduces the risk of mass outbreaks. Experiences from forestry practice and from the monitoring system also show that *Lym* outbreaks often start in large areas covered by pure pine stands, which are inherently poor in structure and productivity (**Figures 7A,B**).

In Brandenburg, *Lym* has been characterized as the most important PPI responsible for widespreading defoliation of pine forests in the past (Gräber et al., 2012). Obviously, warm and dry conditions promote mass outbreaks of *Lym* as shown by the huge number of defoliated FC in 2003 (**Figure 2**). Once a mass gradation has successfully begun, however, high population densities seem to uncouple infestation from climate and severe defoliation can be expected in the following year. The large proportion of pure pine stands in Brandenburg represents favorable stand conditions for *Lym* supporting the observed temporal regularity of mass outbreaks. While stand structures alter with time and may be improved by forest management activities that increase the number of mixed and deciduous forest stands, climate change may again raise the biotic risk of defoliation. In addition, *Lym* might be quite adaptable to changes in the forest structure due to its polyphagous nature. Thus, future feeding events should be analyzed for changes in the feeding preferences of *Lym* and characterization of forest stands' predisposition.

Dendrolimus pini

The pine-tree lappet moth's feeding preferences for middle-aged pure pine stands are similar to those of the nun moth as shown by the variable ranking of the forest_ variables (**Figures 5**, **8B**). In fact, mass outbreaks of *Den* frequently follow the observed outbreaks of *Lym* (**Figure 2**) in almost the same infestation areas (see **Figure 1B**, where orange-colored FC indicate *Den* and *Lim* outbreaks in identical FC with yellow representing *Den* and red color representing *Lim*). Thus, *Den* is a harmful forest pest in Brandenburg feeding on already weakened pine stands. Furthermore, the heavy defoliation of juvenile plants associated with this species may lead to the decline of the entire forest. Similar to *Lym*, the pine-tree lappet moth prefers warm conditions suggesting an increasing risk of defoliation in the future.

The duration of sunshine during May of the previous year (**Figure 5**: clim_sun_pm_05_mean), for instance, is highly relevant for the classification of *Den* feeding events. More sunshine is related to higher temperature and less precipitation. Under these conditions the feeding intensity of the poikilothermic caterpillars increases, and a better vitality of the larvae can be assumed. The pupae stage will be reached earlier and the probability that larval parasitoids find their host is reduced. Additionally, faster growth increases the possibility that eggs of *Tachinidae* will slip off during host molting before penetration (Herting, 1960). Some Tachinid species lay their eggs on pine needles and after hatching the egg larvae must "wait" for host caterpillars. It can also be expected that hot and dry conditions shorten the life span of the parasitoids' eggs and especially the egg larvae. In consequence, a sunny May promotes both a high vitality of *Den* and a reduced biological control by parasitoids.

Warm and dry conditions in late summer during the flight, copulation, and egg deposition phase as reflected by the variable clim_tmax_fly_m2p2_mean (**Figure 5**) further promote a high population density of *Den* (Weckwerth, 1952; Majunke, 2000; Ray et al., 2016). Sunshine during September (clim_sun_pm_09_sd) is positive for the development of the thermophilic egg larvae that represent the most sensitive development stage of the insect's life cycle. Because the development of *Den* starts in one summer and ends after overwintering as caterpillar in the next one, it is biologically plausible that data of the previous year are statistically important for classifying population dynamics.

The effect of maximal temperature in October (**Figure 5**: clim_tmax_m10_sum) is also coherent with individual development requirements: During the last feeding period before hibernation the feeding intensity of the caterpillars is predominantly controlled by temperature. The amount of energy reserves deposited as storage proteins and lipid bodies (Levenbook, 1985) determines individual fitness (Kätzel and Möller, 1993) and is crucially important for survival during winter and the power to climb up the tree in spring.

The identification of sunshine duration in February (**Figure 5**: clim_sun_m02-mean) as a significant climatic window is quite interesting. The caterpillars of *Den* hibernate as L3 or L4 in the soil layer, and soil temperature determines the start of their climbing up the tree at the end of winter. At 1°C the caterpillars start to leave the soil, while at 6°C this process is culminating (Schwenke, 1978). In pine stands, the influence of sunshine on soil temperature is high because reflection and diffusion of incoming radiation by the crowns are comparably weak (Larcher, 1987). Leaving the soil earlier under favorable climatic conditions can significantly increase the survival rate for caterpillars because their overwintering in the soil is associated with numerous risks. These comprise natural enemies such as wild boar, mice, and entomophagous fungi, but also soil moisture promoting mortality by bacteria or fungi. Altum (1881) wrote that warm southeastern winds and low air humidity promote the process of ending hibernation. This knowledge is used in forest protection practice to start monitoring the climbing animals by estimating the number of larvae per crown at the right moment. This approach has proved to be an effective method to forecast *Den* feeding risks.

Diprion pini

In Brandenburg, mass outbreaks of *Dip* including widespreading defoliation events in the past were coupled to a bivoltine life cycle and second generation larvae feeding on needles (Möller et al., 2017). Since a successful development of two generations within 1 year depends on different critical phases for the respective development stages, mass outbreaks were predominatly controlled by climatic drivers. In contrast to *Lym* and *Den*, massive defoliation by *Dip* could almost exclusively be explained by climate variables (**Figure 5** and **Table 2**). We also observed a slightly higher variance of the forest_ parameters (e.g., the mean diameter of the upper stand layer) for FC defoliated by *Dip* in comparison to *Lym* and *Den* (**Figure 7B** and Supplementrary **Figures 6**, **7**). We thus suggest that if the climatic conditions favor a change to bivoltine reproduction, the quality of the host needles might be less important and *Dip* may affect a broader range of forest structures.

The monophaguous *Dip* is restricted to pine forests and population density is influenced by the quality of the feeding and breeding ground, even if forest_ and stand_ variables in our study have only minor explanatory power. For example, and also based on RF methods, Blomqvist et al. (2016) found lichen and lingonberry coverages to be the best predictors for cocoon mortality of *Dip* in Finland. Coverage densities were negatively correlated with mean defoliation intensity. Unfortunately, our data did not include detailed information on the herb layer. We nevertheless have to assume that the sensitivity to climatic drivers and the dependency on optimal forest structures in Finland differ from those in our study area because the life cycle of *Dip* in Finland even in outbreak years is predominantly univoltine (Geri, 1988). In northeast Germany these years are related to bivoltine phases, a situation that occurs rarely and is difficult to predict (Möller et al., 2017).

The prediction of this change from univoltine to bivoltine reproduction cycles might be the key to more reliable risk assessments for *Dip* mass outbreaks and severe defoliation. Shifts of the life cycle are probably related to a changing hierarchy of influential factors which should be considered in both model development and application. Flexible, phenology-oriented climatic "windows" can support the prognoses of critical development stages of *Dip* and of the risk of second generation feeding. In a previous study, we showed that recent mass outbreak events of *Dip* in Brandenburg were related to comparably low temperatures during the flight season, scarce precipitation in the period of BBT, a low variance of the mean temperature of the previous-year growing season and high evapotranspiration during the flight period of the previous year (Möller et al., 2017). Except for the precipitation variable, our results correspond to these findings (**Supplementary Table 1**). Deviating from the data set used in Möller et al. (2017), our analyses considered the complete forest area of Brandenburg. This might explain why precipitation data with their naturally high temporal and spatial variability were less influential here.

As a current example, the massive outbreak in 2016 in southern Brandenburg demonstrated the high risk potential of *Dip* if favorable climatic conditions are met. Therefore, we need to further investigate those climatic phases that are of crucial importance for the shift into the bivoltine life cycle of *Dip*. The results should be applied to the existing monitoring programs supplementing the risk assessment by count data.

Forest Protection

Many findings presented for the individual species are important arguments for management strategies that increase tree-species richness in forests. More and wider differentiated habitats for phytophagous species lead to better conditions for predatory zoophagous species and support an increased diversity in parasitoids as natural antagonists of insect pests like *Lym*, *Den*, and *Dip* (Kratochwil and Schwabe, 2001). A range of studies show the positive effect of deciduous trees in conversed pine forests as detectable in lower infestation by phytophagous insect pests (e.g., Hunter, 2001; Schulz and Dreger, 2003; Jäkel and Roth, 2004). As an example, Rös et al. (2004) found that population densities of the pine beauty moth *Panolis flammea* were strongly influenced by the proximity of deciduous forests, presumably due to the benefits for parasitoids such as tachinids.

In addition to the importance of climatic triggers of PPI outbreaks, we could show that the quality of the feeding ground and the structural forest properties clearly determine the spatial distribution of PPI feeding events. We successfully implemented the large body of information provided by forest inventories into our analyses and derived detailed risk assessments on the level of the individual forest compartment. Thus, we want to emphasize the importance of continuous forest inventory programs and the required collaboration of forest management and forest protection services.

The large areas of poorly structured pine stands of young to middle age, in particular in the South of Brandenburg, provide optimal conditions for PPI. In fact, common preferences of the individual PPI for forests with comparable stand structures may lead to parallel and/or sequential feeding in subsequent years which further elevates the risk for devastating defoliation. We thus need to forecast the development of these stands by forest growth simulations if we want to predict future feeding hotspots. Moreover, we observed a stronger predictive influence of forest properties of the neighborhood compared to the properties of the individual stands. Therefore, the predisposition of a FC toward the biotic risk of PPI may be altered by improving the structural features of the surrounding forest area, e.g., by increasing the proportion of mixed stands. Consequently, a higher number of tree species within one FC (**Figure 8A**) is linked to a reduction of the share of pure pine stands in the total forest area.

In agreement with findings from literature, pine stands at less productive sites as indicated by a higher relative site index have been most prone to defoliation by PPI (**Figure 8B**). Unfortunately, these stands are less suited for forest conversion and active enhancement of forest structural and species diversity due to their limited nutrient and water supply. The significant impact of the conditions in a larger buffer area, however, may help to promote individual FC by forest conversion activities at landscape level decreasing the overall predisposition for PPI feeding.

CONCLUSION

This study provides a novel method analyzing a huge set of environmental data with regard to their influence on mass outbreak and defoliation events of PPIs. We have outlined the most promising environmental parameters for modeling the risk of mass outbreaks of *Lym*, *Den*, and *Dip* in Brandenburg. Hence, other research groups might benefit from our findings in the variable selection and modeling processes when performing risk assessments of PPIs on independent data sets. Our analysis showed both the high impact of the climatic conditions in particular for pest species with irregular mass outbreak patterns (e.g., *Dip*) and the importance of stand structures for the predisposition to defoliation by insects with relatively constant outbreak cycles (e.g., *Lym*). Furthermore, we could demonstrate a successful combination of detailed forest inventory data for large areas, regionalized climatic conditions, and site properties supporting forest protection issues and prognosis of future risk levels. The increasing availability of geo-referenced forest data in combination with novel data mining techniques can enhance our knowledge of forest pest insects' population dynamics on landscape level and risk management, respectively.

We showed that (a) the PPI are sensitive to independent climatic triggers and temporal periods within the year relating to their species specific biology. The investigated PPI exhibited preferences to particular stand structures and specific adaptations to ecological (sub-) niches of pine forest ecosystems. However, mass outbreaks of all three PPI concentrated on low structured, young to mid-aged pine forests on rather poor sites. Therefore, we could not fully validate hypothesis (b) because there was no clear differentiation between the general forest types required by the individual species for successful mass outbreaks; all three insect species obviously prefer pure pine stands of lower dimensions. Furthermore, we demonstrated that PPI share (c) a common preference for warm climate and that (d) a higher tree species diversity of the habitat is counteracting severe mass outbreaks due to the limiting effects exerted by the higher abundance of predators and parasitoids under these conditions.

AUTHOR CONTRIBUTIONS

RH had the original idea for this study and covered the statistical methodology and data bank structuring and analyses. KM provided background knowledge on species biology and population dynamics. AW contributed essential monitoring and ecological data. AD was responsible for forest structure and stand variable integration. JS took care for combining all parts into the final manuscript, for the logical structure of the paper and for language and terminology issues.

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SUPPLEMENTARY MATERIAL

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Bark Beetle-Associated Blue-Stain Fungi Increase Antioxidant Enzyme Activities and Monoterpene Concentrations in *Pinus yunnanensis*

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Yunnan pine is the most important tree species in SW China in both economical and ecological terms, but it is often killed by pine shoot beetles (*Tomicus* spp.). *Tomicus* beetles are secondary pests in temperate regions and the aggressiveness of the beetles in SW China is considered to be due to the warm subtropical climates as well as the beetles' virulent fungal associates. Here, we assessed the virulence of three blue-stain fungi (*Leptographium wushanense*, *L. sinense* and *Ophiostoma canum*) associated with pine shoot beetles to Yunnan pine (*Pinus yunnanensis*) in SW China. Following fungal inoculation, we measured necrotic lesion lengths, antioxidant enzyme activities and monoterpene concentrations in the stem phloem of Yunnan pine. *Leptographium wushanense* induced twice as long lesions as *L. sinense* and *O. canum*, and all three fungi induced significantly longer lesions than sterile agar control inoculations. The activity of three tested antioxidant enzymes (peroxidase, polyphenol oxidase, and superoxide dismutase) increased after both fungal inoculation and control inoculation. However, *L. wushanense* and *L. sinense* generally caused a greater increase in enzyme activities than *O. canum* and the control treatment. Fungal inoculation induced stronger increases in six major monoterpenes than the control treatment, but the difference was significant only for some fungus-monoterpene combinations. Overall, our results show that *L. wushanense* and *L. sinense* elicit stronger defense responses and thus are more virulent to Yunnan pine than *O. canum*. The two *Leptographium* species may thus contribute to the aggressiveness of their beetle vectors and could damage Yunnan pine across SW China if they spread from the restricted geographical area they have been found in so far.

Keywords: *Leptographium wushanense*, *L. sinense*, *Ophiostoma canum*, phloem reaction zone, defense response

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INTRODUCTION

Conifers and bark beetles in the subfamily Scolytinae have developed intricate relationships over millions of years of interaction and co-evolution. Conifers have evolved multi-purpose constitutive and inducible defenses to fend off attackers, whereas bark beetles have evolved different strategies to overcome these defenses (Adams et al., 2011; Krokene, 2015). One beetle strategy is to vector diverse phytopathogenic blue-stain fungi of varying virulence that infect the tree when the beetles tunnel in the phloem (Zhou et al., 2002; Masuya et al., 2003). These fungi are thought to facilitate bark beetle host colonization (Krokene, 2015). Blue-stain fungi are necrotrophic pathogens that gradually colonize phloem and xylem tissues away from the beetle tunnels, capturing tree resources (Six and Wingfield, 2011; Six, 2012), eliciting tree defenses (Franceschi et al., 2000; Nagy et al., 2000; Viiri et al., 2001; Lieutier et al., 2009; Zhao et al., 2010, 2011a,b), and likely helping bark beetles overwhelm trees defenses (Paine et al., 1997; Lieutier et al., 2009; Zhao et al., 2018). In addition, some blue-stain fungi appear to be involved in nutritional supplementation of bark beetle larvae (Ayres et al., 2000; Bleiker and Six, 2007) and production of bark beetle aggregation pheromones (Zhao et al., 2015).

Conifer defense reactions are complex and involve several metabolic pathways, such as the isoprenoid and phenylpropanoid pathways (Iriti and Faoro, 2009). Pines and other members of the family Pinaceae rely heavily on monoterpenes and other products of the isoprenoid pathway in their defense against bark beetles and other biotic challenges (Franceschi et al., 2000; Nagy et al., 2000; Viiri et al., 2001; Klepzig et al., 2005; Fäldt et al., 2006; Zhao et al., 2011a). Monoterpenes stored constitutively in resin ducts in the phloem and sapwood are the first defense barrier (Gershenzon and Dudareva, 2007). Following beetle attack and fungal infection, monoterpene concentrations increase dramatically in the necrotic lesions formed by the trees' induced defenses (e.g., Viiri et al., 2001; Klepzig et al., 2005; Fäldt et al., 2006; Zhao et al., 2011a). Since more virulent fungi induce longer necrotic lesions and stronger monoterpene responses upon infection than less virulent fungi (Krokene and Solheim, 1999; Fäldt et al., 2006), fungal virulence is often assessed by measuring lesion lengths and quantifying monoterpene increases following experimental inoculation.

In addition to terpenes, pines and other plants protect themselves against damage by releasing antioxidative scavenging enzymes such as superoxide dismutases (SOD), polyphenol oxidases (PPO), peroxidases (POD), and catalases (CAT). These enzymes protect plants from reactive oxygen species that are released when plant tissues are damaged by feeding insects colonizing fungi (Liao, 2002; Liu, 2010). Previous studies have demonstrated that salt and drought stress, wounding, beetle attack and fungal infection may alter the activity of these enzymes in plants (Nagy et al., 2004; Pan et al., 2006). However, little is known about antioxidative enzymes in conifers and if they are involved in tree resistance and tolerance against bark beetles and blue-stain fungi (Liao, 2002; Liu, 2010).

Yunnan pine (*Pinus yunnanensis*) is an economically and ecologically important conifer in SW China that is suffering large-scale damage from pine shoot beetles in the genus *Tomicus* (Scolytinae) (Ye, 1992; Lu, 2011). Since the 1980's, 93,000 ha of *P. yunnanensis* has been killed by three native *Tomicus* species: *T. yunnanensis*, *T. minor* and *T. brevipilosus* (Ji et al., 2007; Lu, 2011). In Europe, *Tomicus* species are usually secondary pests which can only breed in the stem phloem of weakened or dead trees, but their maturation feeding in the shoots of healthy trees may cause substantial increment losses (Jankowiak and Kurek, 2006; Jankowiak, 2008). However, *Tomicus* species may behave aggressively and kill healthy individuals of their co-evolved host tree Yunnan pine (Lu, 2011). Virulent fungal associates, together with warm and humid climates, are thought to be the explanation for the beetles' aggressiveness in the area (Duan, 1999).

Tomicus is associated with many different blue-stain fungi in SW China (Zhou et al., 2000, 2013; Paciura et al., 2010; Chang et al., 2017; Pan et al., 2017, 2018) and inoculation studies have demonstrated that at least one of them, *L. yunnanense*, is virulent to Yunnan pine (Liao and Ye, 2002). Recently, we isolated three species of blue-stain fungi with unknown virulence from *Tomicus* breeding galleries: *Leptographium wusanense, L. sinense,* and *Ophiostoma canum*. The objective of this study was to determine if these fungi are virulent to Yunnan pine and assess their potential damage to trees. To this end, we inoculated the fungi into the phloem of mature trees and quantified tree defense responses such as lesion lengths, antioxidative enzyme activities, and monoterpene concentrations.

MATERIALS AND METHODS

Fungal Species and DNA Sequencing

Three species of blue-stain fungi associated with pine shoot beetles (*Tomicus* spp.) in China were included in this study (**Table 1**). *Leptographium sinense* and the newly described species *L. wusanense* were isolated from galleries of *T. armandii* infesting Armand pine (*P. armandii*). *Ophiostoma canum* was isolated from a gallery of *T. yunanensis* in Yunnan pine. Armand pine is usually relatively resistant to *Tomicus* (Zhao and Längström, 2012), but since Armand pine and Yunnan pine often forms mixed stands, blue-stain fungi may be transferred between the two species by *Tomicus* beetles. *Leptographium sinense* has previously been isolated from *Hylobitelus xiaoi,* a weevil causing serious damage to several pine species in China (Yin et al., 2015). The third fungal species, *Ophiostoma canum*, is a common associate of *Tomicus* in Yunnan pine in China (Pan et al. manuscript).

The three blue-stain fungi were identified based on morphology and sequencing of three DNA regions (for details see **Supplementary Figures S1, S2**). DNA was extracted as described in Raeder and Broda (1985). For all three species we amplified partial sequences of β-tubulin using the primer pair Bt2a/Bt2b, and elongation factor 1α (EF-1α) using EF1F/EF2R (Glass and Donaldson, 1995; Jacobs et al., 2004). Furthermore, for *L. sinense* and *L. wushanense* we amplified the internal transcribed spacer 2 region and part of the large subunit (ITS2-LSU), using the primer pair ITS3/LRS (Vilgalys and Hester, 1990; White et al., 1990).

For *O. canum*, we amplified the ITS1 and 2 regions including the 5.8S gene (ITS1-5.8S-ITS2), using the primer pair ITS1F/ITS4 (White et al., 1990; Gardes and Bruns, 1993). All PCR conditions were according to the procedure described by Grobbelaar et al. (2009).

The obtained DNA sequences were compared with relevant *Leptographium* and *Ophiostoma* sequences deposited in GenBank. All sequences were then aligned using ClustalX1.81 (Jeanmougin et al., 1998). Data for the three sequenced DNA regions (ribosomal DNA, β-tubulin and EF-1α) were combined using BioEdit 7.0.1. Phylogenetic relationships were determined based on Bayesian (BI) analysis using MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003) and Maximum likelihood (ML) analysis using RAxML version 7.0.4 (Stamatakis, 2014). Two phylogenetic trees clustered our sequenced *L. sinense* and *O. canum* strains together with other *L. sinense* and *O. canum* sequences from GenBank with high support (93 and 100% Bayesian posterior probabilities (BIPP), respectively, **Supplementary Figures S1, S2**). The *Leptographium* tree placed *L. wushanense* in the *L. lundbergii* complex, as a sister taxon of *L. conjunctum* and *L. yunnanense* with 99% BIPP.

The fungal strains used in this study were deposited in the Herbarium of the Laboratory for Conservation and Utilization of Bio-resources, Yunnan University, Kunming, Yunnan, China (YMF; formerly Key Laboratory of Industrial Microbiology and Fermentation Technology of Yunnan). Sequence data were deposited in GenBank (accession numbers are provided in **Table 1**).

Field Procedures

The experiment was carried out in a naturally regenerated Yunnan pine forest in Yunnan, SW China (25°12′N, 102°77′E) in July and August 2016. Twenty to thirty years old Yunnan pines with no apparent damage and of similar size (diameter at breast height: 13 ± 3 cm, height: 15 ± 3 m (mean ± SD) were selected for inoculation. Each tree was inoculated three times with each fungus and three times with the control using a 1 cm diameter cork borer. Inoculum consisted of fungal cultures (**Table 1**) incubated on 2% malt extract agar for 14 days at 20°C in the dark or sterile malt extract agar as a control. The 12 inoculation sites on each tree were positioned in a spiraling pattern around the stem from 0.7 to 1.7 m height, with alternating positions for each of the four treatments. Lesion lengths induced by the different inoculation treatments were recorded 10, 20, and 30 days after inoculation by carefully removing the cork bark over one set of inoculation holes and measuring the full length of the necrotic lesions in the phloem (one inoculation site per inoculation treatment on each sampling day). At the same time, a phloem sample was taken from each necrotic lesion using a sterilized scalpel and forceps. The phloem samples collected each day were evenly assigned to two groups. One group was used for terpene analysis and another group for determining antioxidative enzyme activity. After sampling, phloem samples were placed individually in 1.5 mL sterilized Eppendorf tubes and immediately stored in liquid nitrogen. Two or three additional phloem samples (12 ± 3 mm × 1.5 ± 0.5 mm) were taken from each necrotic lesion and cultured on 2% malt extract agar to reisolate the inoculated fungi.

Determination of Antioxidative Enzyme Activity

Four antioxidative enzymes (CAT, POD, PPO, SOD) were extracted from control and fungus inoculated phloem samples collected 10, 20, and 30 days after inoculation ($n = 10$ trees). Enzyme activity was determined using specific kits following the manufacturer's instructions (Suzhou Comin Biotechnology Co., Ltd., Suzhou, Jiangsu, China). The specific determination method used for each of the four enzymes is given in **Supplementary Table S1**. Briefly, phloem was ground into a fine power under liquid nitrogen, 0.1 g powder was added to 1 mL reagent (**Supplementary Table S1**) in an Eppendorf tube and placed in an ice bath. The supernatant was then transferred to a new Eppendorf tube and centrifuged at 8000 rpm for 10 min at 25°C. The resulting crude enzyme extract was mixed with defined reagents following the instructions outlined in **Supplementary Table S1**. The absorbance of each post-reaction solution was measured using an ultraviolet visible spectrophotometer (model 752N, Shanghai analytical instrument factory, Shanghai, China) and the activity of each antioxidative enzyme was calculated according to the specific formulas given in **Supplementary Table S1**.

One unit of SOD activity was defined as the amount of enzyme required to cause a 50% inhibition in the reduction of Nitro Blue Tetrazolium (NBT). One unit of POD and PPO activity was defined as the amount of enzyme required to cause a 0.01 change in absorbance at 525 (PPO) and 470 (POD) nm per minute. One unit of CAT activity was defined as the amount of enzyme required to decompose 1 nmol H_2O_2 at 240 nm per minute.

TABLE 1 | Isolates of blue-stain fungi used in this study and GenBank accession numbers for DNA sequences. *L., Leptographium; O., Ophiostoma.*

Fungus	Isolate no.	Host/insect vector	Origin	GenBank accession no.			
				ITS1-5.8S-ITS2	ITS2-LSU	β -tubulin	EF-1 α
L. wushanense	YMF 1. 04934	*Pinus armandii/Tomicus armandii*	Wushan,Chongqin, China	______	MG878407	MG878408	MG878409
L. sinense	YMF 1. 04935	*P. armandii/T. armandii*	Liupanshui, Guizhou, China	______	MH216027	MH216030	MH216033
O. canum	YMF 1. 04936	*P. yunnanensis/T. yunnanensis*	Luliang,Yunnan, China	MG702035	______	MG702062	MG702089

Monoterpene Separation, Identification, and Quantification

All phloem samples from 10 of the 20 experimental trees were submerged individually in 1.5 mL hexane in 8 mL brown sampling vials (Aijiren, China) and kept at 20°C for 48 h. The extract was centrifuged at 5000 rpm for 10 min at 8°C, 1 mL supernatant was transferred to a 2 mL sampling vial (Agilent, United States), and 0.3 mg nonene (99%, donated by Dr. Ehrenstorfer) was added as an internal standard. Extracts were stored at −20°C until gas chromatography-mass spectrometry (GC-MS) analysis. The remaining phloem was dried at 80°C for 6 h and weighted on an electronic balance (Sartorius) to calculate monoterpene concentrations per unit dry weight.

An Agilent 6890 GC connected to a 5973 MS and a HP-5 fused silica capillary column (J&W Scientific^{TM}, 30 m length, 0.25 mm inner diameter and 0.25 μm film thickness) was used for the analysis. One microliter extract was injected to a split/splitless injector with a 30 s splitless injection at a temperature of 250°C. The temperature program started at 40°C, increased to 80°C at a rate of 3°C/min, then to 280°C at a rate of 5°C/min, and then remained at 280°C for 20 min.

Monoterpenes were identified using the Wiley 7 n.l and NIST 98.L reference libraries and confirmed by comparing retention times and mass spectra with available authenticated standards. The absolute amount of each monoterpene (m_x) was calculated using the equation $m_x = k(A_x/A_s)\ m_s/D_w$ (where k is the relative response factor, A_x is the peak area of a monoterpene in the sample, A_s is the peak area of the internal standard nonane, m_s is the mass of nonane and D_w is the dry weight of the sample). The relative response factor (k) was calculated using the equation $k = A_s m_i/(A_i m_s)$ (where A_s is the peak area of nonane, A_i is the peak area of a monoterpene standard, m_s is the mass of nonane, and m_i is the mass of the monoterpene standard).

Statistical Analyses

All analyses were conducted with SPSS 17.0 (SPSS Inc., Chicago, IL, United States), and graphs were generated using Origin 8.5 (Origin Lab Corporation, Northampton, MA, United States). The variation in lesion lengths was analyzed using repeated measures model, and enzymes activities and monoterpene concentrations was tested using a linear mixed model (GLMM) for repeated measures since we had some missing data caused by a few samples that were too diluted for enzyme and monoterpene analysis. Following ANOVA, differences between inoculation treatments at each time point and between time points for each treatment were tested using the Bonferroni test (for data with equal variances) or Dunnett's T3 test (for data with unequal variances) at $P = 0.05$.

RESULTS

Phloem Lesion Length

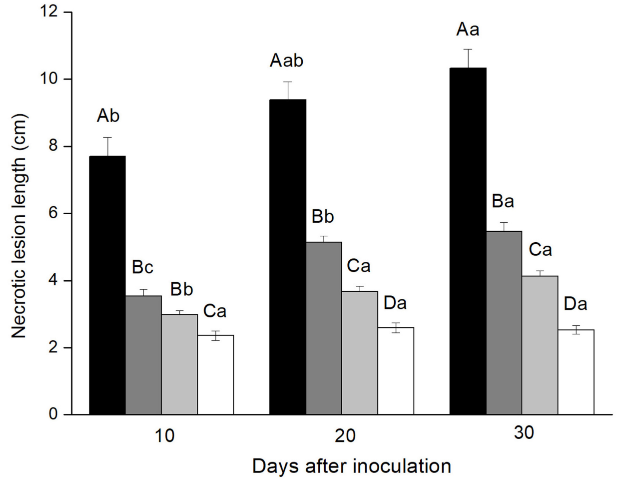

FIGURE 1 | Necrotic lesion lengths in the phloem of *Pinus yunnanensis* following fungal inoculation or mechanical wounding (black bars: *Leptographium wushanense*, gray bars: *L. sinense*, light gray bars: *Ophiostoma canum*, white bars: agar control inoculation). The data are expressed as mean ± SE ($n = 20$). Different letters indicate significant differences between treatments at each time point (capital letters) or between time points for each treatment (lowercase letters), following Bonferroni or Dunnett's T3 test at $P = 0.05$.

All fungi were pathogenic to Yunnan pine and induced significantly longer lesions than agar control inoculations at all three time points [10th day: $F_{(3,\ 39.796)} = 30.321$, $P < 0.001$; 20th day: $F_{(3,\ 40.870)} = 72.155$, $P < 0.001$; 30th day: $F_{(3,\ 39.635)} = 86.683$, $P < 0.001$]. The three fungi showed different virulence to Yunnan pine. *Leptographium wushanense* induced very long lesions (up to 103 mm) that were significantly longer than those induced by *L. sinense* and *O. canum* at all three time points. *Leptographium sinense* induced slightly but significantly longer lesions than *O. canum* 20 and 30 days after inoculation.

Lesion lengths increased significantly over time for *L. wushanense*: [$F_{(2,57)} = 5.658$, $P < 0.01$], *L. sinense* [$F_{(2,35.038)} = 56.403$, $p < 0.01$] and *O. canum* [$F_{(2,57)} = 15.579$, $p < 0.001$; **Figure 1**].

Antioxidative Enzyme Activities in Phloem Lesions

Enzyme activities varied greatly among the four treatments and over time since inoculation. SOD activities in stem phloem inoculated with *L. wushanense* were significantly higher than for the three other treatments 30 days after inoculation [$F_{(3,9.131)} = 6.382$, $P = 0.013$]. There were no significant treatment differences at the other time points. SOD activities increased significantly over time since inoculation for all treatments except *O. canum* (**Figure 2A** and **Supplementary Table S2**).

PPO activities were more variable with few significant treatment differences, but PPO activities in phloem inoculated with *L. wushanense* were significantly higher than for *O. canum* 10 days after treatment [$F_{(3,8.765)} = 6.770$, $P = 0.012$]. The only treatment with a significant increase in PPO activities over time was *O. canum*, but it started out with very low activities 10 days after inoculation [$F_{(2,7.303)} = 10.729$, $P = 0.007$; **Figure 2B** and **Supplementary Table S2**].

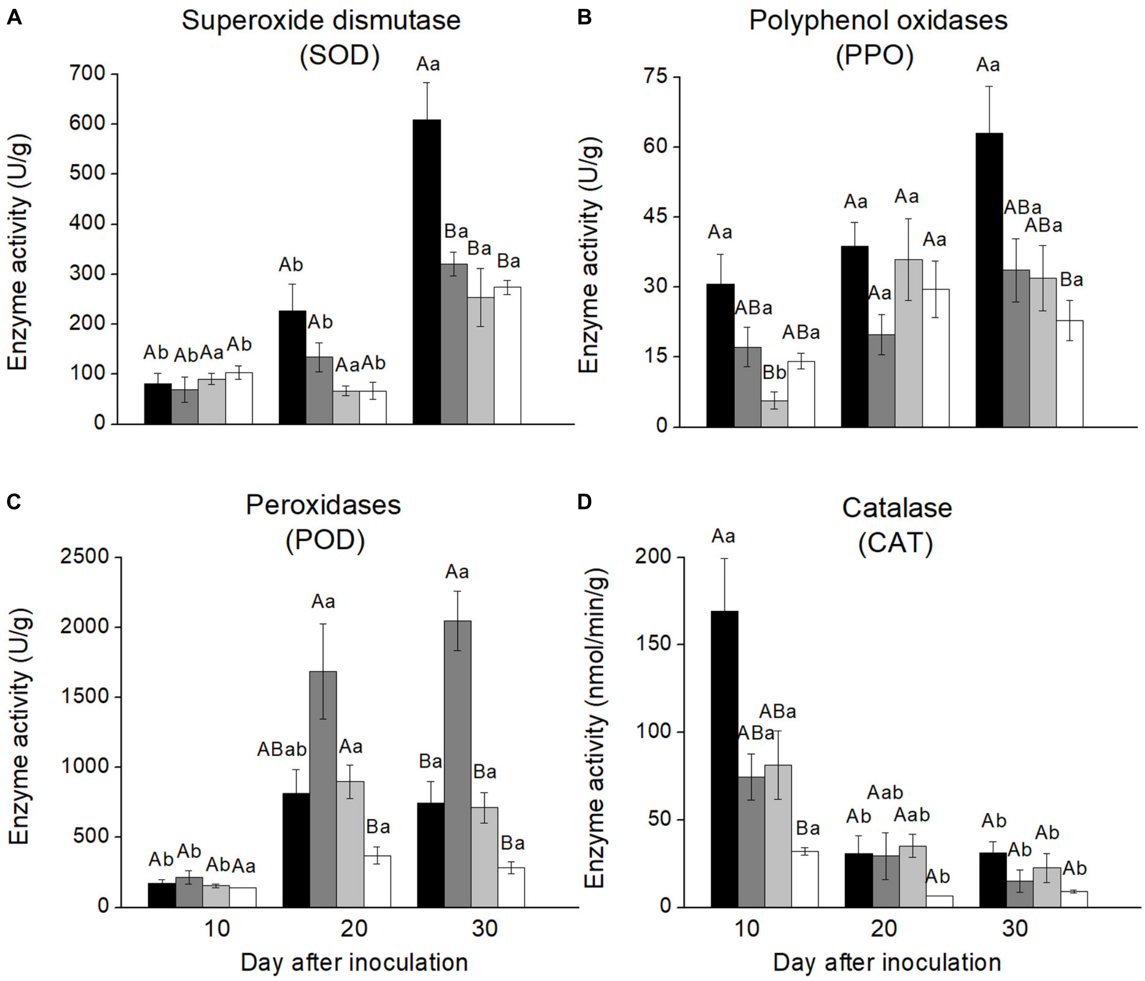

FIGURE 2 | Antioxidative enzyme activities in the phloem reaction zones of *Pinus yunnanensis* following fungal inoculation or mechanical wounding (**A**, SOD; **B**, PPO; **C**, POD; **D**, CAT) (black bars: *Leptographium wushanense*, gray bars: *L. sinense*, light gray bars: *Ophiostoma canum*, white bars: agar control inoculation). The data are expressed as mean ± SE ($n \leq 10$). Different letters indicate significant differences between treatments at each time point (capital letters) or between time points for each treatment (lowercase letters), following Bonferroni or Dunnett's T3 test at $P = 0.05$.

POD activities in phloem inoculated with *L. sinense* were significantly higher than in the control 20 days after inoculation [$F_{(3,9.427)} = 8.802$, $P = 0.004$] and significantly higher than for the other treatments 30 days after inoculation [$F_{(3,11.580)} = 24.407$, $P < 0.001$]. Furthermore, POD activities increased significantly over time for all treatments except control (**Figure 2C**).

CAT activities showed a different temporal pattern than the other antioxidative enzymes, with CAT activities decreasing significantly over time for all four treatments (**Figure 2**). The only significant treatment difference was that *L. wushanense* had significantly higher CAT activities than the control 10 days after treatment [$F_{(3,9.420)} = 10.663$, $P = 0.002$ (C)].

Monoterpene Concentrations in Phloem Lesions

Six monoterpene hydrocarbons (α-pinene, camphene, β-pinene, myrcene, β-phellandrene, and α-terpinolene) were detected from phloem lesions induced by fungal and agar control inoculations (**Figure 3**). The most abundant monoterpenes in all treatments were α-pinene and β-pinene, which constituted more than 83.4% of total monoterpenes in most samples. There was no significant increase in monoterpene concentrations over time since inoculation for any treatment, although the absolute values increased gradually for most monoterpene-treatment combinations (**Figure 3** and **Supplementary Table S3**).

Monoterpene concentrations varied between inoculation treatments. The two *Leptographium* species induced more monoterpene accumulation than *O. canum* and mechanical wounding. At the final sampling 30 days after inoculation, *L. wushanense* induced significantly higher concentrations of α-pinene [$F_{(3,13.193)} = 7.172$, $P = 0.004$], β-myrcene [$F_{(3,28)} = 4.323$, $P = 0.013$], and β-phellandrene [$F_{(3,29)} = 3.542$, $P = 0.027$] compared to the control. In addition, *L. wushanense* also induced higher levels of camphene, β-pinene, and α-terpinolene than the control, but the differences were

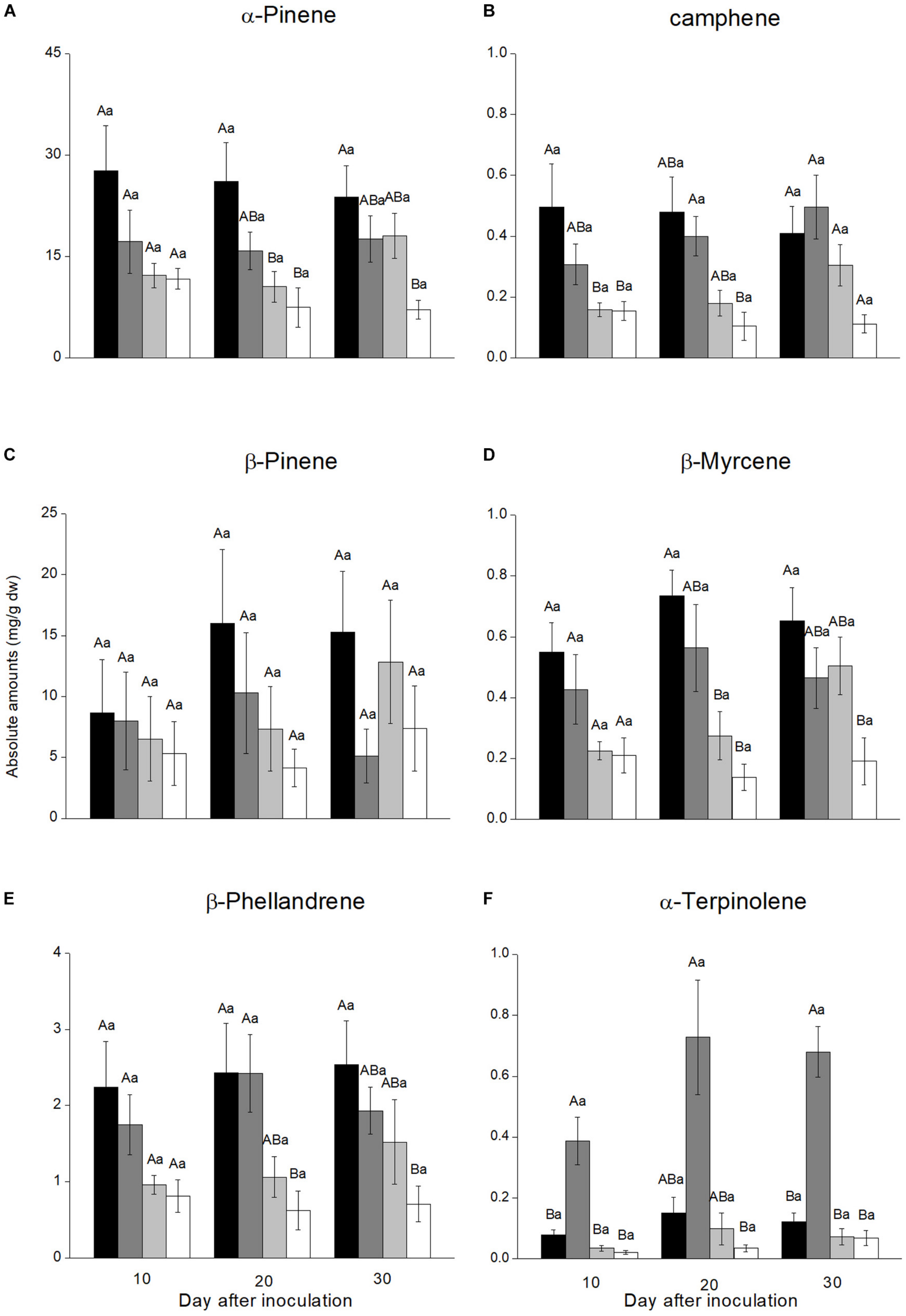

FIGURE 3 | Monoterpene concentrations in necrotic lesions in the phloem of *Pinus yunnanensis* following fungal inoculation or mechanical wounding (**A**, α-pinene; **B**, camphene; **C**, β-pinene; **D**, myrcene; **E**, β-phellandrene; **F**, α-terpinolene) (black bars: *Leptographium wushanense*, gray bars: *L. sinense*, light gray bars: *Ophiostoma canum*, white bars: agar control inoculation). The data are expressed as mean ± SE ($n \leq 10$). Different letters indicate significant differences between treatments at each time point (capital letters) or between time points for each treatment (lowercase letters), following Bonferroni or Dunnett's T3 test at $P = 0.05$.

not statistically significant. Similarly, *L. sinense* induced a stronger accumulation of most monoterpenes than the control, but the increase was only significant for α-terpinolene [10th day: $F_{(3,9.056)} = 9.951$, $P = 0.003$; 20th day: $F_{(3,11.388)} = 5.755$, $P = 0.012$; 30th day: $F_{(3,13.668)} = 15.920$, $P < 0.001$]. *Ophiostoma canum* induced higher absolute concentrations than the control for most monoterpenes, but the differences were not statistically significant for any monoterpene (**Figure 3**).

In addition to the differences observed at the final sampling time, one or both *Leptographium* species induced significantly higher concentrations than the control for α-pinene, camphene, β-myrcene, β-phellandrene, or α-terpinolene at 10 or 20 days after inoculation (**Figure 3**).

DISCUSSION

Blue-stain fungi are well-known associates of scolytid bark beetle species colonizing both hardwoods and conifers. Many blue-stain fungi are pathogenic to host trees, causing necrosis around natural infection points and experimental fungal inoculations. Because virulent fungi kill more host tissues and induce longer necrotic lesions than less virulent species, lesion length after fungal inoculation is used as a standard measure of fungal virulence (e.g., Krokene and Solheim, 1999; Solheim et al., 2001; Viiri et al., 2001; Fäldt et al., 2006). In the present study, inoculation of *L. wushanense* induced the longest lesions in Yunnan pine, followed by *L. sinense* and *O. canum*. This suggests that *L. wushanense* is the most virulent and *O. canum* the least virulent fungus of the three fungi we tested.

Ophiostoma canum is a common associate of *T. minor* and is weakly pathogenic to Scots pine (*P. sylvestris*) and Japanese red pine (*P. densiflora*) (Solheim et al., 2001; Masuya et al., 2003). We found that *O. canum* caused slightly, but significantly, longer lesions than mechanical wounding, suggesting that this fungus is weakly pathogenic also to Yunnan pine. The pathogenicity of *L. sinense* and *L. wushanense* was previously unknown. Our results show that they are moderately (*L. sinense*) or highly virulent (*L. wushanense*) to Yunnan pine. The presence of these pathogenic fungi in SW China might be a contributing factor to the destructiveness of *Tomicus* species in the area.

The two *Leptographium* species we studied were more virulent to our experimental trees than *O. canum* was. Blue-stain fungi in the genus *Leptographium* are primarily associated with bark beetles that attack live pine trees (Jacobs and Wingfield, 2001). These fungi seem to play a major role in decline diseases of several pine species, including red pine (*Pinus resinosa*) and other North American pines. Two pathogenic *Leptographium* species (*L. terebrantis* and *L. procerum*) introduced into red pine by the bark beetle species *Dendroctonus valens* and *Hylastes porculus* are thought to be a major cause of red pine decline in Midwestern United States (Klepzig et al., 1995). Another species, *L. wageneri*, cause black-stain root disease in several pines and other conifers and inflict considerable damage across western North America (Schweigkofler et al., 2005). In China, *L. yunnanense* vectored by *Tomicus* species are implicated in the ongoing decline of Yunnan pine (Liao, 2002). At the peak of the decline, *L. yunnanense* was isolated from 12.5% of the *T. yunnanenesis* individuals (Zhou et al., 2000). Our results suggest that also *L. sinense* and *L. wushanense*, which were isolated from *Tomicus* beetles tunneling in the relatively resistant *P. armandii* (Zhao and Långström, 2012), are virulent to pine trees and may contribute to the decline of Yunnan pine if they are transmitted to the tree by the bark beetles.

Production of antioxidative scavenging enzymes is part of the defense of many plants against biotic and abiotic stresses. Increased PPO activity in response to fungal infection in different plant species (Robert, 1966) is thought to increase plant resistance since PPO is involved in the formation of anti-fungal compounds and lignification process of host plants (Jung et al., 2004; Jaiti et al., 2007). Overexpression of PPO genes also increases resistance to herbivorous insects in tomato and hybrid aspen (Mahanil et al., 2008). Here, we found that inoculation of *L. wushanense* induced significantly higher PPO, SOD and CAT activities at one of the sampling times and *L. sinense* inoculation enhanced POD activity at the last two sampling times, indicating that these enzymes are involved in defense or repair responses of Yunnan pine against blue-stain fungi.

In addition to differences between inoculation treatments, there may be temporal differences in the induction of enzyme activities. After fungal inoculation of Yunnan pine, CAT activities were highest at the first sampling time, whereas SOD, POD, and PPO activities gradually increased over time and reached the highest level mostly at the last sampling time. This suggests that different antioxidative enzymes have different response speeds and that multiple sampling times are needed to get a complete picture of the enzyme activities. It is interesting to note that inoculation of *L. wushanense*, the most virulent fungus, induced the highest activities of SOD, PPO, and CAT activities, whereas the least virulent fungus *L. sinense* induced the highest POD activity. POD is involved in many physiological processes in plants, including responses to biotic and abiotic stress and lignin biosynthesis. These observations suggest that Yunnan pine activate different antioxidant enzymes depending on the nature of the attacking pathogens and the infestation stages.

Contrary to the increase observed after inoculation for the other antioxidant enzymes, CAT activity decreased significantly over time in all inoculation treatments (**Figure 2**). Very similar responses, with increased POD and SOD activities and decreased CAT activity, have been observed in liquorice (*Glycyrrhiza uralensis* Fisch) exposed to drought and salt stress (Pan et al., 2006). The underlying mechanism may be that SOD activity can increase concentrations of H_2O_2, which can react with superoxide to form hydroxyl radicals and cause a reduction in CAT activity (Pan et al., 2006). Different stress factors such as wounding, drought, pathogen infection and insect attack may induce similar responses (Nagy et al., 2004), and we speculated that the significant decline in CAT activity over time in our study might be due to the same mechanism.

Though small amounts of sesquiterpenes and diterpenes were found, monoterpenes were the main terpenoid class detected in our phloem samples. These 10-carbon terpenes are generally toxic to insects and may increase bark beetle mortality during host tree colonization (Gershenzon and Dudareva, 2007). Monoterpenes can also inhibit the growth of fungal pathogens (Cobb et al., 1968; Novak et al., 2014), such as the blue-stain fungi vectored by bark beetles. Terpene concentrations increase dramatically in the phloem of Norway spruce (*Picea abies*) following inoculation with the bark beetle-associated blue-stain fungus *Endoconidiophora polonica* (Zhao et al., 2011a). This terpene increase inhibits colonization by the spruce bark beetle (*Ips typographus*) in a dose-dependent manner (Zhao et al., 2011a). Norway spruce also produces monoterpenes with antifungal properties in response to infection by blue-stain fungi (Novak et al., 2014). Similarly, Scots pine trees produce more monoterpenes in the phloem following inoculation with the virulent fungus *L. wingfieldii* than with the less virulent species *O. canum* (Fäldt et al., 2006). We found the same pattern in the present study: *L. wushanense* and *L. sinense* induced a more intense monoterpene response than the less virulent *O. canum*. These observations suggest that Yunnan pine can distinguish between more and less virulent blue-stain fungi and activate appropriate inducible defenses according to the virulence of the attacker.

Conifers often have a very strong increase in terpene concentrations following fungal infection. For example, monoterpene levels in Scots pine phloem increase several 100-fold 4 weeks after inoculation with *L. wingfieldii* and *O. canum* (Fäldt et al., 2006) and terpene concentrations in Norway spruce phloem increases dramatically in response to *E. polonica* inoculation (Zhao et al., 2011a). Compared to Scots pine and Norway spruce the monoterpene response we observed in Yunnan pine to fungal inoculation was quite moderate, with small and mostly non-significant differences between sampling times. The different monoterpene response of Yunnan pine to other conifers may be a result of biological differences between both tree and fungal species In addition, the dry and warm weather in Yunnan may have impaired the terpene defense in Yunnan pine and made the trees more susceptibility to infection.

We inoculated our experimental trees with three fungal species and sampled the trees multiple times to compare fungal pathogenicity and disease symptoms over time. There is a possibility that the inoculations and sampling could have induced tree defense and thereby influence the results. However, many previous studies that have used a similar experimental design (Krokene et al., 1999; Fäldt et al., 2006; Zhao et al., 2018) have found negligible interference between treatments. Systemic priming or induction of tree defenses have been documented in Norway spruce (Krokene et al., 1999), but this require more time to become effective and more extensive and concentrated wounding than we used in our study. We thus trust that our experimental design have not interfered with our results. The considerable variation we observed within treatments may be due to the fact that we used naturally regenerated Yunnan pine trees of unknown genetic background. Extensive genetic differences between trees might explain why differences in terpene concentrations and antioxidant enzyme activities sometimes were unclear between treatments or time points.

Based on our observations we conclude that the three blue-stain fungi we studied are potentially pathogenic to Yunnan pine. The fact that *L. wushanense* and *L. sinense* elicited longer lesions and stronger monoterpene and antioxidant enzyme responses in Yunnan pine phloem than *O. canum* suggests that the two *Leptographium* species are more virulent to Yunnan pine than *O. canum*. So far, *L. wushanense* and *L. sinense* have only been isolated from restricted areas in SW China. However, they might be vectored by *Tomicus* beetles to Yunnan pine stands and cause serious damage in the future. Further studies are needed on their geographical distribution and pathogenicity mechanisms to better understand the risk they pose to Yunnan pine and to determine if they may facilitate tree-killing by *Tomicus* bark beetles.

AUTHOR CONTRIBUTIONS

HY and PC conceived the experiments. YP and JL designed and performed the experiments. Z-fY, MQ, HY, and PC contributed reagents, materials, and analysis tools. YP, TZ, and PK analyzed the data. TZ, YP, and PK wrote the paper. All authors revised the paper.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2018.01731/full#supplementary-material

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Terpene Synthase Genes in *Quercus robur* – Gene Characterization, Expression and Resulting Terpenes Due to Cockchafer Feeding

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Root herbivory caused by larvae of the forest cockchafer (*Melolontha hippocastani*) enhances the impact of drought on trees, particularly in oak forest rejuvenations. In Germany, geographically distant oak stands show differences in infestation strength by the forest cockchafer. While in Southwestern Germany this insect causes severe damage, oak forests in northern Germany are rarely infested. It is known that root-released volatile organic compounds (VOCs) are perceived by soil herbivores, thus guiding the larvae toward the host roots. In this work, we exposed seedlings of two distant oak provenances to forest cockchafer larvae and studied their population genetic properties, their root-based VOC chemotypes, their attraction for larvae and terpene synthase gene expression. Based on nuclear and chloroplast marker analysis, we found both oak populations to be genetically highly variable while showing typical patterns of migration from different refugial regions. However, no clear association between genetic constitution of the different provenances and the abundance of cockchafer populations on site was observed. In contrast to observations in the field, bioassays revealed a preference of the larvae for the northeastern oak provenance. The behavior of larvae was most likely related to root-released volatile terpenes and benzenoids since their composition and quantity differed between oak populations. We assume repellent effects of these compounds because the populations attractive to insects showed low abundance of these compounds. Five different oak terpene synthase (TPS) genes were identified at the genomic level which can be responsible for biosynthesis of the released terpenes. TPS gene expression patterns in response to larval feeding revealed geographic variation rather than genotypic variation. Our results support the assumption that root-released VOC are influencing the perception of roots by herbivores.

Keywords: root volatile, terpene synthase (TPS) gene, root herbivore, oak provenance, climate change

INTRODUCTION

Global climate change will most likely lead to higher mean annual air temperatures as well as seasonal and spatial changes of precipitation patterns (Forster et al., 2007; Spellmann et al., 2007; IPCC, 2014). In Central Europe, these changes will result in considerably reduced summer precipitation and, therefore, in a higher probability of drought stress in forests. It is assumed that drought periods will at least partially be accompanied by heat waves. In addition to such expected more frequent and severe abiotic stress events, which weaken the vitality of trees, there might be an enhanced risk for the occurrence of biotic stressors. For example, there are hints that climate change will cause an increased abundance of certain insect herbivores (Bolte et al., 2009; Delb, 2013). One of these insects is the forest cockchafer (*Melolontha hippocastani* Fabr.). The adult beetles of this species are consumers of tree and shrub leaves and are potentially able to migrate long distances while the larvae or "grubs" can move horizontally in the soil within a range of 1.5–5.5 m (Hasler, 1986; Weissteiner et al., 2012). Larvae have been reported to cause severe root damage to a range of economically important trees and in particular to oak (Delb, 2004; Huiting et al., 2006); it is assumed that due to global warming the reproduction cycle of this insect species will decrease from 5–6 years to only 3–4 years (Delb, 2013; Kolář et al., 2013), which will further enhance the frequency of biotic stress for their host tree species in the future.

In Central Europe, the oak species *Quercus robur* and *Q. petraea* belong to the main forest trees with enormous economic and ecological value. These oak species show a wide geographic distribution in Europe as well as in Germany and they are dominant tree species in many forests growing under a broad range of climatic and edaphic conditions (Gailing, 2008). *Q. robur* is the most widespread oak species in Europe and it preferentially grows on clay or acidic, deep and well-watered soils. Moreover, it is resistant to strong winter frosts and tolerant to high summer temperatures and high light exposure (Jones, 1959). Since the studies of Petit et al. (2002) with maternally inherited chloroplast DNA markers much is known about the genetic differentiation of oaks in Europe as a result of the recolonization from different glacial refugia. Further investigations (Gailing et al., 2003, 2007; König and Stauber, 2004; Liesbach et al., 2006; Neophytou et al., 2010, 2015; Hosius et al., 2012) draw a very detailed picture of genetic variation and differentiation of oak stands in Germany. These investigations underline the function of oak species as an excellent model species to study the adaptation of forest trees to changing environments (Gailing et al., 2009). However, these findings also highlight that the genetic background of oak populations has to be considered in field studies when interpreting their stress tolerance.

It is well known that aboveground plant parts emit a large variety of volatile organic compounds (VOCs) into the atmosphere. Terpenoids, including monoterpenes and sesquiterpenes, represent the largest and most diverse class of volatiles released by plants (Dudareva et al., 2006). These compounds exert multiple functions in the interaction of plants with their environment. For example, they serve as attractants for pollinators and fruit dispersers or play a role as repellents in the defense against herbivores and pathogens (Huang et al., 2010; Irmisch et al., 2014). The biosynthesis of terpenoids is mediated either by the chloroplastic MEP pathway or the cytosolic mevalonate pathway (Dudareva et al., 2006). These biochemical routes provide the C_5 compounds isopentenyl diphosphate and dimethylallyl diphosphate, which are used to synthesize the larger terpenes including monoterpenes (C_{10}) and sesquiterpenes (C_{15}). The final structure of terpenes is eventually formed by terpene synthases (Degenhardt et al., 2009).

Compared to aboveground plant parts, much less is known on the function of root-released VOCs. Nevertheless, specific information can be transferred over long distances in the soil by root-derived VOCs. For example, several studies indicate that they seem to be of significance for plant–fungi (Ditengou et al., 2015), plant–bacteria (Del Giudice et al., 2008) and plant–animal communication (Rasmann et al., 2005; Johnson and Gregory, 2006; Johnson and Nielsen, 2012). For example, Weissteiner et al. (2012) showed that root volatiles can be perceived by the olfactory system of cockchafer larvae and that this signal might be used by the animals for host plant location. Similarly, also other soil-inhabiting insects use root-released volatiles as a signal to orient themselves toward this food source (Nordenhem and Nordlander, 1994; Thomas et al., 2008). Interestingly, the blend of volatiles released by plant parts can change in response to herbivore attack because herbivore-induced plant volatiles (HIPVs) are synthesized (Robert et al., 2012; War et al., 2012). HIPVs are thought to be part of the plant's defense arsenal against plant feeding herbivores. Nevertheless, it has been demonstrated that soil-dwelling larvae were especially attracted to roots of plants which were infested with conspecifics (Robert et al., 2012).

In the present study we aimed to describe the interaction of *Q. robur* populations with *M. hippocastani* based on the release of VOCs from plant roots. Several studies have demonstrated that distant populations or "provenances" of a given tree species form specific blends of terpenes which can be released into the environment (Staudt et al., 2008; Loreto et al., 2009; Welter et al., 2012; Kleiber et al., 2017). However, a clear correlation between provenances of a species and terpene chemotypes might be blurred by underlying evolutionary forces like paleographic fragmentation or hybridization and introgression events (Staudt et al., 2008; Loreto et al., 2009; Welter et al., 2012). Still, intraspecific variability of blends of volatile terpenes (i.e., chemotypes) in oaks has rarely been studied (Staudt et al., 2001, 2008; Loreto et al., 2009; Welter et al., 2012). Interestingly, investigations between geographically distant oak stands in Germany have revealed clear differences in the extent of infestation by the forest cockchafer. While oak stands in Northeast Germany are rarely infested with cockchafer larvae (Subklew, 1937), comparable stands in Southwest Germany regularly experience severe damage by larval feeding, which can even put the survival of oak progeny into danger (Delb, 2004). In spite of that, some stands in close vicinity to severely damaged stands were observed to be far less affected. In the present work, we therefore characterized the genetic structure of *Q. robur* populations in Germany that showed different degrees of cockchafer infestation.

We tested the hypotheses (i) that different *Q. robur* populations from distant locations in Germany are genetically differentiated and that genetic differentiation is associated with infestation strength, and (ii) that these populations form distinct VOC chemotypes. We further postulate (iii) that the attraction of cockchafer larvae is different among these oak populations and (iv) that it is associated with the release of specific terpenes and with the expression of terpene synthase genes. We also assume that (vi) terpene synthase single nucleotide polymorphisms (SNPs) differentiate between oak populations with high and low cockchafer population density. Furthermore, we hypothesize that oak populations in northern Germany have evolved no tolerance to cockchafer feeding, since the insect has been absent from this region. Therefore, the response of such oak populations to herbivory should differ from populations regularly attacked by cockchafer larvae. For this purpose, we quantified the composition of volatile blends in response to cockchafer feeding and studied the expression of terpene synthase genes in roots of the oak trees.

MATERIALS AND METHODS

Site Characteristics and Plant Material

We selected tree individuals from a total of 19 pure and mixed oak stands in Germany (**Supplementary Table S1**). For population genotyping, leaf tissue was randomly sampled from at least 50 individuals per stand, either on site or directly from trees used in the experiments. For the experiments under controlled conditions, 3- to 5-year-old pedunculate oak (*Quercus robur* L.) seedlings of four populations were used. Two populations ("R36," "R45") originated from the Upper Rhine valley in Southwest Germany; two others ("BB1," "BB2") from the Federal State of Brandenburg in Northeast Germany. Either, they were grown in the greenhouse from seeds collected from a range of mother trees randomly distributed over the respective populations (SW German stands), or they originated from a local tree nursery (NE German stands). Consequently, none of the seedlings experienced cockchafer interaction prior to experiments and hence we could rule out any priming effects.

The major difference between those oak-dominated stands is their infestation strength. To the best of our knowledge, "BB1" and "BB2" have never experienced any infestation by the forest cockchafer, while population density is high in "R36" and low in "R45." Cockchafer infestation strength in the Baden-Württemberg stands was inferred from a long-term monitoring program which assesses cockchafer population density at an areal level (Delb and Mattes, 2001).

Insects

For the experiments, some 100 third instar larvae ("white grubs") of the forest cockchafer (*Melolontha hippocastani*) were used. The animals were collected from a field site in the Upper Rhine Valley (48°49′36.3″N 8°10′30.5″E) by digging to a depth up to 60 cm. The grubs were stored in natural soil from the field site at 8°C for a maximum of 1 week until they were used in the experiments.

Experimental Setups

Bioassays

To test if forest cockchafer grubs are particularly attracted by roots of specific *Q. robur* provenances, oaks of three provenances ("R36," "R45," "BB2") were tested against each other. Plants were grown for 3 months in 50 L plastic pots (diameter 52 cm)[1] with burnt quartz sand (Ø = 0.07–0.2 mm; 0.2–0.7 mm and 1.2–2 mm in relation 8:10:1) as a substrate, supplied with 4.5 g l^{-1} long term NPK fertilizer (Manna Cote 8M; Manna; Ammerbuch-Pfäffingen; Germany). The seedlings were grown under long day conditions (16 h light/8 h dark, PAR: 250–300 μmol m^{-2} s^{-1}). They were watered daily with tap water. Four trees (two per provenance) were planted crosswise into the pots, placing trees of different origins opposite to each other (**Supplementary Figure S1**). Each of the three possible combinations was replicated five times resulting in a total of 15 pots. For the bioassays, self-constructed glass tubing systems (inner diameter 45 mm, length of each tube 100 mm) were dug 20 cm deep into the center of each pot. The tubing system was equipped with (i) an opening in the center facing upward for entering the larvae and (ii) four exits facing the root systems of the four oak seedlings. The whole glass tubing system was filled with the same quartz sand substrate as the pots. To increase the possible number of independent replicates, two opposing exits were closed with an air-impermeable film for the first set of experiments; for a second set of bioassay experiments, the impermeable film was shifted to the remaining two opposing exits. The two open exits were closed with a net to hinder grubs from escaping the glass tubing system. For starting the bioassays, one grub was placed carefully into the middle entrance of the system. After a 12 h period the glass tubing system was removed from the soil and checked for the location of the grub. Each grub was only used once. In the course of the experiment, the root-released VOCs were collected in 10 cm depth in the rhizosphere of each single tree with passive samplers as described below. After each experiment, the substrate was completely removed from the tubing system, replaced by new substrate and placed again into the center of the pot.

Herbivory Effects

To study the effects of herbivory on the gene expression of terpene synthases and on the release of VOC by oak roots, a "feeding experiment" using populations "R45" and "BB1" was conducted. For this purpose, 12 seedlings per population were individually planted into 5.5 L plastic pots (AgrarPeter, Teningen, Germany) and grown for 8 months in the same substrate and under the same conditions as given above for the bioassays.

For the experiment, two forest cockchafer grubs were transferred to each pot. For each of the three provenances six pots were infested with grubs ($n = 6$) and six trees of each provenance were kept uninfested as controls. At the same time, we placed three passive samplers (details see below) for adsorption of root-released VOCs in 10 cm depth in each pot. After 8 days of

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[1] www.meyer-shop.com

infestation, the passive samplers were collected, carefully cleaned from soil particles and stored at 8°C until analysis of VOCs which was done within 1–2 days after collection.

Population Genetic Analysis: Microsatellite Genotyping With Chloroplast and Nuclear Single Sequence Repeats (cpSSR, nSSR)

In order to comprehensively characterize the genetic background of the plant material used for this study, we characterized the tree individuals from the different field sites in Germany at species-discriminating nuclear SSRs (Neophytou et al., 2010). Specifically, we used these markers for species assignments to make sure that only *Q. robur* trees were included in our experiments and to test whether genetic differentiation is associated with differences in infestation strength.

For population genotyping leave tissue was randomly sampled from at least 50 individuals for each of the 19 stands (**Supplementary Table S1**), either on site or directly from trees used in the experiments. Total DNA was extracted using the DNeasy 96 Plant Kit (Qiagen, Hilden, Germany). All samples were genotyped in six multiplexed PCR reactions amplifying the 11 nuclear SSRs QpZAG1/5, QpZAG9, QrZAG11, QpZAG16, QpZAG15, QrZAG30, QrZAG87, QrZAG96, QrZAG101, QpZAG110, QrZAG112 (Steinkellner et al., 1997; Kampfer et al., 1998) and the 10 chloroplast SSRs μkk4, μkk3, μcd5, μcd4, μdt4, μdt3, μdt1, ccmp10, ccmp6, ccmp2 (Weising and Gardner, 1999; Deguilloux et al., 2003) following the protocol of Neophytou et al. (2010). Capillary electrophoresis was performed in an ABI-PRISM-3130 Genetic Analyzer (Applied Biosystems) and the GeneMapper 4.0 Software (Applied Biosystems) was used for allele calling.

Population genetic analysis was performed in order to test for an association between genetic differences and differences in cockchafer abundance between populations. Each individual was assigned to one of the three oak species *Q. robur*, *Q. petraea* and *Q. pubescens* by cluster analysis in STRUCTURE v2.3.4 (Pritchard et al., 2000; Falush et al., 2003). Species-discriminant markers were used according to Neophytou et al. (2010) and four known pure stands (R36, V, F) served as reference. Only pure-bred *Q. robur* individuals ($q \geq 0.875$) were chosen for further experiments, resulting in 14 pure *Q. robur* stands consisting of more than 10 individuals. Genetic variation within and among the resulting *Q. robur* populations was calculated using the software GenAlEx 6.5 (Peakall and Smouse, 2006, 2012). The Bayesian approach in STRUCTURE was used to reveal genetic structures corresponding to provenances or cockchafer population density. The admixture model was applied and analysis parameters were set to 100,000 runs in the burn-in period, followed by 100,000 MCMC repetitions. K was set to 1–20 with 10 iterations per K. The highest ΔK was calculated based on the rate of change between successive K values (Evanno et al., 2005). Chlorotypes were defined according to Neophytou and Michiels (2013). The refugial ancestry of the original seed material was inferred by comparison with the corresponding refugial linages (Petit et al., 2002). Chlorotype frequency within and genetic variation among all populations were calculated in GenAlEx.

Identification of Genomic Terpene Synthase Genes by PCR

The orthologous sequences of several terpene synthase genes of *Populus trichocarpa* available in the EMBL database were used to find corresponding sequences by a BLAST search in the *Q. robur* transcriptome database publicly available at https://urgi.versailles.inra.fr/blast/blast.php and with a BLASTx search of the translated nucleotide sequences within the NCBI BLAST+ database (**Supplementary Table S2**). Primers (**Supplementary Table S3**) were designed by using the program Primer 3 (Koressaar and Remm, 2007; Untergasser et al., 2012; from S. Rozen & Whitehead Institute/MIT Center for Genome Research)[2]. Primers were quality checked using the program Oligo calc: Oligonucleotide Properties Calculator[3]. PCR amplification was performed in a 25 μl volume containing 10 ng template DNA, 10 mM Tris/HCl pH 9.0, 0.2 mM of each dNTP, 1.5 mM $MgCl_2$, 50 mM KCl, 0.2 mM each of forward and reverse primer and 1 unit Taq polymerase (Qiagen, Hot Star Master Mix, Hilden, Germany). All amplifications were performed in a Peltier Thermal Cycler (PTC-0200 version 4.0, MJ Research) under the following conditions: an initial denaturation at 95°C for 15 min, followed by 35 cycles of 1 min at 94°C, a 45 s annealing step at 53°C, a 1 min extension step at 72°C, with a final 10 min at 72°C. The PCR products were cloned into a pCR2.1 vector using the TOPO TA cloning® kit (Invitrogen, Carlsbad, CA, United States). The inserts were amplified with colony PCR using M13 forward and reverse primers, and the sequencing reaction was carried out with the Big Dye® Terminator v3.1. cycle sequencing kit (Applied Biosystems, Darmstadt, Germany). Sequencing reactions were run on an ABI 3100 genetic analyzer (Applied Biosystems). Three to six different clones of the fragments were sequenced using both M13 forward and M13 reverse primers in order to identify the presence of different haplotypes within individuals (heterozygotes) and to control for Taq polymerase sequencing errors. The cloned and sequenced fragments were identified with a TBLASTX search, in order to analyze their structural features (exons and introns) and their nucleotide diversity in the different oak samples. In case of TPS1 and TPS6 the sequences were divided into two fragments since the overlap of 10 bp between the sequences generated by the forward and reverse primers was not sufficient.

Analysis of Sequences

The allelic variation of the terpene synthase genes was analyzed by comparative sequencing of the samples used in the herbivory experiment and additional eight individuals of each population. Thus, populations “R45” and “BB1” were each represented by up to 12 individuals and each individual was represented by two haplotypes (**Table 1**). For editing and visual inspection of the sequences, as well as for the analysis of single nucleotide polymorphisms (SNPs) and indels (insertions/deletions) within

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[2] http://primer3.ut.ee/

[3] http://www.basic.northwestern.edu/biotools/oligocalc.html

TABLE 1 | Allelic variation of the terpene synthase genes in the populations "BB1" and "R45."

Gene	*N*	bp	SNP	*h*	Hd	π	Tajima's *D*
*TPS*1 fr1	46	527	30	21	0.951	0.0106	−0.672
*TPS*1 fr2	46	611	30	22	0.961	0.0081	−0.922
*TPS*6 fr1	46	640	72	36	0.986	0.0220	−0.523
*TPS*6 fr2	46	592	25	19	0.929	0.0070	−0.890
*TPS*7 fr1	44	626	65	37	0.993	0.0318	1.161
*TPS*12	46	1062	50	36	0.989	0.0128	0.674
*TPS*13	42	1114	45	31	0.976	0.0062	−1.202

N, sample size; bp, B = length in base pairs; SNP, number of SNPs (without singletons); h, number of haplotypes; Hd, haplotype-diversity; π, nucleotide diversity (per site); Tajima's D, neutrality test.

the gene, the sequences were aligned with Codon Code Aligner (Codon- Code cooperation)[4] and BioEdit version 7.0.0 (Hall, 1999) using clustalw multiple alignment (Thompson et al., 1994). Only polymorphisms with Phred scores above 25 in the chromatograms were considered. For estimating the standard population genetic parameters (e.g., number of segregating sites S, nucleotide diversity p, population differentiation F_{ST}), DnaSP 5 (Rozas et al., 2003) was used. The number of Single Nucleotide Polymorphisms (SNPs), nucleotide diversity (π) and allelic variation measured as haplotype diversity (Hd) are summarized in **Table 1**. Tajima's *D* neutrality test was used in order to find alleles or SNPs under selection. Negative values are usually interpreted as a sign of negative or purifying selection, while positive values are a signature of positive, balancing or diversifying selection. Singletons were excluded from all analyses.

Gene Expression Analysis by Quantitative Real-Time Polymerase Chain Reaction

RNA was extracted from roots ($N = 6$) of infested and non-infested plants of populations "BB1" and "R45" according to Le Provost et al. (2007). RNA (15 mg) from each sample was treated with DNase (RNasefree) at 37°C for 30 min to eliminate contamination with genomic DNA, then extracted with phenol/$CHCl_3$ (1:1) and $CHCl_3$ and precipitated overnight at −80°C with NaAc 1:10 and 2.5 volumes of absolute ethanol. cDNA was synthesized from 1 μg of the treated RNA samples using oligo(dT) primers and Superscript III reverse transcriptase (Invitrogen) at 50°C overnight. Based on the genomic sequences of the terpene synthase genes and of Actin, primers for quantitative real-time PCR were designed within the exon regions of the identified genes (**Supplementary Table S3**) using primer 3 (see above) and checked by PCR with genomic DNA as template. Quantitative real-time PCR was carried out on a LightCycler 480 (Roche) and monitored with SYBR-Green I dye (Roche). Melting curve analysis was performed for each primer pair by default and did not indicate off-target sequencing. Triplicates of 10 μl PCR reactions of each sample were done. Relative expression levels were analyzed using the $\Delta\Delta$cp method (Livak, 1997, 2001; Livak and Schmittgen, 2001). The relative expression of the terpene synthase genes was normalized with the expression of the reference gene Actin in order to compensate for inter-PCR variations between the runs.

VOC Analyses

To identify the VOCs released from oak roots (herbivory and bioassay experiments), we used self-made passive samplers as originally described by Kallenbach et al. (2014) which were furthermore tested particularly for their suitability for soil systems (Eilers et al., 2015). For this purpose, PDMS tubing (inner diameter 1.0 mm, outer diameter 1.8 mm, Roth, Germany) was cut into 5 mm long pieces and subsequently washed exactly as given by Kallenbach et al. (2014). For conditioning, passive samplers were placed in empty thermodesorption tubes (Gerstel, Germany) which were heated to 200°C under a N_2 flux of 30–40 ml min^{-1} (N_2 5.0, Messer, Germany) in a Gerstel tube conditioner unit (Gerstel, Germany) for 6 h.

To test the suitability of such passive samplers for our purposes, a preliminary experiment was conducted in which the recovery rates of the passive samplers were characterized. We placed a piece of quartz wool spiked with authentic standards of the monoterpene (R)-(+)-limonene (123 nmol), the oxygenated monoterpenoids linalool (111 nmol) and the sesquiterpene β-farnesene (79 nmol) in the center of the same pots ($n = 4$) as used for plant experiments. Passive samplers were placed in a circle around this VOC source. After 7 days of exposure, the passive samplers were removed from the soil and analyzed as described above. It became evident, that all terpenes present in the source were detectable with our system (**Supplementary Figure S2a**). However, this experiment also indicated that the efficiency to trap a compound strongly depends on its chemical properties. In a second approach, we tested the linearity of trapping different amounts of volatiles. For this purpose, passive samplers were placed in solutions containing different amounts of limonene, linalool and farnesene. Importantly, we observed a linear correlation between the amount of the compound present in the solution and the compound trapped on the passive sampler (**Supplementary Figure S2b**).

The volatiles adsorbed on the passive samplers were analyzed by GC-MS (GC 6890, MS 5975C, both Agilent Technologies, Waldbronn, Germany) equipped with a thermodesorption unit (TDU, Gerstel, Müllheim an der Ruhr, Germany) coupled

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[4] https://www.codoncode.com

with a cold injection system (CIS, Gerstel, Müllheim an der Ruhr, Germany) (Eilers et al., 2015) For this purpose, the thermodesorption tubes containing passive samplers were inserted into the TDU by a Multipurpose Sampler (MPS 2, Gerstel, Müllheim an der Ruhr, Germany). After thermodesorption of analytes at 220°C for 4 min, analytes were cryofocussed in the CIS at −50°C. After heating up the CIS to 240°C for 3 min, analytes were flushed onto the separation column (DB-5MS Ui, 30 m, 0.25 mm ID, 250 μm, Agilent Technologies, Waldbronn, Germany). Details of GC and MS settings are provided by Kleiber et al. (2017). Raw data of GC-MS analysis were further processed by the MassHunter Quantitation software (Agilent, Waldbronn, Germany). Calibration was realized by using terpene standards, which were solved in hexane at different concentrations ranging from 0 to 20 nmol μl^{-1}.

Statistical Analysis

All statistical analyses were performed using Origin 2018G (OriginLab Corporation, Northampton, MA, United States). For the comparison of two groups the non-parametric Mann–Whitney *U* test was conducted (with significance level from $p < 0.01$–0.001) and to check more than two groups against each other we used the non-parametric Kruskal–Wallis ANOVA ($p < 0.05$). Gene expression and volatile data were log transformed before they were further analyzed. Differences between the volatile pattern released by roots of different origins were highlighted using a multivariate partial least squares discriminant analysis (PLS-DA) in MetaboAnalyst 4.0[5] (Xia et al., 2015).

In order to calculate putative associations between genetic difference and infestation strength, we performed a Mantel test in GenAlEx (Peakall and Smouse, 2006). To do so, a dissimilarity matrix was created between the 14 *Q. robur* populations. Infestation strength was defined by a yes/no decision of infestation absent (0) and infestation present (1), corresponding to a low/not relevant and high cockchafer population density, respectively (**Supplementary Table S1**). When performing the Mantel test, cockchafer condition was correlated with unbiased Nei's genetic distances using 999 permutations.

RESULTS

Population Genetic Analysis With Nuclear and Chloroplast SSRs

The genetic characterization of the studied populations based on the nSSRs showed a clear differentiation between the different species *Q. robur, Q. petraea, Q. pubescens* present at the different field sites (**Supplementary Figure S3**). Importantly, for further studies only pure-bred *Q. robur* individuals were selected.

The molecular variation among the 14 *Q. robur* populations was relatively low (2%, data not shown) and no strong differentiation between the provenances was observed if tested by PCA (**Supplementary Figure S4**). Only populations "BB2" and "R36" differed from the others (**Supplementary Figure S4**). Also, no correlation between geographic and genetic distance was observed (Mantel test, $R^2 = 0.0277$). Similarly, no association between Nei's unbiased genetic distance and the presence of infestation could be detected in a Mantel test ($R^2 = 0.0014$).

In accordance with the results reported by Neophytou et al. (2010), all investigated nuclear SSRs were highly polymorphic with a total number of alleles ranging from 9.09 in "SA4" to 18.91 in "R36" (**Supplementary Table S4**). High allelic diversity and heterozygosity were observed in our samples. Overall unbiased expected heterozygosity varied between 0.802 in population "BB2" and 0.831 in population "BY1."

Chloroplast analyses were performed to test for autochthony of stands, since different susceptibility to cockchafer infestation could be associated with different geographic origins. All analyzed chloroplast microsatellite loci were variable possessing two to four alleles each, except for μkk3 which was monomorphic. In total, nine chlorotypes could be defined by combining the different genetic variants at each locus (**Supplementary Table S5**). For the purpose of comparability, chlorotypes were assigned to those described earlier (Petit et al., 2002; Neophytou and Michiels, 2013) (**Supplementary Table S5**). The most commonly found chlorotypes were chlorotypes 9 and 2, corresponding to chlorotype 9 (Lineage C, Italian) and chlorotype 6 (Lineage A, Balkan) as observed by Neophytou and Michiels (2013), respectively. Spatial distribution of the chlorotypes differed among populations, with those in the Upper Rhine Valley (Baden-Württemberg) being the most variable comprising chlorotypes from all lineages (Balkan, Iberian, Italian). Two populations in Bavaria ("BY1" and "BY2") and three in Saxony-Anhalt showed only one chlorotype. While the Iberian lineage was absent from the populations of Bavaria, Brandenburg and Saxony-Anhalt, chlorotype 4 (Lineage A, Balkan) was present only in one population in Saxony-Anhalt (**Figure 1**). However, the analysis of the cpSSRs did not detect a correlation between Nei's unbiased genetic distance and the presence of infestations in the populations (Mantel test, $R^2 = 0.0238$).

Oak Populations Are Differently Attractive for Cockchafer Grubs

In an experiment under controlled conditions ("olfactory bioassay"), we tested if roots of the different oak populations from distant sites in Germany (Northeast Germany vs. Southwest Germany) are differently attractive to cockchafer larvae. The bioassays clearly indicated that trees from population "BB2" (NE Germany) were significantly more attractive to cockchafer larvae than trees from the SW German population "R45" (**Figure 2**). Interestingly, the reactions of the insects were comparable when the populations "R36" and "R45" from SW Germany were tested against each other in the bioassay. Similarly, there was no preference when "R36" was compared to "BB2."

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[5] http://www.metaboanalyst.ca

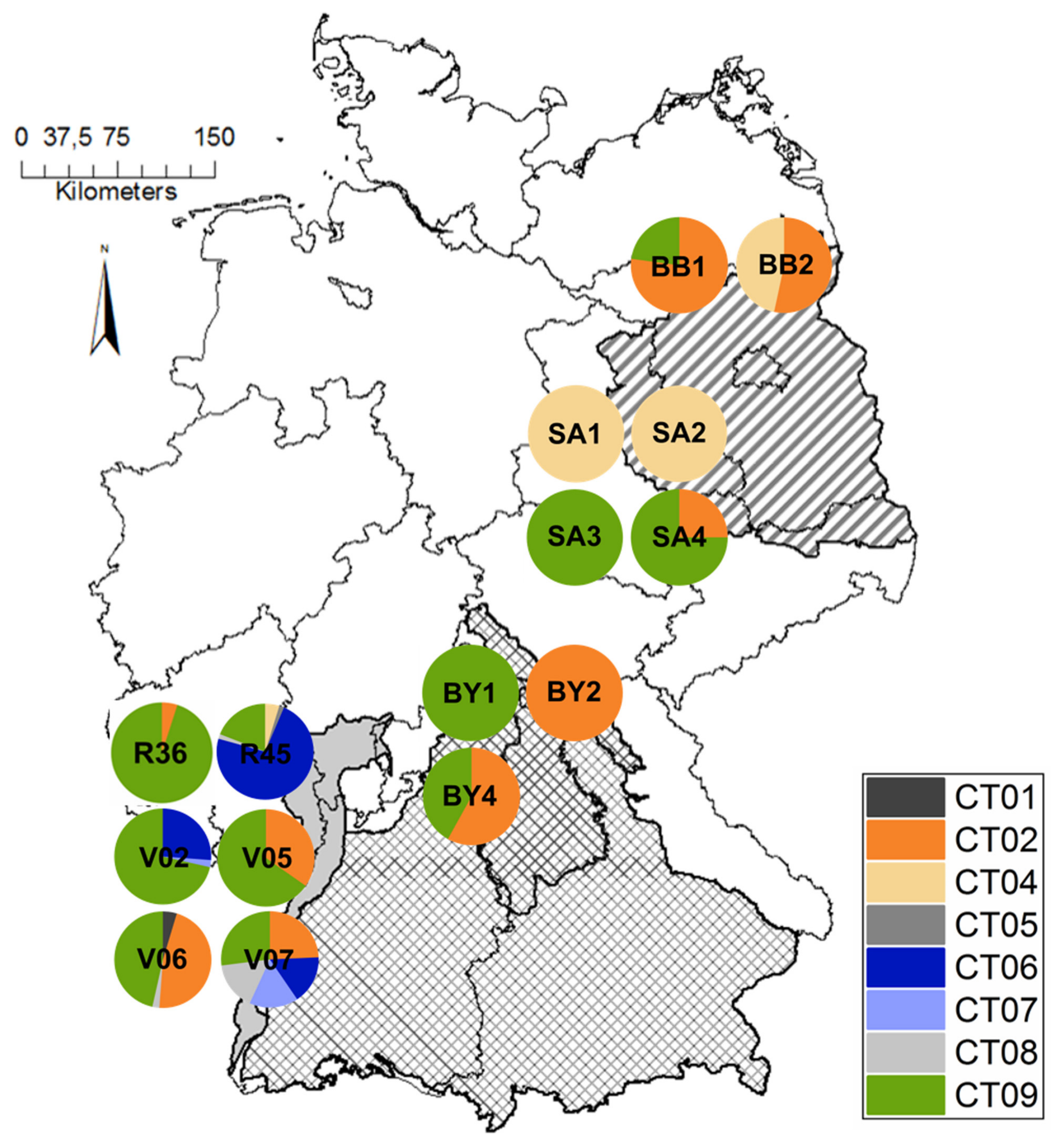

FIGURE 1 | Distribution of found chlorotypes (CT) in *Q. robur* individuals within sampled populations in Baden-Württemberg (R36, R45, V02, V05, V06, V07), Bavaria (BY1, BY2, BY4), Saxony-Anhalt (SA1, SA2, SA3, SA4) and Brandenburg (BB1, BB2). The three relevant German *Q. robur* provenances 'Oberrheingraben' (81707, gray uni), 'Süddeutsches Hügel- und Bergland sowie Alpen' (81709, checkered) and 'Ostdeutsches Tiefland' (81704, lined) are highlighted. The map of Germany is based on a map from Esri Germany (https://opendata-esri-de.opendata.arcgis.com/).

Oak Populations Differ in Volatiles Released by Roots

To test the hypothesis that different blends of root-released volatiles are responsible for the observed different preferences of the insects, the volatiles present in the soil of the trees were analyzed (**Figure 3** and **Supplementary Table S6**). We observed that oak trees originating from different stands specifically affect the abundance of certain volatiles in their substrate. This

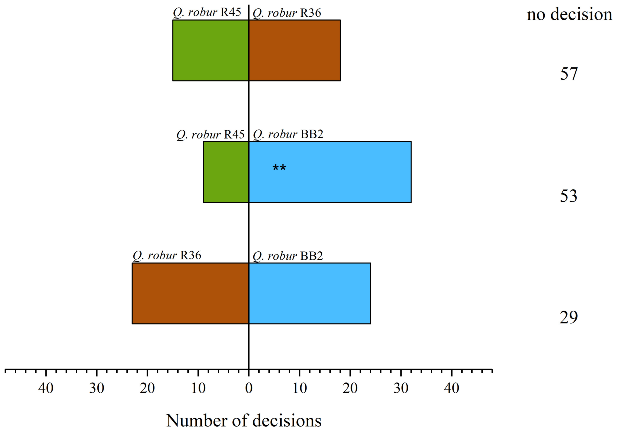

FIGURE 2 | Olfactory bioassay with *M. hippocastani* larvae and three *Q. robur* populations. Two trees, one of two populations each, were tested against each other. Each of the three possible combinations was planted 10 times. Individual larvae were placed in the central inlet of the bioassay system for 12 h. Data are total numbers of animals selecting specific trees, or with no decision. Statistically significant differences were calculated with the Wilcoxon rank sum test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

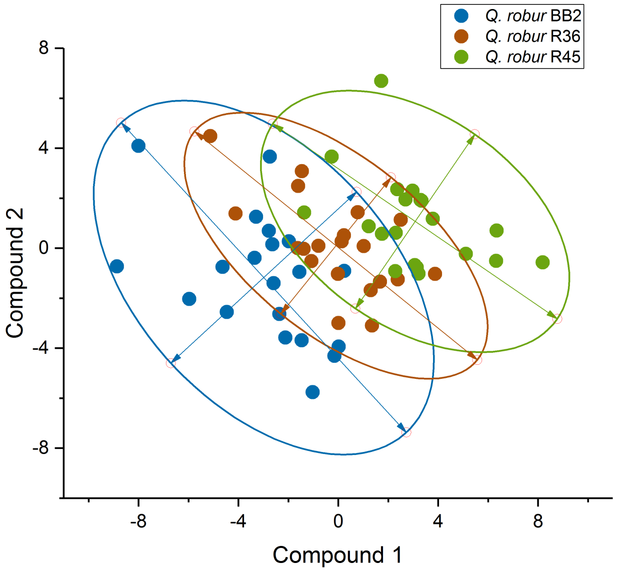

FIGURE 3 | PLS-DA of the VOC patterns in the soil of three different *Q. robur* provenances ($N = 20$). The trees were kept for 3 months on the identical soil substrate and were used for the bioassays (see **Figure 2**). During the bioassays, volatiles abundant in the soil were collected on passive samplers and analyzed by GC-MS. Ellipses indicate the 95% confidence level.

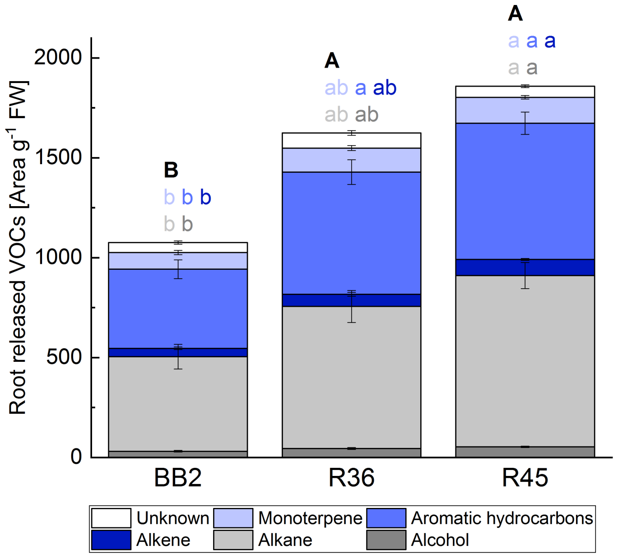

FIGURE 4 | Root-released VOCs from trees of three *Q. robur* populations ($N = 20$). Data shown in **Supplementary Table S6** were summarized according to main compound classes. Statistically significant differences between populations were calculated by Kruskal–Wallis-ANOVA ($p < 0.05$) for each compound class and are indicated by different letters (individual colures represent the different compound classes).

became obvious when classifying the volatiles into different compound classes (**Figure 4**). Particularly, populations “R45” and “BB2” showed clear differences, whereas “R36” and “R45” did not greatly differ in their composition of volatiles. This pattern correlated with the preferences of the cockchafer larvae in the olfactory bioassay (**Figure 2**). Interestingly, many of the compounds differing in abundance between “BB2” and “R45” belonged to the classes of benzenoids and terpenes (**Supplementary Table S6**).

Identification of Oak Terpene Synthase Genes and Their Structural Features

We amplified, cloned, sequenced and identified five different oak terpene synthase genes at the genomic level. They represent partial gene sequences and their structural features are described in **Table 2**. Following the nomenclature of Irmisch et al. (2014), *TPS*1 and *TPS*7 represent sesquiterpene synthases, whereas *TPS*6, *TPS*12 and *TPS*13 represent monoterpene synthases. At the genomic level they consisted of 1050–1450 bp, containing three to four introns and three to four exons (**Table 2**); they showed typical conserved elements like the DDxxD and the NSE/DTE motifs (data not shown). In genes *TPS*1, *TPS*6 and *TPS*12 also alternative splicing sites were found belonging to the class of U12 Introns (Lewandowska et al., 2004).

Differentiation of the Terpene Synthase Genes Analyzed by Comparative Sequencing of Individuals of the Populations “BB1” and “R45”

In general, a high total nucleotide diversity (π) was found in all analyzed *TPS* genes ranging from 0.0062 to 0.0318, but no variation was found in the conserved motifs DDxxD and DxxDD. The genetic differentiation between populations, F_{ST}, was calculated according to Wright (1951). Depending on the gene, a differentiation of up to 12.5% (*TPS*1) was found

TABLE 2 | Structural features of the identified oak terpene synthase genes in the populations "BB1" and "R45."

	Intron	Exon 1	Intron	Exon 2	Intron	Exon 3	Intron	Exon4	Intron
TPS 1		1–192	193–321*	322–417	418–596	597–845	846–946	947–1237	
TPS 6	1–37	38–250	251–338	339–716	717–933*	934–1161	1162–1234	1235–1345	1346–1385
TPS 7	1–221	222–581	582–671	671–899	890–1225	1226–1363			
TPS 12		1–42	43–152	153–398	399–516	517–807	808–1055*		
TPS 13		1–141	142–252	253–501	502–723	724–1026			

**Represents alternative splicing sites AT/AC instead of GT/AG.*

because some alleles were found in population "BB1" but not in population "R45." However, *TPS*12 showed no differentiation between the two populations. Tajima's *D* values did not differ significantly.

Expression Rates of the Terpene Synthase Genes in Roots of Tree Samples of the Stands "BB1" and "R45" After Feeding

In a controlled herbivory experiment, cockchafer grubs were allowed to feed on roots of oak seedling of different populations ("BB1," "R45"). The RT-qPCR analysis showed distinct differences in the ΔCt values ($Ct_{TPS} - Ct_{house-keepinggene}$) and therewith in the relative expression rate as analyzed as $2^{-\Delta\Delta Ct}$ for the identified TPS genes (**Figure 5**). In roots, while relative gene expression varied between individuals, the relative expression levels of all identified TPS genes generally increased in response to feeding. The expression ratio was generally higher in provenance "BB1" where upregulation after larval feeding was significant for *TPS*1, *TPS*6, and *TPS*12 (**Figure 5**).

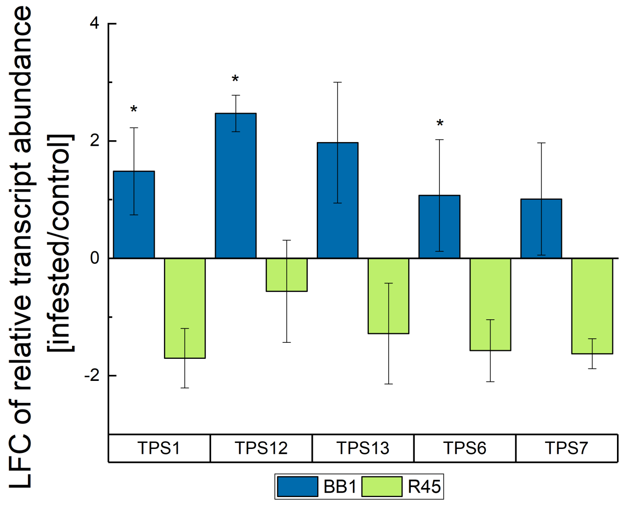

FIGURE 5 | Effects of larvae-induced root damage on the expression of terpene synthase genes in roots ($N = 6$) of trees of two *Q. robur* populations ("BB1" and "R45"). Displayed are the means ± SE of the log2 values of the fold-changes (LFC) of relative gene expression between infested and control plants. Statistically significant differences between populations were calculated by Mann–Whitney *U* test for each TPS and are indicated by *$p < 0.05$.

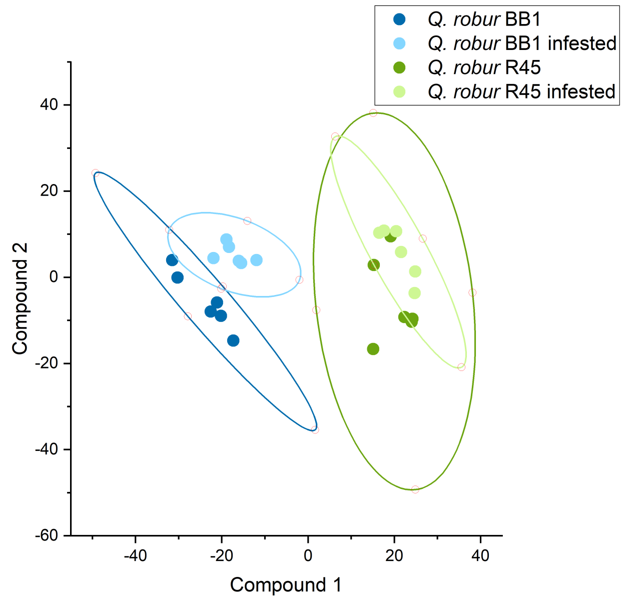

FIGURE 6 | PLS-DA of root-released VOC patterns of two different *Q. robur* populations ($N = 6$). Six trees of each population were infested for 7 days by two cockchafer grubs each. Volatiles in the soil were trapped by passive samplers which were placed in the soil during the course of the experiment. Ellipses reflect the 95% confidence levels.

Herbivory Affects Terpene Blends

We investigated the effect of root herbivory on the release of VOCs from the roots of the non-infested site "BB1" in NE Germany and "R45" from SW Germany, which revealed the strongest differences in VOC release to the control trees (**Figures 3**, **4**) and the largest differences in the interaction with cockchafer larvae in the bioassay (**Figures 2**, **6** and **Supplementary Table S7**). Trees of both populations showed changes in the release of VOCs from roots after infestation by cockchafer grubs. The differences between non-infested controls and cockchafer-infested plants were most prominent in population "BB1" where many compounds showed an altered pattern of VOC released into the soil (**Figure 6**). Among the compounds with increased levels in response to root herbivory were several benzenoids but also many compounds of other chemical classes (**Supplementary Table S7**). Roots of population

"R45" also responded to exposure to cockchafer larvae but the effects were less pronounced than the reactions of "BB1."

DISCUSSION

In the present study, we performed experiments under controlled conditions in which we tested the attraction of soil-dwelling cockchafer larvae by the root system of *Q. robur* trees from different populations. We compared trees from non-infested sites of NE Germany (populations "BB1" and "BB2") with trees from a region in SW Germany where the forest cockchafer in general is quite abundant (populations "R36" and "R45"). However, despite their proximity, stand "R36" is highly infested, whereas stand "R45" is far less infested by cockchafer larvae as assessed by a long-term monitoring program (Delb and Mattes, 2001; Delb, 2004). Therefore, the question arises if these differences are caused by environmental factors or rather by plant internal factors such as the genetic constitution resulting in different physiological responses.

When compared in the olfactory bioassays, the cockchafer larvae preferentially selected trees from NE Germany ("BB2") compared to population "R45" from SW Germany. This result was unexpected because of the low natural abundance of the cockchafer in stand "BB2" but the general high presence of this insect in SW Germany. We therefore speculate that oak trees from SW Germany evolved some kind of adaptation to the occurrence of the forest cockchafer in this region including, e.g., a repellent effect of the roots mediated by root-released volatiles. Since the forest cockchafer is not present in NE Germany, such a defense mechanism against the larvae might not have evolved in local oak provenances.

Due to the design of our bioassays, only volatile signals released by the root systems of the experimental trees could have determined the choice of the animals. The olfactory detection system of cockchafer larvae has been studied intensively and it was demonstrated that the chemosensory capacities of the larvae of this species are well developed (Eilers et al., 2012). Electroantennograms and electropalpograms indicated that cockchafer larvae are able to perceive a broad range of volatiles including alcohols, acids, amines, esters, aldehydes, ketones and monoterpenes (Eilers et al., 2012). Such compounds can be attractive or repellent to soil-dwelling insects (Johnson and Gregory, 2006). Movement toward a tree of population "BB2" in the present experiments can therefore be caused by perception of an attractant released by the root system or a repellent from the opposing tree (i.e., a tree of population "R45"). Considering the low natural rates of infestation at stand "R45" despite the high abundance of the forest cockchafer in this region, we hypothesize that "R45" releases trace gasses into the soil which act as repellents, most likely contributing to the constitutive defense against herbivore attacks. This assumption is in accordance with the observed distinct differences in the volatile scent released by roots of the three *Q. robur* populations (**Figure 3** and **Supplementary Table S6**). In good agreement with the results of the bioassays, populations "R45" and "BB2" showed the strongest differences in the abundance of volatile compounds in the soil, whereas only small differences were identified between the two SW German populations on the one hand and between "R36" and "BB2" on the other hand.

The volatile compounds released by the root system can provide hints for such assumed repellent properties. The very low levels of VOCs in the soil of "BB2" might indicate the absence of repellent compounds, leading to the preferential choice by cockchafer larvae in the bioassays. Notably, trees of population "R45" in general released much higher amounts of a large variety of different compounds such as aromatics, terpenes as well as alkenes and alkanes than trees of "BB2" (**Figure 4**). Among the aromatics, we identified several compounds which have been discussed to be involved in plant defense. For example, the benzenoid benzothiazole, which was more abundant in the soil of "R45" than of "BB2," seems to be released during induced defense in response to wounding (Bruin et al., 1992; Ping et al., 2001; Mithöfer et al., 2005; Hu et al., 2009) and was suggested as a volatile signal mediating plant–plant communication (Hu et al., 2009). Similarly, benzyl alcohol which is involved in defense against biotic stress (Widhalm and Dudareva, 2015; Szewczyk et al., 2016; Wang et al., 2016) was more abundant in the soil of "R45" than in "BB2." Deterrent effects against herbivores have also been ascribed to members of the chemical class of terpenes (War et al., 2012). Among the 7 terpenoids identified in the soil of the trees (α-pinene, camphene, δ-3-carene, limonene, sabinene, *p*-cymene, isomenthol; **Supplementary Table S6**), the contents of δ-3-carene, the aromatic monoterpenoid *p*-cymene and the oxygenated compound isomenthol were significantly higher in the soil of "R45" than of "BB2." Eilers et al. (2012) demonstrated that from the compounds present in the soil of our oak trees, at least α-pinene, limonene and camphene can be perceived by the cockchafer larvae. The monoterpenes α-pinene and camphene, which did not show significant differences in abundance between "BB2" and "R45," exerted attractive effects on larvae of the common cockchafer (Eilers, 2012). Unfortunately, information on the perception and effects of δ-3-carene, *p*-cymene and isomenthol is lacking, and should be in the focus of future work.

It is well understood that herbivory induces a plethora of plant defense mechanisms at different levels including numerous plant secondary compounds (Walling, 2000; War et al., 2012). Induced defense often includes the release of herbivore-induced plant volatiles (HIPVs) (Robert et al., 2012). HIPVs are non-polar, volatile compounds which are released either into the atmosphere mainly from leaves, or into the soil from the roots in response to herbivore attack (Dudareva et al., 2006, 2013). These gasses can directly impair the animal's vitality, act as feeding deterrent, or indirectly attract predators of the herbivore in tritrophic interactions (Farag and Paré, 2002; Dudareva et al., 2006; Arimura et al., 2009; Dicke and Baldwin, 2010; War et al., 2012; Kaling et al., 2018). As important part of a plant's defense arsenal against herbivore attack we therefore aimed at clarifying if *Q. robur* trees of different populations differ in their response to infestation by cockchafer larvae. Because of the clear differences between the NE German population "BB2" and the SW German population "R45" in the bioassays (**Figure 2**) and in the release of volatiles from the roots system (**Figures 3**, **4**), we included two populations from these origins in the analysis on the effects of root herbivory. Due to the lack of trees from "BB2," in this experiment we had to work with "BB1" which originates in close vicinity to "BB2" with assumed very similar properties. Indeed, trees of population "BB1" showed clear effects on the VOC release after 8 days of cockchafer infestation whereas trees of "R45" did not show such strong reactions as indicated by multivariate analysis (**Figure 6**). Interestingly, the release of some volatile compounds decreased in response to exposure to the herbivore. In "BB1" this was most pronounced for undecylcyclopentane, isopropyl myristate, heptadecane and propanetrial triacetate. In contrast, roots of "R45" released lower amounts of mainly β-pinene, trimethylbenzene, and cyclododecane in response to herbivory (**Supplementary Table S7**). There is not much information on these compounds in the literature, although emission of some of them has been reported for other plants. Isopropyl myristate, trimethylbenzene, and heptadecane, for example, are emitted by several plant species, but their functions are not known yet (Thompson and Gornall, 1995; Weissteiner, 2010; Yang et al., 2014; Martins et al., 2017). According to the electrophysiological studies of Eilers et al. (2012), the common cockchafer is not able to perceive β-pinene so that lower emission of this compound cannot be related to the interaction with this herbivore, supposing that the forest cockchafer used in the present study has similar capabilities.

However, we also observed elevated abundance of several compounds in the soil of infested trees of "BB1." It was obvious that the abundance of some aromatic compounds strongly increased. Several studies reported that herbivory on aboveground plant parts activates the jasmonic acid-induced phenylpropanoid pathway, presenting an important defense reaction of plants against biotic stress (Kazan and Manners, 2008; Rasmann and Agrawal, 2008; Lanoue et al., 2010). The present study provides hints that similar processes also occur in belowground parts of oak trees. The observed reactions in *Q. robur* roots might therefore be interpreted as an induced stress defense response in this tree species. Accordingly, some of the compounds showing elevated abundance have been reported to fulfill defense functions in other species. As mentioned above, benzenoids such as benzaldehyde, benzyl alcohol, and benzothiazole most likely play a role in defense against biotic stress (Bruin et al., 1992; Ping et al., 2001; Mithöfer et al., 2005; Hu et al., 2009; Widhalm and Dudareva, 2015; Szewczyk et al., 2016; Wang et al., 2016). Similarly, 1,2,3-trimethylbenzene was abundant in approximately 2-fold higher amounts in the soil of infested "BB1" compared to controls. This result is consistent with increased emission rates of this volatile from tropical plants infested by leaf herbivores (Martins et al., 2017). In good agreement with our results, these authors also observed higher release of the aldehydes nonanal and decanal, which therefore also seem to be involved in biotic stress defense. Moreover, in our study the abundance of the alcohol 2-ethyl-1-hexanol was significantly increased in the soil of infested trees of population "BB1." This compound has been shown to be released from leaves of the tea plant in response to treatment with the plant hormone methyl jasmonate which acts as an efficient elicitor of secondary metabolite production under biotic stress (Shi et al., 2015). The emission of similar compounds from the roots of apple trees was also induced by the forest cockchafer (Abraham et al., 2015). Consistent with the work of Eilers et al. (2012), we also observed increased emission of 2-ethyl-1-hexanol in oak roots damaged by cockchafer larvae. In addition, Eilers et al. (2012) observed enhanced emissions of 1-octen-3-ol, anisol, octan-3-one and the oxygenated monoterpene eucalyptol in response to infestation of oak roots. However, none of these compounds were detected in our study which might be caused by the different sampling techniques used in both studies. Such compounds might have been adsorbed by soil particles and, thus, did not reach our passive samplers (Ramirez et al., 2010) or the efficiency of the samplers was too low for such compounds. Since our test experiments indicated that a broad range of compounds with different polarities was efficiently trapped by the samplers, the former assumption seems to be more likely.

Among the compounds abundant in higher amounts in the soil of infested trees were also some terpenes (**Supplementary Table S7**). Increased emission of terpenes has often been observed in herbivore-infested plants (Unsicker et al., 2009; Dudareva et al., 2013; Martins et al., 2017). Terpene release is assumed to protect the roots by their antimicrobial and antiherbivore properties. In tritrophic interactions, they can also attract natural enemies of the root feeding insect pests. For example, the sesquiterpene β-caryophyllene which is synthesized in corn roots due to infestation by the larvae of *Diabrotica virgifera* attracts the nematode *H. megidi* (Rasmann et al., 2005). However, sesquiterpenes were not detected in the soil during our studies which might be due to the short half-life time of these compounds. Nevertheless, strong hints for stimulated production of mono- and sesquiterpenes is provided from our gene expression studies where an upregulation of several terpene synthases occurred in "BB1" in response to infestation which was not the case for "R45." This finding is in good agreement with an increased release of β-pinene and δ-3-carene from roots of infested of "BB1." We propose that one of the assumed monoterpene synthases TPS13, TPS12, or TPS6 is responsible for the biosynthesis of these compounds. Other studies identified monoterpenes like myrcene (TPS13), terpinolene (TPS12 and TPS13), linalool (TPS12), and ocimene (TPS6) as the natural products of these genes. These terpene products have also been described as typical stress monoterpenes in green leaves of *Q. robur* after infestation with oak powdery mildew (Copolovici et al., 2014). The sesquiterpene synthases (TPS1 and TPS7) use farnesyl diphosphate (FPP) as substrate and the larger carbon skeleton of FPP increases the structural diversity of the products. In poplar TPS1 is known as germacrene D synthase and as a multiproduct enzyme (Eberl et al., 2017); also, TPS7 can produce more than 15 different sesquiterpenes with elemol being the main product (Irmisch et al., 2014). As mentioned above, we identified other terpenes in control trees of the different populations (**Supplementary Table S6**) which might be the products of the TPS of the *Q. robur* trees studied here. Therefore, one can assume that the allelic variation and also the alternative splicing sites found in three (*TPS*1, *TPS*6, and *TPS*12) of the identified TPS genes contribute to the terpene diversity. Similar results were also described by Huang et al. (2010) who analyzed the herbivore-induced volatile terpenes in different ecotypes of *Arabidopsis*. They also found that allelic diversification of TPS genes in wild gene pools contributes to the natural variation in terpene formation.

The genetic characterization of the studied populations regarding their origin as well as possible local adaptation processes permit a better understanding of their putative resilience to stresses, such as the herbivore effects of cockchafer larvae. Therefore, we characterized the genetic diversity of the plant material used in this study using nuclear and chloroplast SSRs. Those highly variable nuclear microsatellite loci are an effective tool for studying population genetic diversity, gene flow and genetic structure as well as for gathering information about adaptive processes (Neophytou et al., 2010). To date, they have been extensively used for studying the genetic properties of oak populations in European forests (Petit et al., 2002; Neophytou et al., 2010; Gerber et al., 2014). The main findings describe a higher genetic variation within than among oak populations (Kremer and Petit, 1993; Neophytou et al., 2010). Accordingly, the nuclear SSRs showed no distinct spatial genetic structuring of the studied populations. The same was true for the association between infestation strength and genetic properties where we could not detect a correlation between the presence of cockchafer infestation and Nei's genetic distance at putatively neutral SSRs. The characterization of a large array of genes involved in defense against biotic stress is necessary to identify associations between genetic variation in genes and infestation strength in natural populations. The observed physiological differences in response to cockchafer feeding between the Brandenburg populations "BB1"/"BB2" and "R45" from Baden-Württemberg are most likely due to the absence of the forest cockchafer from Brandenburg so that defense mechanisms against the larvae have not evolved. In contrast, this cannot be an explanation for the differences in population density of cockchafer larvae of the neighboring stands "R45" and "R36."

Contrarily, the chloroplast SSR analyses did reveal a distinct geographic structure of chlorotypes. This suggests a post-glacial recolonization of the studied populations by oaks originating from different refugial regions as described by Petit et al. (2002) (**Figure 1**). These results are in accordance with those stated by Neophytou and Michiels (2013), showing a high chlorotype variation in the Upper Rhine Valley and confirming the hypothesis of a merging point of the refugial migration routes from the West, the South and the Southeast. Consistently, the populations further east possess chlorotypes of the southeastern lineage (Balkan) in higher frequencies. In spite of their proximity, the two Baden-Württemberg populations "R45" and "R36" reveal a different composition of chlorotypes. The highly infested population "R36" is dominated by chlorotype 09 from the southern (Italian) lineage, whereas "R45" is dominated by chlorotype 06 from the western (Iberian) lineage (**Figure 1**).

There are studies showing that populations originating from different genetic lineages possess different local adaptation capacities (Anderson et al., 2011; Prunier et al., 2012; de Lafontaine et al., 2018). This might also be true for areas of high cockchafer population density such as the Upper Rhine Valley, where populations with chlorotype 09 show higher cockchafer infestation levels than populations with chlorotype 06. However, measured TPS gene expression and VOC emission were rather similar in populations "R36" and "R45." Therefore, we assume that other genes are involved in the defense mechanisms against cockchafer infestation or other factors such as specific habitat characteristics regulate cockchafer infestation on those sites. But this remains to be confirmed by more detailed studies.

CONCLUSION

The present study has demonstrated that *Q. robur* trees from spatially distant populations with differing genetic background induce different reactions in soil dwelling forest cockchafer larvae. Surprisingly, trees originating from regions with low abundance of this herbivore species were more attractive to the insect larvae than trees from SW Germany where cockchafer density is high. Olfactory bioassays clearly suggested that the difference in insect attraction was caused by different patterns of root-released VOCs. Since the SW German population released higher amounts of VOCs into the soil, we assume a repellent effect against cockchafer larvae of at least some of these compounds. The presence of several benzenoids and terpenes, both groups of chemicals known to be involved in plant defense, support this view. Interestingly, we also observed that the release of such compounds was stimulated by herbivory, underlining their role in plant defense against root feeding insects. As seen from gene expression studies as well as from the analysis of root abundant volatiles, the oak populations obviously differed in the production of HIPVs. This finding suggests that plant defense against herbivory is population specific and therefore under genetic control.

Different strengths of infestation with the cockchafer of closely neighboring stands ("R45," "R36") can have several reasons. Root related processes such as differences in root attraction as affected by the release of plant volatiles as well as differences in plant defense after wounding by a herbivore can play a role only at a small spatial scale. However, at larger scale the selection of oviposition sites is of greater importance. Location of suitable feeding and oviposition sites by host-seeking insects might be mediated by volatile chemical cues (Webster and Cardé, 2017). Such volatiles are released from habitats in greater amounts than from single host plants and are detectable by the insects over longer distances (Webster and Cardé, 2017). Future studies should clarify if volatile chemical cues released from different oak forest stands differ in composition and, consequently, influence the selection of oviposition and feeding sites of the forest cockchafer.

AUTHOR CONTRIBUTIONS

FC performed the sampling, carried out the GC-MS analysis, evaluated the data, and wrote the manuscript. IC performed the sampling and the genotyping. HD, AD, OG, and LL were involved in writing the manuscript. JK performed data analysis and wrote the manuscript. JT-S and BV performed the sampling, genetic characterization, data analysis, and wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2018.01753/full#supplementary-material

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Genetic-Based Susceptibility of a Foundation Tree to Herbivory Interacts With Climate to Influence Arthropod Community Composition, Diversity, and Resilience

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Understanding how genetic-based traits of plants interact with climate to affect associated communities will help improve predictions of climate change impacts on biodiversity. However, few community-level studies have addressed such interactions. Pinyon pine (*Pinus edulis*) in the southwestern U.S. shows genetic-based resistance and susceptibility to pinyon needle scale (*Matsucoccus acalyptus*). We sought to determine if susceptibility to scale herbivory influenced the diversity and composition of the extended community of 250+ arthropod species, and if this influence would be consistent across consecutive years, an extreme drought year followed by a moderate drought year. Because scale insects alter the architecture of susceptible trees, it is difficult to separate the direct influences of susceptibility on arthropod communities from the indirect influences of scale-altered tree architecture. To separate these influences, scales were experimentally excluded from susceptible trees for 15 years creating susceptible trees with the architecture of resistant trees, hereafter referred to as scale-excluded trees. Five patterns emerged. (1) In both years, arthropod abundance was 3-4X lower on susceptible trees compared to resistant and scale-excluded trees. (2) Species accumulation curves show that alpha and gamma diversity were 2-3X lower on susceptible trees compared to resistant and scale-excluded trees. (3) Reaction norms of arthropod richness and abundance on individual tree genotypes across years showed genotypic variation in the community response to changes in climate. (4) The genetic-based influence of susceptibility on arthropod community composition is climate dependent. During extreme drought, community composition on scale-excluded trees resembled susceptible trees indicating composition was strongly influenced by tree genetics independent of tree architecture. However, under moderate drought, community composition on scale-excluded trees resembled resistant trees indicating traits associated with tree architecture became more important. (5) One year after extreme drought, the arthropod community rebounded sharply. However, there was a much greater rebound in richness and abundance on resistant compared to

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susceptible trees suggesting that reduced resiliency in the arthropod community is associated with susceptibility. These results argue that individual genetic-based plant-herbivore interactions can directly and indirectly impact community-level diversity, which is modulated by climate. Understanding such interactions is important for assessing the impacts of climate change on biodiversity.

Keywords: pinyon pine, pinyon needle scale, drought, climate change, arthropod community, community resilience, direct and indirect genetic effects, herbivore resistance and susceptibility

INTRODUCTION

Despite a growing understanding of the importance of intraspecific genetic variation within foundation plant species in structuring their associated communities (Whitham et al., 2006; Des Roches et al., 2018; Koricheva and Hayes, 2018), few studies have incorporated both genetics and climate when quantifying these interactions (Johnson and Agrawal, 2005; Sthultz et al., 2009b; Gehring et al., 2017). Through direct and indirect genetic-based influences, foundation species have the potential to have impacts on biodiversity at many trophic levels as well as on ecosystem scale processes (reviews by Ellison et al., 2005; Whitham et al., 2012; Crutsinger, 2016). Furthermore, climate change is likely to have major consequences for the interactions among foundation species and their dependent communities (Parmesan and Yohe, 2003; Whitham et al., 2006; Gilman et al., 2010; Stone et al., 2010; Nooten et al., 2014). Taking a genetic-based approach allows researchers to place the impacts of climate change within an evolutionary framework (e.g., Grant and Grant, 2002; Franks et al., 2007; Aitken et al., 2008; O'Neill et al., 2008; Sthultz et al., 2009a; Grady et al., 2011, 2015; Busby et al., 2014; Gehring et al., 2017).

Foundation tree species influence the structure of associated communities in several ways. There are direct interactions with herbivores or species that use the trees for habitat that are often mediated by plant architecture and chemistry, and there are indirect influences that extend over many trophic levels as the species that are directly associated with the trees interact with other trophic levels or physically alter the plant. Genetic influences that modulate the direct interactions have the potential to cascade up to higher trophic levels and indirectly influence extended community structure (Bolnick et al., 2011; Bailey et al., 2014). These influences are referred to as interspecific indirect genetic effects (IIGEs; Shuster et al., 2006). Often these indirect interactions between plants and higher trophic levels come from plant modifications induced by herbivores because the effects of herbivory persist after initial herbivore activity has ended (Jones et al., 1994).

Herbivore–plant interactions can be genotype dependent (Crawford et al., 2007) or contingent on a plant's resistance or susceptibility to specific herbivores, which often have a genetic basis (Dickson and Whitham, 1996; Gehring et al., 1997; Agrawal, 2004; Keith et al., 2017). Most studies on plant mediated indirect effects have focused on interactions of just a few species or trophic groups, while interactions between foundation species (e.g., a foundation herbivore and its host plant) that may indirectly affect a much larger associated community have been less studied (Ellison et al., 2005; Bailey and Whitham, 2007; Keith et al., 2010; Busby et al., 2015). Furthermore, few studies have addressed this issue in the context of genetic-based resistance and susceptibility to a foundation herbivore that physically alters the architecture of its host plant (but see Sthultz et al., 2009b). Quantifying whole community responses to plant mediated indirect effects is important because it represents the end result of multiple species interactions that can be difficult to isolate but are important for understanding community structure (Bangert et al., 2006), and the underlying mechanisms that maintain biodiversity (Ohgushi, 2005).

We quantified the arthropod community response to herbivory by the pinyon needle scale (*Matsucoccus acalyptus*) on juvenile pinyon pines that were resistant and susceptible to this insect. Scale herbivory on pinyon induces physical changes to susceptible trees and their local environment such as reduced foliage retention and mycorrhizal fungal colonization (Del Vecchio et al., 1993) and dramatically altered architecture (Cobb and Whitham, 1993). At high densities, scale herbivory causes extensive chlorosis and premature needle abscission (**Figure 1A**) leaving only the current year's needles intact resulting in a "poodle tail" growth form (Cobb and Whitham, 1993) (**Figure 1B**). Increased needle senescence associated with susceptibility also alters micro-site characteristics such as soil temperature, litter decomposition, and nutrient cycling (Classen et al., 2005, 2007) and slows soil development (Classen et al., 2013).

Three lines of evidence argue that resistance and susceptibility to the scale insect is under genetic control. (1) Scale-infested trees with millions of scales commonly grow with their branches interdigitated with resistant trees with few or no scales (**Figure 1B**). (2) Long-term monitoring and tree ring analyses showed that patterns of scale infestation on individual trees in a stand remained consistent for 15 years such that susceptible trees support high scale loads (e.g., >6 scales per cm of needle) while resistant trees support few to no scales on the entire tree (Cobb and Whitham, 1993). Furthermore, these differences are reflected in the long-term tree ring record in which resistant trees produce rings 25% wider than susceptible trees (Trotter et al., 2002). Importantly, when scales are experimentally removed from susceptible trees, tree ring width rebounds to resemble resistant trees (Trotter et al., 2002). (3) Scale survival on resistant trees was only 9% compared to 50% on susceptible trees (Cobb and Whitham, 1993; Del Vecchio et al., 1993), and scales experimentally transferred onto resistant trees suffered 3-4X higher scale mortality than scales transferred onto susceptible trees (Gehring et al., 1997). When scales were transferred onto susceptible trees that had scales experimentally excluded for

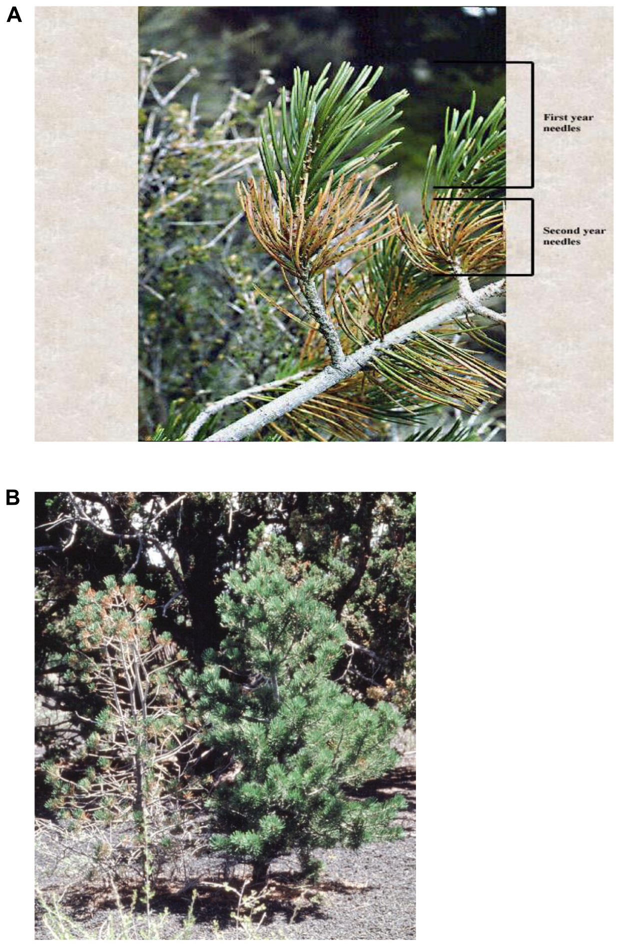

FIGURE 1 | Pinyon needle scale attacks second year and older needle cohorts, but not the current year growth (scales are small black dots on chlorotic needles on the second year needles) **(A)**. Trees that are resistant and susceptible to pinyon grow side by side. On 1–2 m tall trees, scale densities on susceptible trees can exceed 500,000, while resistant trees with interdigitated branches may support 0 scales (Cobb and Whitham, 1993) **(B)**.

8 years, scale survival did not differ from survivorship on the control susceptible trees despite differences in tree architecture (Gehring et al., 1997). In other words, long-term removal of scales resulted in a change in the architecture of susceptible trees to resemble resistant trees, but this did not alter their susceptibility to subsequent scale attack.

We predicted that indirect effects associated with changes in architecture and micro-site characteristics resulting from scale herbivory would affect other arthropod community members that reside on these trees. However, there might be direct genetic influences resulting from resistance and susceptibility to scales that also affect the associated community independent of the presence of scale insects. For example, differences in needle chemistry or resin production that may deter scale insects might also negatively impact other herbivorous insects. Because pinyon pine is habitat for a diverse arthropod community comprised of several hundred species (Trotter et al., 2008), differentiating between these two possibilities is important for understanding how a single genetically based interaction can influence higher-order diversity.

We sought to determine if arthropod community structure was more influenced by the indirect genetic influence of scale induced changes in tree architecture and associated traits, or the direct genetic influence of resistance and susceptibility acting on the associated community independent of the presence of scale. To separate the direct and indirect genetic influences of susceptibility to scale, we utilized an ongoing long-term scale exclusion experiment. For at least 15 years prior and throughout our study, scales were physically excluded from individual susceptible trees by removing the scale egg masses at the base of the tree (see Materials and Methods for more details). With long-term scale removal, these susceptible trees had recovered to exhibit the architectural phenotype of resistant trees (**Figure 1B**). By comparing resistant, susceptible and susceptible scale-excluded trees, we were able to separate the direct and indirect genetic effects of susceptibility to scale herbivory on the arthropod community.

Although direct and indirect influences of the scale-pinyon interaction on the arthropod community is genetic-based, the effects may not be consistent through time. Climate change is associated with declining insect biodiversity worldwide (Warren et al., 2018), and changes in climate are known to influence genetic-based interactions (Gehring et al., 2014a; Cooper et al., 2018). Indirect genetic effects under one set of environmental conditions might switch to direct genetic effects under another set of conditions. For example, community interactions that may depend more on foliage and habitat availability when environmental conditions are favorable, tree vigor is high, and communities are large and diverse may switch to be more dependent on tree genetics when drought conditions reduce both tree vigor and the overall richness and abundance of the arthropod community.

One predicted outcome of climate change in the southwestern US is an increase in the frequency and intensity of drought events (U.S. Global Change Research Program [USGRP], 2017). Pinyon pine is a foundation tree in this region that has been shown to be especially sensitive to drought (Breshears et al., 2005; Mueller et al., 2005; Gitlin et al., 2006). Because extreme climate events such as drought not only affect foundation tree species, but their associated arthropod communities, quantifying the effects of extreme drought on communities can add resolution to long-term predictions of the effects of climate change on arthropod diversity.

In this study, we sought to determine if the direct and/or indirect genetic influences of susceptibility to scale herbivory were influenced by climate by comparing arthropod community data on scale resistant and susceptible trees during an extreme drought year (2002), to data collected during a moderate drought year (2003) (NOAA/NCEI, 2018). We hypothesize that variation in drought intensity across years could impact both the direct and indirect genetic influence of pinyon on the arthropod community. If confirmed, such findings would suggest that interactions between climate and the genetic-based traits of foundation species are important to consider in understanding the effect of global change on biodiversity (Johnson and Agrawal, 2005; Gehring et al., 2017). Furthermore, arthropod richness and abundance were greatly depressed in the first year of the study due to extreme drought. By comparing those results to the subsequent moderate drought year we could address how resilient the arthropod community might be to drought stress. Particularly, we could determine if resistance or susceptibility to scale herbivory affects the ability of the arthropod community to recover from drought. The potential for community recovery from extreme environmental stress is likely to become a major issue as it is expected that with climate change, periods of record drought are likely to be interspersed with near normal or even wet periods (Cook et al., 2004; Gray et al., 2006).

MATERIALS AND METHODS

Study Site and Drought Severity

Research was conducted near Sunset Crater National Monument in Flagstaff, AZ (elev. 2,000 m). The study area is co-dominated by pinyon pine (*Pinus edulis*) and one-seed juniper (*Juniperus monosperma*). Soils in the area consist of lava, ash, and cinders resulting in water limited and nutrient poor conditions (Gehring and Whitham, 1994; Cobb et al., 1997; Classen et al., 2005, 2007). Over the course of this study, northern Arizona experienced extreme drought conditions resulting in one of the hottest and driest summers on record in 2002. In 2003 the severity of the drought decreased, and this area received a 19% (29.57–35.26 cm) increase in annual precipitation which, coupled with slightly cooler temperatures caused the Palmer Drought Severity Index (PDSI) for northern Arizona to decrease from −4.08 to −2.55 (NOAA/NCEI, 2018). The PDSI is a region-specific index of meteorological drought calculated from precipitation, temperature and potential evapotranspiration (Palmer, 1965; Alley, 1984). The PDSI compares current year drought conditions to long-term average values and assigns positive values to above average wetter conditions and negative values to below average drought conditions. Because our study site fell on the border of two drought regions in Arizona (regions III and IV), we averaged the yearly PDSI values from these two regions in each year. Index values < -4 indicate extreme drought and index values between −3 and −2 indicate moderate drought conditions (Alley, 1984). Comparing these 2 years of differing drought intensity gave us the unique opportunity to quantify the impact of an extreme drought event on arthropod communities associated with pinyon, to assess the resiliency of the arthropod community, and to detect if there were interactions with genetic variation among the trees.

Scale Lifecycle and Outbreak

Under conditions of nutrient and water stress, pinyon pine express resistance and susceptibility to a mesophyll feeding scale insect (*Matsucoccus acalyptus*). In the early spring, the scale insects emerge from egg clusters at the base of the trees and climb up to the branches where they attach themselves to the existing needles. Feeding usually begins in May and continues 6 months for males and 10 months for females (Cobb and Whitham, 1993). At high densities, scale feeding results in extensive chlorosis and premature needle abscission leading to an altered architecture where only the current year's needles are retained (Cobb and Whitham, 1993) (**Figure 1B**).

Subsequent to a record drought in 1996, scale insects were able to expand their range out of small pockets of high nutrient and water stress in the ash and cinder fields around northern Arizona. In 1998 scales began to be observed along much of the western edge of the Colorado Plateau in the four corners region. This outbreak and range expansion was quantified by following the distribution of pinyon along the Mogollon rim and making observations at one-mile increments. Observational data was collected 1–3 times per year from 1990 to 1998. Pinyon were located by observations along major roads that followed pinyon-juniper woodland boundary lines as determined by U.S. Forest Service vegetation maps. Scale distribution boundaries were then further defined by using Forest Service roads to access trees. Where pinyon was observed, trees were driven or walked to until trees were encountered that had scales present on greater than 33% of their needles. Point observations of scale infested trees were cataloged with GPS and overlaid on the existing range of pinyon in ArcMap (version 8.0.1).

Tree Selection and Sampling

To quantify the effect of extreme drought on the associated arthropod community on pinyon pine 60 trees were chosen from a group of trees associated with a long-term monitoring study which began in 1985. These trees were classified as resistant, susceptible or scale excluded at the time that monitoring began, and all trees used in this study had retained their original classification throughout the course of the monitoring period. Twenty trees from each of the three tree categories (scale susceptible, resistant, and scale excluded trees) were chosen for this study. Annual removal of scales began at the outset of the monitoring study in 1985 (Cobb and Whitham, 1993). Scales were completely removed from trees that supported high-density scale populations. Trees were defaunated by removing egg masses laid at the base of the trees in the early spring and Tanglefoot was placed approximately 5 cm above the ground to prevent any remaining scales from ascending the tree. The Tanglefoot was placed on duct tape so that it could later be removed in order to prevent other insects from potentially being affected. Tanglefoot was kept on the tree for several weeks to ensure that all of the eggs had hatched. Nearby non-treated trees were used to monitor when the eggs hatched. After hatching, egg masses turn from yellow to white as larvae move away from the eggs, leaving the white egg casings and filamentous fibers extruded by females behind. Within 10 years of having scales excluded in this way, scale excluded trees had similar amounts of foliage as scale resistant trees (Gehring et al., 1997). All three classes of trees were intermixed in an area of approximately 1 km^2 and were juvenile pinyons between the ages of 40–60 years as determined by tree cores. The trees were blocked by height and basal trunk diameter to control for differences in tree age.

Due to the severity of the drought in 2002, only 16 of our 60 study trees survived over the winter. Because there were not enough surviving trees to do a repeated measures analysis, 20 new triplets were chosen in 2003 from the same study area that closely matched the height and basal trunk diameter of the original trees so that we could keep the age classes constant across years. These newly selected trees came from the same pool of trees that were associated with the long-term monitoring study. Like the first set of trees, the newly selected trees had been categorized as resistant, susceptible, or scale excluded when monitoring began in 1985 and had retained their original classifications throughout the period of this study. To address the hypothesis that the new trees selected from this pool might be physiologically different from the triplets used in the previous year, the 16 trees that survived both years were surveyed to compare their responses relative to the new trees.

To census arthropods, all branches on each tree were sampled using a muslin sweep net. The contents were emptied into sealable bags and stored in a freezer for subsequent analyses. Abundance, richness, and compositional data were recorded. In each year, arthropods were sampled over a 2-week period in late May in order to capture the greatest diversity within the community (Trotter et al., 2008; Stone et al., 2010). Because our sampling technique removed the arthropods from the tree, each tree was sampled only once during this period. All arthropods were categorized by morphotype and assembled into a reference collection that is stored at the Colorado Plateau Arthropod Museum at Northern Arizona University.

Data Analysis

Richness and abundance data were analyzed using a blocked one-way analysis of variance (ANOVA) in the program JMPin (SAS, 2000; JMP 4.0). Trees were blocked by size across the three treatments to control for age differences among trees. Differences among treatment means were determined using Tukey HSD comparisons. To determine if there was an interaction between herbivore susceptibility and year-to-year variation in climate, a two-factor split plot design was implemented using the factors treatment (resistant, susceptible, scale excluded), year (2002, 2003), and the interaction between treatment and year.

For the 16 trees that survived both years, we plotted reaction norms for total arthropod richness and abundance on each tree to evaluate individual genotype responses to a reduction in drought intensity across years. Because these 16 trees were not included in the analyses of the newly selected trees in 2003, they represent an independent assessment of how drought interacts with resistance and susceptibility at the genotype level to affect the associated arthropod community. Differences in community composition were visualized using non-metric multidimensional scaling (NMDS) in the program PC-ord (McCune and Mefford, 1999). The community composition analysis was done with arthropod abundance relativized to species maximum. Relativization of the abundance data enabled us to equalize the importance of common and rare species in the analysis so that very abundant species would not overly influence differences among the groups. We did not include the scale insect in this analysis so that potential differences in community composition between susceptible and resistant trees would not be influenced by this one species. All pairwise comparisons among treatments were analyzed using a multi-response permutation procedure (MRPP) within PC-ord. Species accumulation curves were generated using EstimateS (Colwell, 2004). The species accumulation curves were generated according to Gotelli and Colwell (2001) using a randomized resampling technique of 1000 iterations with our samples defined as the individual trees from each of the three treatments.

RESULTS

Scale Range Expansion With Drought

Prior to 1996, our study area experienced above average rainfall in 16 of the previous 18 years (NOAA/NCEI, 2018). During this time scale insects were concentrated only in the high stress cinder soils associated with Sunset Crater National Monument. Starting in 1996 and continuing until the present time, this same region has experienced levels of drought ranging from abnormally dry to extreme (NOAA/NCEI, 2018). During this drought period scales have expanded their distribution into areas not formerly occupied (**Figure 2**). This range expansion with drought and the associated architectural changes

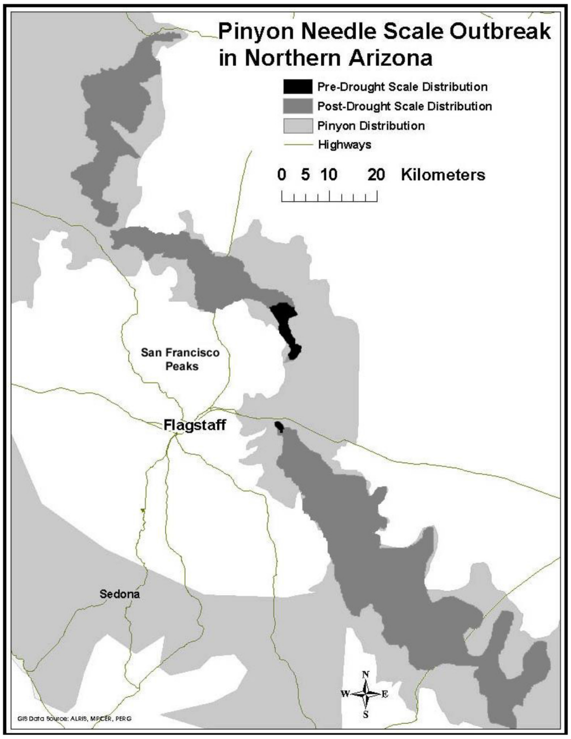

FIGURE 2 | Range expansion of pinyon needle scale after a record drought in 1996. Dark gray represents pre-drought distribution of the scale insect, medium gray represents its post-drought distribution, and light gray represents the current distribution of pinyon pine.

associated with susceptibility has the potential to affect the associated community of arthropods representing several trophic levels.

Scale Impacts on Associated Arthropod Community

In 2002, a total of 325 individuals comprising 90 morphospecies were collected. To determine if year-to-year variation in climate interacted with tree genetics we collected in the same manner in 2003. In 2003, 4046 individuals comprising 202 morphospecies from at least 30 families and 12 orders were collected. The number of families and orders are similar to another arthropod community study on pinyons in this area by Trotter et al. (2008), which found 287 species from 14 orders and 80 families. Across all treatments mean species richness per tree was 4X lower (3.8 ± 0.47 to 14.6 ± 0.99) and mean abundance was 11X lower (5.4 ± 0.80 to 58.8 ± 8.11) in the record drought year (2002) compared to the moderate drought year (2003). There was a consistent relationship across years in which susceptible trees had 2-3X lower richness (2002– $F = 4.87$, $p = 0.01$; 2003– $F = 6.32$, $p = 0.016$) and 3-4X lower abundance (2002– $F = 4.15$, $p = 0.02$; 2003– $F = 18.16$, $p < 0.0001$) than resistant and scale-excluded trees which did not differ from each other (**Figure 3**).

Consistent with the above findings species accumulation curves showed that, in both years, alpha diversity (mean richness, which is indicated by the first point of each curve) and gamma diversity (total richness, which is indicated by the final point on each curve) were higher on resistant and scale-excluded trees compared to susceptible trees (**Figure 4**). The accumulation curve for the susceptible trees was shallower, showing a rate

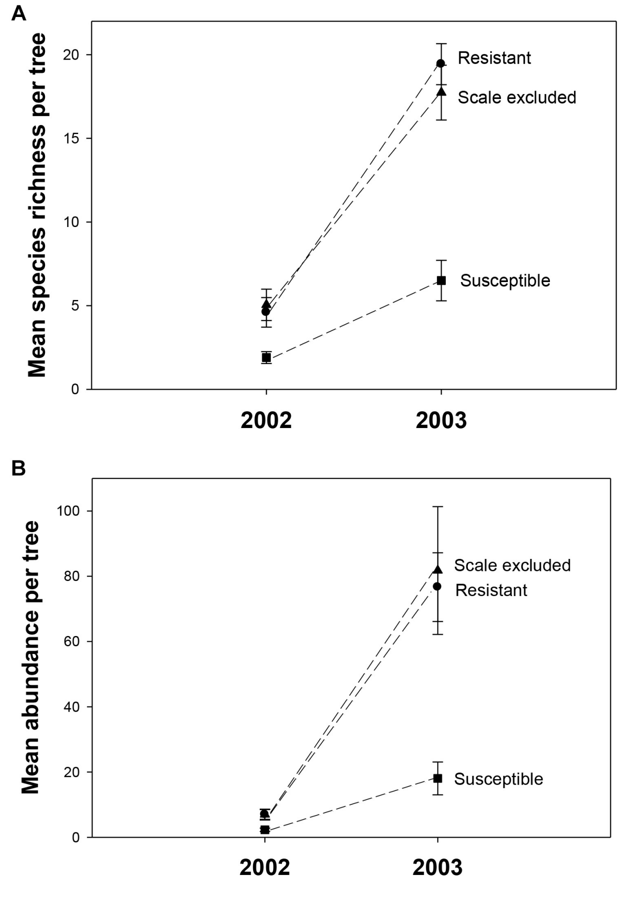

FIGURE 3 | Mean species richness **(A)** and abundance **(B)** on scale excluded, scale resistant and scale susceptible trees in an extreme drought year (2002) and subsequent moderate drought year (2003). Dashed lines connect treatments between years.

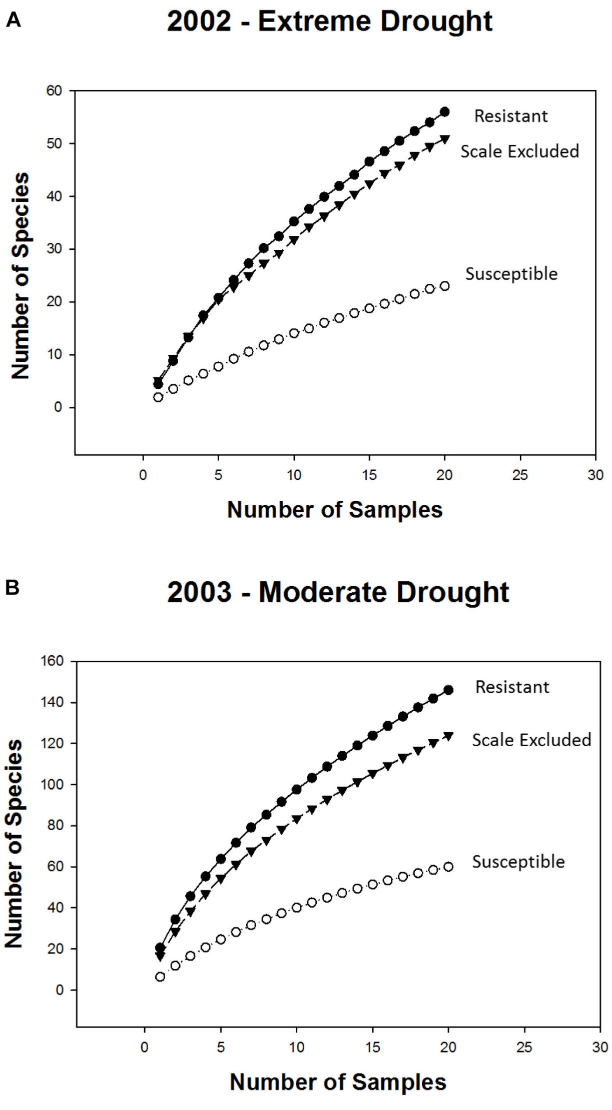

FIGURE 4 | Sample based species accumulation curves for resistant, susceptible and scale excluded trees in the extreme drought year of 2002 **(A)** and the subsequent moderate drought year of 2003 **(B)**. Mean richness is represented by the first point of each curve. The rate at which new species appear in relation to sampling effort is represented by the shape of each curve where flatter curves indicate more consistent species composition across samples. Total richness is represented by the final point of each curve.

of species accumulation that was much slower than either the scale-excluded or resistant trees.

Interactions Between Tree Genetics and Climate

Although there was consistency across years in the pattern of the response, we detected a significant interaction between treatment and year in which the differences in arthropod abundance ($p = 0.007$) and richness ($p = 0.002$) between resistant and susceptible trees was more pronounced in the moderate drought year compared to the extreme drought year (**Table 1**). Between 2002 and 2003 abundance increased 11X on resistant trees compared to 8X on susceptible trees while richness increased 4X on resistant trees compared to 3X on susceptible trees (**Figure 3**). These results demonstrate an interaction between tree genetics and climate in which the increase in richness and abundance associated with a decrease in drought severity is more pronounced on resistant trees compared to susceptible trees.

Because a 73% mortality rate in our study trees prevented us from analyzing the same trees in both years at the treatment level, we had to choose a new set of 60 trees to use for the 2003 comparison. However, we did qualitatively examine the arthropod communities of the 16 surviving trees in 2003 to determine if patterns seen at the scale of individual tree genotype were similar to those seen at the treatment level in the newly selected trees. For the 16 surviving trees, reaction norms of total per tree species richness and abundance are plotted in **Figure 5**. These reaction norms show a more pronounced increase in both species richness and abundance on resistant and scale excluded trees compared to susceptible trees. These results are very similar to the findings at the treatment level (**Figure 3**) in that scale susceptible trees show a much reduced response to a reduction in drought stress. In addition, the reaction norms of individual tree genotypes show large tree-level variation in the response of the arthropod community to climate change. This indicates that some tree genotypes are more plastic in the way that their arthropod community responds to decreasing drought stress than other tree genotypes.

TABLE 1 | Richness and abundance were significantly different among treatments in both years.

Source	SS	F-ratio	*p*-value
Richness			
Year	87.24	53.84	<0.0001
Treatment	44.90	34.11	<0.0001
Year × Treatment	9.22	7.01	0.002
Abundance			
Year	638.35	61.03	<0.0001
Treatment	207.74	13.51	<0.0001
Year × Treatment	82.89	5.39	0.007

There was also a significant interaction between treatment and year in which the increase in richness and abundance between 2002 and 2003 was greater for resistant and scale excluded trees compared to susceptible trees.

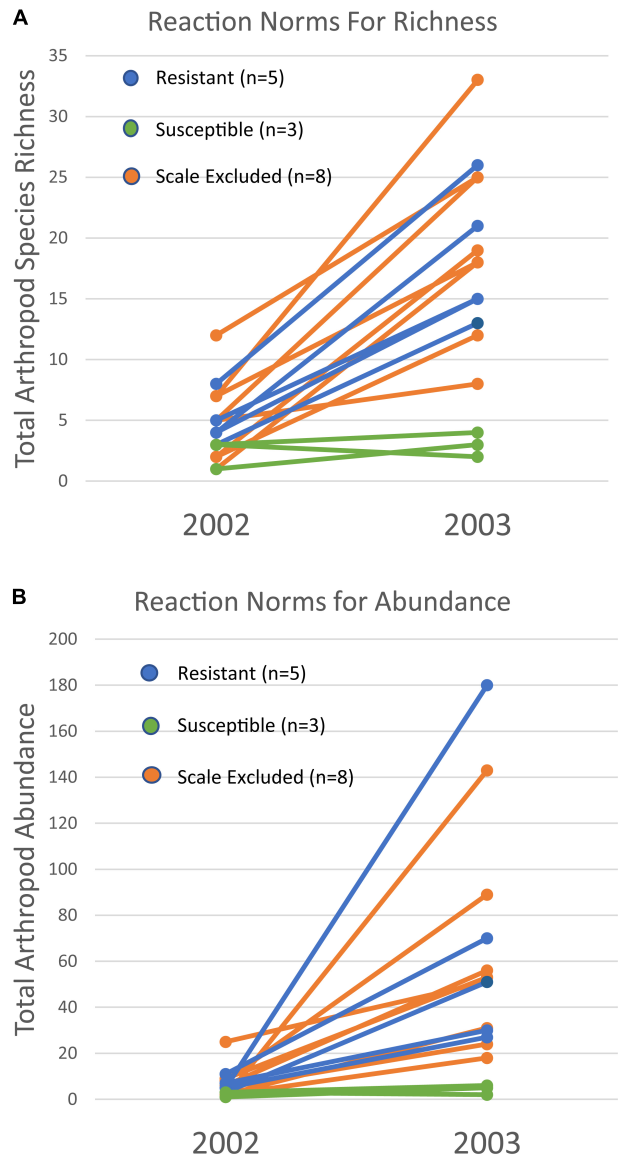

FIGURE 5 | Reaction norms for 16 individual tree genotypes that survived both years of the study. Total species richness **(A)** and abundance **(B)** per tree show variation within and among susceptibility classes in their community plasticity responses to the extreme drought year of 2002 and moderate drought year of 2003. Each line represents the response of a single tree genotype across years.

In the extreme drought year, comparisons of arthropod communities on resistant, susceptible, and scale-excluded trees indicated that the direct influence of scale susceptibility was driving arthropod community composition (**Figure 6A**). This is because the composition of the arthropod communities on scale-excluded trees did not differ from susceptible trees, and both were different from resistant trees (Chance-corrected within group agreement, $A = 0.027$; $p = 0.013$). However, in the subsequent moderate drought year, the pattern switched

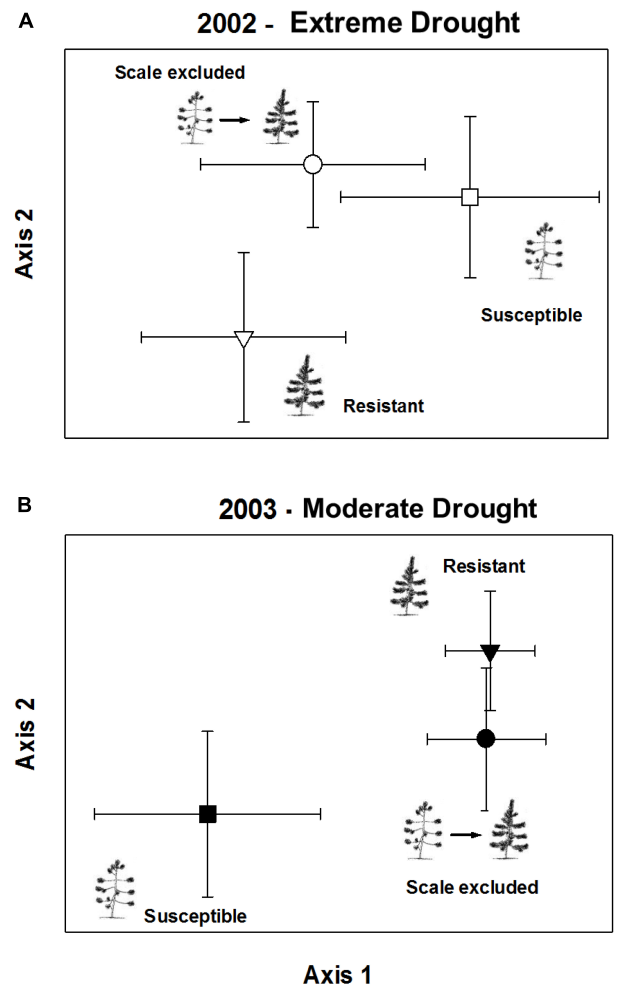

FIGURE 6 | Non-metric multi-dimensional scaling ordination of arthropod community composition on scale excluded, scale resistant and scale susceptible trees in an extreme drought year 2002 **(A)** and subsequent moderate drought year 2003 **(B)**. Arthropod abundance is relativized to species maximum. Symbols represent the mean axis value of the community of arthropods on all trees within treatments ± 2 standard errors.

and composition of the arthropod community on scale-excluded trees no longer resembled the susceptible trees and did not differ from resistant trees (Chance-corrected within group agreement, $A = 0.033$; $p = 0.0001$) (**Figure 6B**). This demonstrates an interaction between tree genetics and climate that affects community composition. When the abundance of the arthropod community is low, traits related to tree architecture do not seem to have as much of an impact and the direct genetic influence of susceptibility to scale is more important in structuring communities. However, when the abundance of the arthropod community is high, factors related to tree architecture have a larger impact. Note that scale insects were not included in these analyses as our main goal was to examine how resistance and susceptibility affected the rest of the community independent of the scale insect.

DISCUSSION

Indirect Genetic Influences of Susceptibility on Community Structure

The indirect influences of plants on community structure which are mediated through genetic-based interactions with herbivores have received increased attention (Werner and Peacor, 2003; Ohgushi et al., 2007; Keith et al., 2017), although most studies focus on chemically induced changes in plants (Denno et al., 1995; Van Zandt and Agrawal, 2004; Viswanathan et al., 2005) and not on morphological changes (but see Gehring et al., 1997; Crawford et al., 2007; Sthultz et al., 2009b). Susceptibility to scale affects both the resource quantity and the quality of pinyon pine by altering the tree's architecture (Gehring et al., 1997; Chapman et al., 2003; Classen et al., 2005), as well as affecting microsite characteristics associated with individual trees such as soil moisture and temperature (Classen et al., 2005). We expected that morphological changes in pinyon such as a greater than 50% reduction in foliage (Gehring et al., 1997) would result in reduced food resources as well as increased exposure to predation that would deter many insects from using susceptible trees as habitat (Gonzalez-Megias and Gomez, 2003). This is supported by the result that in both years species richness was 2-3X higher, abundance was 3-4X higher and the composition of the community differed on resistant trees compared to susceptible trees (**Figures 3**, **6A**). In addition, species accumulation curves demonstrate that susceptible trees accumulate new species much more slowly than resistant trees. In both years alpha diversity (mean richness) and gamma diversity (total richness) were all reduced on scale susceptible trees relative to resistant trees (**Figure 4**). This is in agreement with other research suggesting that morphological changes occurring as a consequence of susceptibility to herbivory can have as large of an impact on associated communities as physiological or chemical changes (Johnson and Agrawal, 2005). These findings also demonstrate that plant genetics can affect sampling effort as scale resistant trees require more sampling to characterize their communities relative to susceptible trees. If generally common, researchers may underestimate the importance of insect resistant trees in promoting biodiversity. Although our findings relate mainly to scale-altered architecture (e.g., a single cohort of needles rather than 6+ years of needle cohorts), it is important to note that plant phytochemistry, phenology, nutrient availability and other important plant traits could also be affected by susceptibility to the scale insect, which deserve future study.

Direct Genetic Influences of Susceptibility on Community Structure

Many differences in arthropod community structure between resistant and susceptible trees are based on the indirect influence of susceptibility mediated by a change in tree architecture and associated traits. However, it is likely that there are also direct influences on community structure that stem from genetic differences related to resistance and susceptibility. To separate the indirect influence of architectural changes induced by scale insects from the direct influence of tree genetics we utilized a scale exclusion treatment in which scales were experimentally excluded from susceptible trees for 15 years. Excluding scales for this amount of time allowed the tree architecture of the susceptible trees to grow back 6+ years of needle cohorts to resemble resistant tree architecture. When we compared scale-excluded susceptible trees to resistant and scale-altered susceptible trees we found that richness, abundance, and the accumulation of species did not differ between resistant and scale-excluded susceptible trees but both were significantly different from scale-altered susceptible trees. This pattern was observed in both the extreme drought year of 2002 and the subsequent moderate drought year of 2003. These results argue that metrics such as arthropod richness and abundance are more tied to the morphology of the trees and that arthropods are responding, for example, to the amount of foliage as a resource for factors such as food or predator avoidance. These findings are in agreement with other studies that have found that morphological changes associated with herbivory are more important in structuring communities than the direct influence of tree genetics (i.e., susceptibility to scales might also indicate susceptibility to other arthropods). Dickson and Whitham (1996) showed that with the removal of the galling aphid, *Pemphigus betae*, from susceptible cottonwood trees, species richness and abundance declined to resemble resistant trees, arguing that the plant genetic effects on the community acted indirectly (i.e., through the presence of aphid galls) rather than directly on the rest of the community. However, our studies are different in that we found that the presence of scales negatively affected species richness and abundance, whereas Dickson and Whitham (1996) found that the aphid positively affected species richness and abundance. This difference is likely because the galling aphids provided food for predators and parasitoids, did not severely damage their hosts, and created habitat for inquiline species that used the galls as habitat (Martinsen et al., 2000). Scale insects, however, damage their host plants causing most needles to prematurely abscise thereby eliminating resources for other arthropods (Cobb and Whitham, 1993; Trotter et al., 2002).

When we compared the composition of the arthropod communities among the three treatment groups in the extreme drought year of 2002, we found that the composition of the community on scale-excluded trees differed from that of resistant trees but was not significantly different from susceptible trees. This result argues that metrics such as richness and abundance are more influenced by tree architecture and associated traits, whereas the composition of the community is more directly influenced by tree genetics. Certain arthropod species were more likely to be found on the susceptible genotype whether or not the scale insect was present. Despite large differences in tree architecture, richness, and abundance between scale-excluded and scale-altered susceptible trees, they had similar community compositions. This is in agreement with Wimp et al. (2005) who found that on two species of cottonwoods and their F_1 hybrids, species richness and abundance did not differ among the groups, but they all had unique community composition. The findings of Wimp et al. (2005) and this study suggest that because of the complex interactions that create community structure, genetic influences that directly impact even a few species can cascade up to create large differences in the composition of the whole community.

Interactions Between Tree Genetics and Climate

From 2002 to 2003 total species richness of the arthropod communities on pinyon quadrupled and abundance increased 11-fold. This corresponded with a decrease in drought severity from extreme to moderate drought. While this result shows remarkable resilience in the arthropod community, this resilience was not similarly experienced by resistant and susceptible trees. Although arthropod richness and abundance increased on both resistant and susceptible trees from 2002 to 2003, there was a treatment × year interaction in which the increase in both richness and abundance was greater on resistant trees compared to susceptible trees (**Table 1**). Although the arthropod community has the potential to rebound on both resistant and susceptible trees after an extreme drought event, the increase in richness and abundance on susceptible trees did not keep pace with resistant trees. The resilience of the arthropod community after drought is impeded by the genetic-based interaction between pinyon and scales. It is important to consider how different community responses to stress (i.e., linear versus non-linear) might affect these findings. For example, if richness and abundance increased exponentially rather than linearly, what would appear to be a treatment by year interaction in a linear model might be explained by a different rate of increase in richness and abundance on resistant and susceptible trees along an exponential curve. Regardless, genetic influences that affect even just the rate of recolonization of species can be important to the resiliency of the arthropod community.

The patterns demonstrated with resistant, susceptible and scale excluded trees at the treatment level (**Figure 3**) are also reflected in our plots of the reaction norms of 16 individual tree genotypes that survived both years (**Figure 5**). These reaction norms show that individual tree genotypes within treatments differ greatly in their response to drought severity, and that even within a treatment reaction norms can be different. These community-level reaction norm findings are similar to those observed by Keith et al. (2017) who found significant within group variation of arthropod richness and abundance on cottonwood genotypes with aphids and with aphids excluded in a common garden. Because the arthropod community associated with pinyon participates in many trophic interactions across multiple taxa, our findings argue that direct genetic-based interactions such as between pinyon and the scale insect have important community-level effects. To our knowledge, no other study has demonstrated the impact of a fundamental genetic-based plant-herbivore interaction on the resiliency of the associated arthropod community after extreme drought, but these interactions are important to consider when predicting the impacts of climate change on biodiversity.

These community level effects have been shown to be associated with large ecosystem-level effects as well. For example, studies by Classen et al. (2006) at the same study site showed that scales significantly increase needle litter nitrogen (N) and phosphorus (P) concentrations by 50%, as well as litter inputs to soil by 21%. In addition, microbial biomass was 80% lower in soils beneath scale-susceptible trees when compared to resistant trees. Classen et al. (2005) also showed that scale herbivory reduced leaf area index (LAI) of susceptible trees by 39% and that scale herbivory increased soil moisture and temperature beneath susceptible trees by 35 and 26%, respectively. The authors concluded that the magnitude of scale effects on soil moisture and temperature is similar to global change scenarios, and sufficient to drive changes in ecosystem processes that ultimately slow soil development (Classen et al., 2013). Since the expression of susceptibility is mediated by climate, the expansion of scale insects with drought (**Figure 2**) is likely to have important ecosystem-level consequences.

In the extreme drought year community composition on scale-excluded susceptible trees did not differ from scale-altered susceptible trees and both were different from resistant trees. However, in the subsequent moderate drought year when environmental conditions improved and the arthropod community rebounded, the pattern switched and the composition of the community on scale-excluded susceptible trees became similar to resistant trees and both were different from susceptible trees (**Figure 6**). Under poor environmental conditions such as the record drought of 2002 when trees suffered very high stress, the abundance of the arthropod community was very low. With low abundance, we hypothesize that extrinsic factors such as decreased interactions among the arthropods may make it more likely that they respond to the genetics of the trees and we find similar communities on susceptible genotypes (with and without scales) regardless of their architecture. However, when conditions improve and arthropod abundance and richness increase, traits associated with tree architecture become more important in structuring communities.

Our findings link the effects of climatic stress with genetic-based susceptibility to herbivory and show a strong negative impact on the diversity and resilience of arthropod communities. This study fits in to a broader context of community-level research which deals with the effects of environmental stress on arthropod communities. Within a stand, Stone et al. (2010) found that insect diversity is greatest on vigorously growing pinyons and declines to near zero on stressed trees exhibiting slow growth, branch dieback and high needle abscission. Similarly, across stands varying in soil type, Trotter et al. (2008) found that trees growing under high stress in cinder soils supported about 1/10th the number of arthropods, and roughly half the species as trees growing under more favorable conditions in sandy-loam soils. The median ratio of herbivores to predators at low stress sites was 17.8:1 while in the high stress sites the ratio dropped to 1.7:1. This order-of-magnitude shift herbivore-predator ratio indicates both a change in community composition and trophic structure in which predator pressures on herbivores may dramatically increase in stressed environments. Thus, at multiple levels of study (i.e., genetic differences among trees within a stand in their susceptibility to a common herbivore, among trees within a stand that differ in tree vigor, and across stands that differ in stress levels associated with different soil types), chronic herbivory, climatic stress, and soil stressors each have similar negative impacts on arthropod community metrics. All three of these stressors combine to impact scale susceptible trees growing in poor soils during drought years. To understand the effects of global change on arthropod communities, we need to integrate the impacts of multiple and simultaneously occurring biotic and abiotic stressors that often occur together. To better understand these observationally and experimentally derived patterns, more mechanistic studies are needed.

These results are among the first to demonstrate tree genetics interacting with climate to affect the richness, abundance, and composition of arthropod communities. The results of this study demonstrate that climatic changes have the potential to alter fundamental genetic-based interactions between foundation tree species and their associated arthropod communities (Maddox and Cappuccino, 1986; Gilman et al., 2010). We found that the arthropod community response to both the direct genetic influence of resistance and susceptibility to scale and the indirect genetic influence of susceptibility manifested in altered tree architecture can be influenced by climatic changes that occur over the span of a single year. We are aware of only one study that has utilized an herbivore removal experiment to separate direct and indirect genetic influences of a foundation tree on its dependent community under two different environmental conditions to determine if the community response remained the same. Sthultz et al. (2009b) found that the composition of ectomycorrhizal fungal (EMF) communities on mature pinyon pine (*Pinus edulis*) were very different on trees that were resistant to the stem and cone-boring moth (*Dioryctria albovittella*) and trees that were susceptible. Susceptible trees exhibit a more shrub-like and closed architecture, but long-term removal of moths from susceptible trees allowed the susceptible trees to recover and resemble the architecture of resistant trees. They found that during both wet and drought years, plant genetic effects on EMF community composition were direct. In other words, plant genetics was more important than tree architecture in determining EMF community composition across both a drought year and a wet year. Our results do not show the same consistent pattern across climatically variable years. This could be because mycorrhizal fungi are generally mutualists, whereas arthropod communities generally contain many antagonistic herbivores. Furthermore, mycorrhizal fungi are physically attached to their host, while most arthropods are mobile. The direct interactions between a host tree and the mycorrhizal fungal community compared to the arthropod community are fundamentally different and are likely to be affected differently by changes in tree condition in response to environmental change. Regardless, if climate change alters fundamental genetic-based interactions of some species but not others, then it becomes especially important to understand and predict which interactions might be most sensitive to climate change.

In their review of community and ecosystem genetics, Whitham et al. (2006) argued that changes in global climate will create new selection pressures on foundation species that can affect the evolution of associated communities. Current predicted patterns of climate change involve not only increasing mean temperatures, but increased year-to-year variation in weather patterns and more frequent extreme climatic events such as drought (U.S. Global Change Research Program [USGRP], 2017). Recent climate change studies show that the American southwest is undergoing severe drought (U.S. Global Change Research Program [USGRP], 2017) that is resulting in widespread mortality of foundation tree species (Breshears et al., 2005; Gitlin et al., 2006; van Mantgem et al., 2009; Whitham et al., 2019). In 2002 the southwest experienced drought of an intensity that had not been observed in 50 years (Breshears et al., 2005). Although droughts of this severity are rare, they provide unique opportunities to quantify short-term ecological responses to extreme events (Holmgren et al., 2001; Reusch et al., 2005; Gehring et al., 2014b). Quantifying the impact of drought and the biotic response are especially important because the intensity and frequency of drought in this region is predicted to increase (Seager et al., 2007; U.S. Global Change Research Program [USGRP], 2017), which is likely to result in major shifts in the geographic distribution of many foundation tree species (Parmesan, 2006; Rehfeldt et al., 2006; O'Neill et al., 2008; Ikeda et al., 2017), their associated communities (Gilman et al., 2010) and outbreaks of herbivores such as bark beetles which attack trees that have compromised defenses due to environmental stressors (Raffa et al., 2008). Mortality and range shifts in these important tree species due to drought is worldwide (Allen et al., 2010) and likely to have major consequences for the communities they support (Ikeda et al., 2014). Because the expression of susceptibility is often stress dependent (i.e., in the absence of climate stress all trees appear to be resistant, but with stress, susceptible and resistant phenotypes become apparent) (Cobb et al., 1997) (**Figure 2**), and additional outbreaks may be more frequent with increasing drought, our findings suggest that climate change in the southwest is likely to result in a substantial loss of arthropod diversity at a regional scale.

AUTHOR CONTRIBUTIONS

AS designed the study, collected and analyzed the data, wrote the manuscript. NC, CG, and TW initiated the scale removal experiment, helped design the study, and revised the manuscript.

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Adaptive Diversity of Beech Seedlings Under Climate Change Scenarios

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The ability of beech (*Fagus sylvatica* L.) populations to adapt to the ongoing climate change is especially important in the southern part of Europe, where environmental change is expected to be more intense. In this study, we tested the existing adaptive potential of eight beech populations from two provenances in N.E. Greece (Evros and Drama) that show differences in their environmental conditions and biogeographical background. Seedling survival, growth and leaf phenological traits were selected as adaptive traits and were measured under simulated controlled climate change conditions in a growth chamber. Seedling survival was also tested under current conditions in the field. In the growth chamber, simulated conditions of temperature and precipitation for the year 2050 were applied for 3 years, under two different irrigation schemes, where the same amount of water was distributed either frequently (once every week) or non-frequently (once in 20 days). The results showed that beech seedlings were generally able to survive under climate change conditions and showed adaptive differences among provenances and populations. Furthermore, changes in the duration of the growing season of seedlings were recorded in the growth chamber, allowing them to avoid environmental stress and high selection pressure. Differences were observed between populations and provenances in terms of temporal distribution patterns of precipitation and temperature, rather than the average annual or monthly values of these measures. Additionally, different adaptive strategies appeared among beech seedlings when the same amount of water was distributed differently within each month. This indicates that the physiological response mechanisms of beech individuals are very complex and depend on several interacting parameters. For this reason, the choice of beech provenances for translocation and use in afforestation or reforestation projects should consider the small scale ecotypic diversity of the species and view multiple environmental and climatic parameters in connection to each other.

Keywords: ***Fagus* sp., phenology, survival, height, adaptation, common garden experiment**

INTRODUCTION

The European beech (*Fagus sylvatica* L.) is one of the most important tree species in the continent, both economically and ecologically. Its geographical range extends from Scandinavia to the Mediterranean covering various habitats (Bolte et al., 2007; Willner et al., 2017). It is generally considered as an oceanic species that can grow in areas with mild winters and moist summers, sensitive to intense drought periods in the growing season (Fotelli et al., 2001; Leuschner et al., 2001; Bolte et al., 2007; Granier et al., 2007; Pšidová et al., 2015). Several studies report that beech populations in Southern Europe have faced strong selective pressures during the last decades (Jump et al., 2006; Piovesan et al., 2008), which are expected to become more intense because of future changes in rainfall patterns and temperatures under the forthcoming climate change (Charru et al., 2010; Rita et al., 2014), arising concerns about the survival dynamics of the European populations (Bréda et al., 2006; Geßler et al., 2007). Besides increased drought, climate change is expected to cause temporal shifts in the occurrence of cold events, too early or too late in respect to the winter period. To overcome these negative consequences on the future performance of beech forests in afforestation programs in Europe, several authors suggest testing and using beech ecotypes that are adapted to a less oceanic climate (Schraml and Rennenberg, 2002; Nielsen and Jørgensen, 2003; Meier and Leuschner, 2008; Rose et al., 2009), such as the refugial beech populations from Southern Europe (Rennenberg et al., 2004; Geßler et al., 2007) that are expected to be adapted both to cold events and extended periods of drought during their growing season (St Clair and Howe, 2007; Fotelli et al., 2009; Eilmann et al., 2014; Thiel et al., 2014; Dounavi et al., 2016).

The adaptive potential of tree populations can be described through various parameters such as growth, survival and shifts in phenology (Eckhart et al., 2017). Seedling growth can be severely affected by abiotic stressors such as temperature and water deficiency. As climate becomes warmer and summer precipitation is expected to decline, beech populations may face intense drought periods (Geßler et al., 2007). Under water stress, plants usually decrease growth in terms of both height and biomass accumulation because of minimization in carbon fixation through photosynthesis. In addition, low soil water potential affects hydraulic traits (e.g., conductivity) and can create xylem cavities leading to plant mortality (Bolte et al., 2016). For this reason, survival under environmental stress is an important adaptive trait, since it reflects the regeneration dynamics of populations (Ngulube, 1989; Sexton et al., 2002; Matías and Jump, 2014).

Leaf phenology is a key adaptive trait that determines carbon balance (production and accumulation) and the overall growth of plant species, while also affecting ecosystem productivity (Kramer et al., 2000; Larcher, 2003). Bud burst, and leaf senescence are the most important leaf phenological traits used in studies, since they mark the onset, duration and ending of a species growth period. Bud burst reflects the transition phase from the winter dormancy to the onset of next year's growth period and requires a preceding chilling period (Heide, 1993; Kramer et al., 2017). Late bud burst can protect trees from late frosts but can also shorten their growth period (Lechowicz, 1984; Višnjić and Dohrenbusch, 2004). Bud burst is referred to be under genetic-provenance control (Robson et al., 2011, 2018), while it is can be also directly affected by environmental factors such as temperature and photoperiod (e.g., Heide, 1993; Yan and Wallace, 1996; Basler and Körner, 2014; Schüler and Liesebach, 2015; Kramer et al., 2017). However, a limited number of studies exist on the possible effect of water availability on bud burst timing of temperate forest trees (e.g., Morin et al., 2010; Kuster et al., 2014). The time of leaf senescence determines the end of the growing period and the onset of winter dormancy and strongly depends on the environmental factors during the current year. For example, premature leaf senescence can be observed under low summer and autumn precipitation, to mobilize leaf nutrients (Sedigheh et al., 2011; Chen et al., 2015; Gill et al., 2015; Tombesi et al., 2015), while leaf senescence can be delayed by higher autumnal temperatures (Fu et al., 2017), as well as by an increased photoperiod (Way and Montgomery, 2014; Gill et al., 2015). Furthermore, it is also influenced by spring leaf phenology (Fu et al., 2014; Keenan and Richardson, 2015; Panchen et al., 2015) and at the same time it can affect leaf flushing in the next year (Heide, 2003).

Since the overall response of plants to abiotic and biotic stressors is determined by both environmental and genetic factors that act in combination, common garden experiments are needed in order to separate environmental from genetic effects on plant adaptive traits and to describe their interactions (Scheepens et al., 2010; Malyshev et al., 2014; de Villemereuil et al., 2016). In addition, provenance tests may contribute to the selection of suitable sources of reproductive material for future forest restoration and management activities (Bezděčková and Matějka, 2015; Carón et al., 2015). Several provenance tests exist in Europe for beech, under field or glasshouse conditions (e.g. von Wühlisch et al., 1995; Nielsen and Jørgensen, 2003; Czajkowski and Bolte, 2006; Gömöry and Paule, 2011; Kreyling et al., 2012; Liesebach, 2012; Schüler et al., 2012; Thiel et al., 2014; Harter et al., 2015; Dounavi et al., 2016; Petkova et al., 2017; Robson et al., 2018). Despite the possible adaptive importance of Greek beech populations for European forestry, no common garden experiments have been established for this species in the country so far. Furthermore, no provenance test in Europe has so far evaluated the adaptive response of beech provenances to expected rainfall distribution patterns under future climate change scenarios.

The aim of our study was to describe the adaptive potential of beech populations in the southeastern part of Europe (N.E. Greece) to climate change. Two hypotheses about the adaptive potential of beech were tested: (a) Beech provenances from sites with longer drought intervals in summer should be better adapted to the expected environmental conditions under climate change and (b) differences in temporal distribution patterns of precipitation should trigger different physiological responses in beech trees. In order to test these hypotheses, a provenance test was established in a growth chamber, where controlled climate change conditions of temperature and precipitation for the year 2050 were simulated. Seedling survival, growth, and leaf phenology were used as adaptive traits for 3 years. Seedling survival was also tested for the same provenances in a field trial under natural conditions allowing comparisons between the adaptability of provenances under both current environmental conditions and the ones anticipated under climate change.

MATERIALS AND METHODS

Area of the Study and Selection of Populations

Northeastern Greece is a mountainous region, with a topographically diverse landscape covering various altitudes. Mt. Rodopi, a long massif shared between Greece and Bulgaria, dominates this region and extends from the east to the west. In the lowlands, the climate is subhumid and submediterranean, with harsh winters and dry summers, while it becomes more humid and temperate with harsh winters and no summer drought in higher altitudes (Mavromatis, 1980). Beech forests are present all over this region, covering habitats with a large variety of environmental conditions (Bergmeier and Dimopoulos, 2001; Tsiripidis et al., 2007). According to genetic and ecological studies, beech populations in N.E. Greece have a complex biogeographic background, since they represent multiple postglacial lineages, originating from different glacial refugia (Hatziskakis et al., 2009; Papageorgiou et al., 2014). At the same time, this region is suggested to be part of a possible introgression zone between two beech species *F. sylvatica* and *F. orientalis* (Gömöry et al., 1999; Hatziskakis et al., 2009, 2011; Kandemir and Kaya, 2009; Papageorgiou et al., 2014; Houston Durrant et al., 2016), with the former species being present in the western part of the region (west Mt. Rodopi, Mt. Falakro, Mt. Menikio) and the latter characterizing the eastern part of the Mt. Rodopi (Christensen, 1997; Tsiripidis and Athanasiadis, 2003; Papageorgiou et al., 2008).

Two beech provenances were chosen in N.E. Greece, "Evros," on the eastern side and "Drama" on the western side of the study area (**Figure 1** and **Supplement Table 1**). Four populations were selected in each provenance (E1–E4 and D1–D4 respectively), representing different postglacial lineages, based on genetic studies (Hatziskakis et al., 2009; Papageorgiou et al., 2014). Due to the absence of long term meteorological data from the area of the selected populations, we used current climatic data available from worldclim.org in a 30-acr seconds resolution (version 1.4) (Hijmans et al., 2005; Souto et al., 2009). Four basic climatic and 19 bioclimatic variables were extracted for the coordinates of each sampled seed parent and the average values were used to describe each population (**Supplement Tables 1**, **3** and **Figures 2A,B**). Provenance Evros represents a climatic environment with moist and cold winters, warm and dry summers with an intense (but not long) drought period. The climate in Drama provenance appears to be more continental with relatively moist and more severe winters as well as warm summers with less intense dry periods (Gouvas and Sakellariou, 2011).

Seed Collection and Germination

Seed collection occurred in autumn 2012, a non-mast production year for beech in the study area, since <50% of the trees produced nuts. In each population, 60–80 seeds per seed tree were collected from 30 trees, totaling 240 families. We consider seeds and later seedlings originating from the same seed tree as a half-sib family. After their transfer to the laboratory, seeds were air-blown to remove the empty ones. The remaining seeds were immersed for 2 min in 35% H_2O_2 (Anand and Chanway, 2013) for disinfection and thoroughly rinsed with tap water for several minutes. After the cleaning procedure, seeds were subjected to cold stratification for 90 days (Baskin and Baskin, 2001) at 0°C in petri dishes filled with sterilized sand. Each dish contained 10 randomly selected seeds per family, totaling 300 seeds per population and 1,200 seeds per provenance. Germination was completed during the stratification stage. Seeds that germinated were transferred to plastic pots filled with turf, sand and perlite in a ratio of 4:2:1, respectively, for further development and evaluation. The emerging seedlings were evaluated as normal or abnormal according to ISTA (2018) specifications. Only normal seedlings were included in the following experiments.

Common Environment Test in a Growth Chamber

In March 2013, the surviving containerized normal seedlings of both provenances were put in a growth chamber under simulated temperature and precipitation levels estimated from the CSIRO MK3 CGM model, according to the expected conditions in the year 2050 (downloaded from Climong.org) (Kriticos et al., 2011) (**Supplement Table 2**). The specific model was selected for its relevance with the summer drought periods in the Mediterranean region (Marcos and Tsimplis, 2008; Syktus et al., 2011; Ziv et al., 2013; Pulvento et al., 2015). The reference area for the climate simulation in the growth chamber was the location "Agios Georgios" (Drama, Greece) that corresponds to population D4 in this study. Climate change scenario A1 and storyline A1B were selected, with the assumption that the industrial development in the areas of the populations of this study will remain minimal and that there will be a balanced use of all energy sources until 2050 (IPCC, 2014). This model incorporates the indirect effects of greenhouse gases in the change of the estimated future bioclimatic parameters (Kriticos et al., 2011).

For each month, the estimated maximum, minimum and mean temperatures, as well as the precipitation data were extracted from the above dataset (CSIRO MK3 CGM) using the DIVA-GIS software (www.diva-gis.org) (**Supplement Table 2**). Using these values, a different temperature schedule (change of daily values at 3-h intervals) for each month was created and applied in the growth chamber. Each separate monthly schedule was stable for all the days of each respective month. Light intensity during the day inside the chamber was set according to *in situ* observations that took place in the reference location under clear sky. Annual height growth between the experimental years 2014-2015 and 2015-2016 was also calculated. Light intensity values inside the chamber were set to a range between 0.010 and 0.025 μmol x s^{-1} following the course of natural daylight. The lengths of day and night for each month were adjusted to those under natural conditions in the reference area.

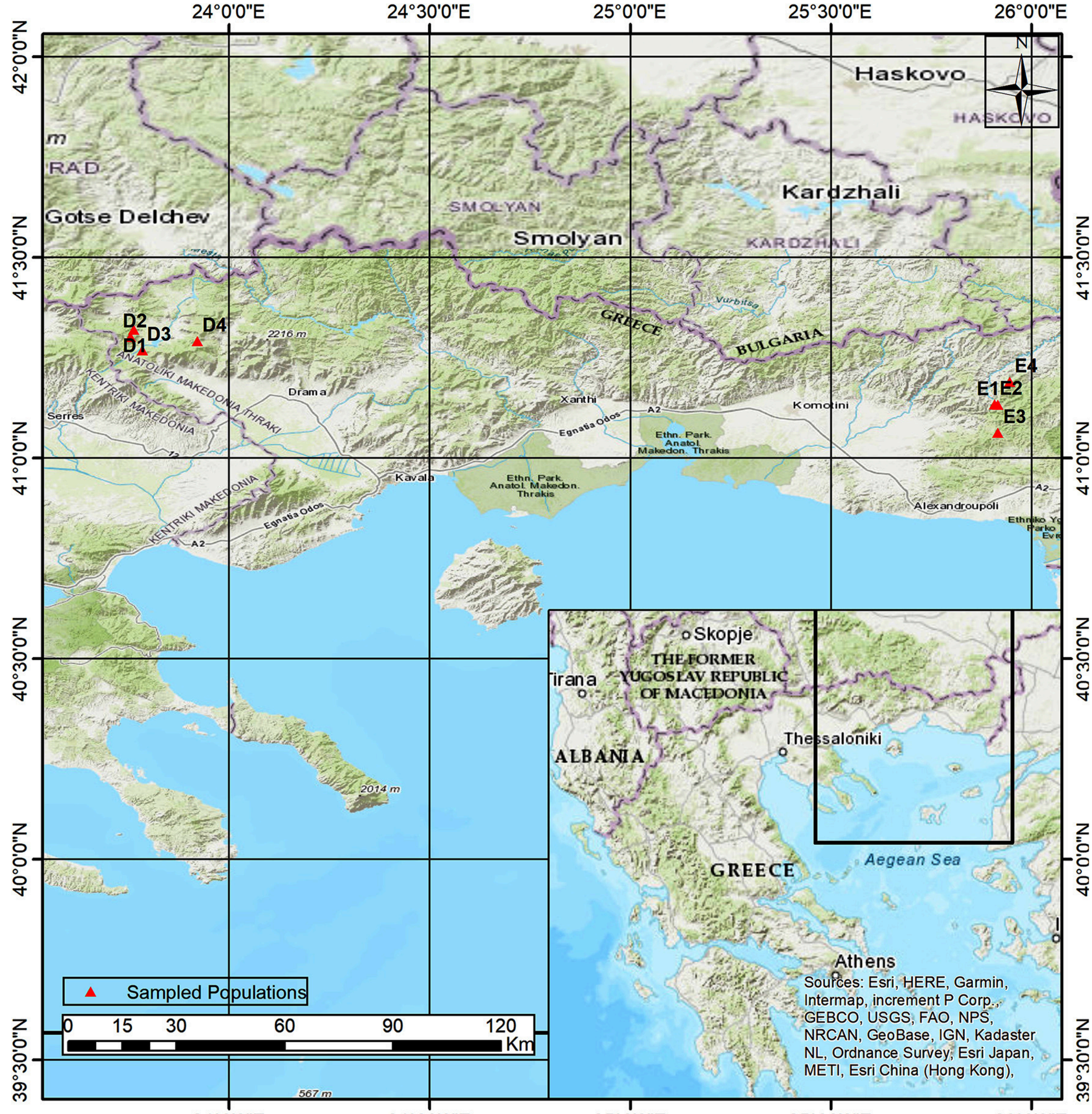


FIGURE 1 | Map of the area of study.

The simulated monthly precipitation height (mm) was converted into water volume (ml) to regulate seedling irrigation, following Brouwer et al. (1985). Besides precipitation height, we tested the effect of precipitation distribution within a month on seedlings, especially during the summer period, since climate change is expected to destabilize the current precipitation frequency and intensity and cause longer drought periods, as well as climate extremes in the Eastern Mediterranean region (Alpert et al., 2002; Gao et al., 2006; Lelieveld et al., 2012). For this reason, precipitation was distributed within each month according to two different irrigation schemes:

1. Irrigation scheme A1 (non-frequent): irrigation of seedlings every 20 days with the relevant amount of water of the corresponding month (50% of monthly precipitation height when irrigation occurred twice a month, or 100% of monthly precipitation height when irrigation occurred once a month).
2. Irrigation scheme A2 (frequent): irrigation of seedlings every seven days with 25% of the simulated monthly precipitation height.

One seedling per family and per irrigation scheme was included in the experiment. Seedlings representing population D1 were abnormal in a high ratio (66%), producing finally a limited number of viable normal seedlings that were not enough to represent D1 in both irrigation schemes. For this reason, D1 was excluded from the experiment in the growing chamber and the

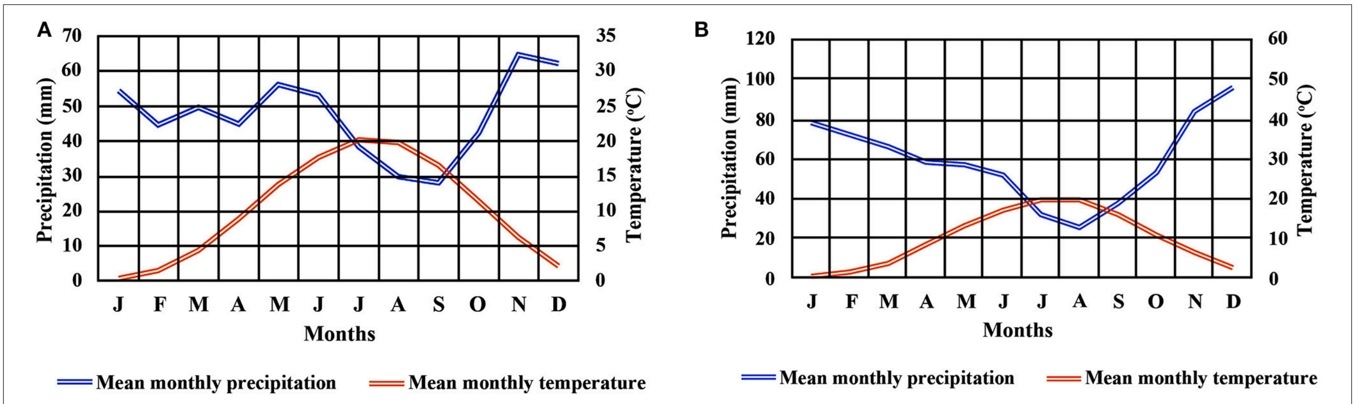


FIGURE 2 | Climate diagram for provenances Drama **(A)** and Evros **(B)**.

viable normal seedlings from this population were used only in the common garden test under field conditions.

The growth chamber experiment lasted for 3 years (2013–2016). Measurements included seedling height, survival and leaf phenological traits (Madsen, 1994; Minotta and Pinzauti, 1996; Hiura, 1998; Whiteley et al., 2003; Kanaga et al., 2008). Seedling survival was monitored every week and the non-surviving ones were removed. Seedling height was measured at the end of each growing season from October 2014 until October 2016. Annual height growth between the experimental years 2014–2015 and 2015–2016 was also calculated. Phenology measurements included bud burst (leaf emergence), leaf senescence and duration of the growing period. Both parameters were expressed in number of days. The beginning of bud burst was considered to occur when the bud scales opened in a way that the newly emerged leaves were visible. Individual plants were considered entering the senescence stage when at least 50% of their leaves were discolored (Gömöry et al., 2015). Measurements of phenological traits were conducted in 3 day–intervals. The plastic response between subsequent experimental years was expressed for all traits as the difference of the annual mean values between these years.

Provenance Experiment Under Field Conditions

A common environment provenance test under natural field conditions was set up in Agios Georgios (Drama, Greece), the same location that was used as a reference for the climate simulation in the growth chamber. An area of 100 m^2 was fenced and a total number of 480 seedlings (two seedlings/family/population) were planted in a natural beech stand with dense crown closure (70–80%). Seedlings were planted with the soil substrate of their original pots (4 turf/2 sand/1 perlite). Survival measurements were taken two times per year during the months of October and April for 3 years. The survival percentage at the end of the 3 years experiment was used in this study. This experimental site was established in order to provide a direct comparison of seedling survival between the simulated future conditions applied in the growth chamber and the current natural forest conditions. Due to the absence of meteorological stations in the broader area and the lack of ecological studies for the specific site, the local environmental conditions were not described in detail and they were not monitored throughout the experiment.

Basic Statistical Analysis

A comparison of means among populations and between provenances, was performed for the variables measured in the seedlings growing in the growth chamber and in the field. Differences in seedling survival among populations were described through a repeated pairwise chi-square test, since survival was scored as a binary variable. The same test was also used to evaluate the differences between the irrigation schemes, separately for each population. Mean differences in seedling height and annual growth were tested for significance using the analysis of variance (ANOVA) and the LSD criterion, since normal distribution and homoscedasticity of data were proven. This comparison was performed separately for each irrigation scheme and year of the study. Pairwise differences in all phenological traits between populations for each irrigation scheme and year were tested for significance, using the non-parametric Mann-Whitney U test, since normal distribution assumptions were not met. For all comparisons, the software STATISTICA v.10 (STATSOFT inc) was used.

Hierarchical Linear Multilevel Models (HLM)

To assess the effect of provenance, population and irrigation scheme on the dependent variables (all traits used in this study), we utilized a three-level hierarchical linear modeling approach (HLM; Raudenbush and Bryk, 2002), that considers the nested structure of the data in this study. The implementation of this modeling approach is standard in a variety of disciplines (Afshartous and Wolf, 2007) with varying terminology depending on discipline (the hierarchical model is also known as the mixed-effects model, the random-coefficient model, and in the context of panel data, the repeated-measures or growth-curve model). A major advantage in this type of model over the standard regression models, is the within group and between groups comparison and the improved accuracy of point estimates in model parameters (e.g., Katahira, 2016). The framework of the model considers n_{ijk} responses on the dependent variables, nested within the populations ($i = 1, 2, \ldots, 8$) which are again nested within the provenances of Evros and Drama ($j = 1, 2$). The last level of this nested modeling structure is completed with the inclusion of the two different irrigation schemes ($k = 1, 2$). The first level of the model is described as:

$$\mathbf{y_{ijk}} = \boldsymbol{\beta}_{\mathbf{0ijk}} + \boldsymbol{\beta}_{\mathbf{1ijk}} \times \mathbf{X_{ijk}} + \mathbf{e_{ijk}} \tag{1}$$

where $\mathbf{y_{ijk}}$ is the trait as a continuous response variable, $\mathbf{X_{ijk}}$ denotes the level-1 predictor variable of populations nested within each provenance and $\mathbf{e_{ijk}} \sim \mathbf{N}(\mathbf{0}, \boldsymbol{\sigma}^2_{\mathbf{e}})$ is the observation-level deviation which is normally distributed. The β - coefficients of the slope in equation (1) are subsequently used as a response variable (second level):

$$\boldsymbol{\beta}_{\mathbf{1ijk}} = \mathbf{b_{0jk}} + \mathbf{b_{1jk}} \times \mathbf{X_{jk}} + \mathbf{r_{jk}} \tag{2}$$

where $\mathbf{X_{jk}}$ is the level-2 predictor factor (provenance) and $\mathbf{r_{jk}} \sim \mathbf{N}(\mathbf{0}, \boldsymbol{\sigma}^2_{\mathbf{r}})$ expresses the normally-distributed deviations at the provenance level. Finally:

$$\mathbf{b_{1jk}} = \boldsymbol{\gamma}_{\mathbf{0k}} + \boldsymbol{\gamma}_{\mathbf{1k}} \times \mathbf{X_k} + \mathbf{u_k} \tag{3}$$

where X_k denotes the irrigation scheme factor and $u_k \sim N(0, \boldsymbol{\sigma}^2_{\mathbf{u}})$.

Variables "population," "provenance," and "irrigation scheme" were the fixed effects in our models, whereas random effects terms were the corresponding errors not explained by the three fixed effects, i.e., $\mathbf{e_{ijk}}$, $\mathbf{r_{jk}}$ and $\mathbf{u_k}$. For the provenance factors, "Evros" was used as a reference category, while the first population within each provenance was used as a reference category for the population factors. Finally, we have used the irrigation scheme A1 as a reference category of the irrigation scheme.

Acknowledging the universal principle that no true model exists (Box and Draper, 1987), we choose not to fit a unique model but instead fit several candidate (nested) models in terms of varying the number of covariates included as predictors, in order to select a final optimal model among these candidates, based on appropriately balancing goodness-of-fit with simplicity and utilizing the appropriate evaluation criterion. We did not opt for stepwise (either forward/backward) elimination methods for variable selection since the latter have been recognized to suffer from significant drawbacks (Hurvich and Tsai, 1990; Roecker, 1991). Hence, model fit was assessed by initially fitting the null model (Model 1), which includes only the grand mean as predictor. One new predictor variable is added for each subsequent model. While Model 1 includes only the intercept, Model 2 introduces the factor of the populations and Model 3 adds the component of provenances. Finally, Model 4 additionally includes the factor of irrigation scheme and essentially corresponds to the full model presented in equations (1–3). Every next model is compared in terms of fit performance with the previous one. The overall significance of each model is then evaluated through the likelihood ratio statistic (LRT) (based on the likelihood of each model), which acts as a stand-alone measure of goodness-of-fit, as well as through the model comparisons. The LRT is only valid if used to compare hierarchically nested models, as applied in our research. LRT is calculated through the following likelihood ratio statistic:

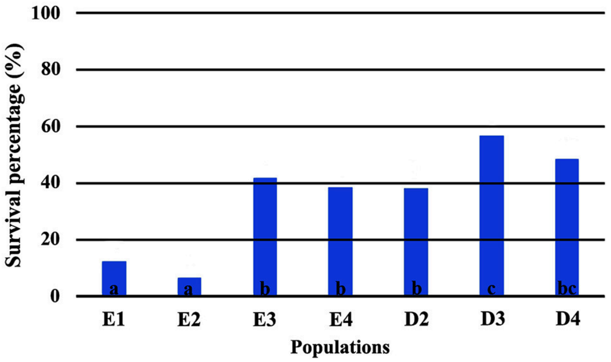


FIGURE 3 | Survival percentages in the field*. *Values followed by the same letter do not differ among populations at 0.05 level of significance.

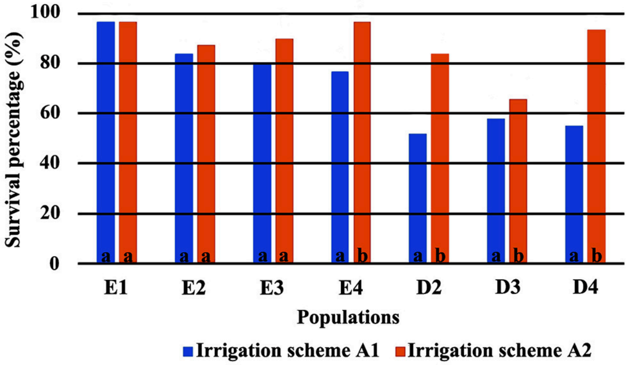


FIGURE 4 | Survival percentages in the growth chamber *. *Values followed by the same letter do not differ between irrigation schemes for each population separately.

$$\mathbf{D} = \mathbf{2} \times \left(\mathbf{ln}\left(\mathbf{likelihood}_{\mathbf{M_i}}\right) - \mathbf{ln}\left(\mathbf{likelihood}_{\mathbf{M_{i-1}}}\right)\right) \tag{4}$$

where M_{i-1} denotes the reduced model and M_i the model with the additional parameter. The lme4 library (Bates et al., 2012) of the R software was used to fit the HLM models.

RESULTS

Seedling Survival

The direct comparison of survival percentages and the HLM model comparison produced similar results as far as seedling survival is concerned. The lowest survival rates were observed under field conditions for all populations, in comparison with the ones observed in the growth chamber (**Figures 3**, **4**). Populations E1 and E2 showed the lowest and population D3

TABLE 1 | Significance of differences (*p*-values) in final seedling survival among populations under irrigation schemes.

Population	E1	E2	E3	E4	D2	D3	D4
IRRIGATION SCHEME A1							
E1	–						
E2	ns	–					
E3	0.047	ns	–				
E4	0.024	ns	ns	–			
D2	0.000	0.010	0.029	ns	–		
D3	0.000	0.027	ns	ns	ns	–	
D4	0.000	0.016	0.044	ns	ns	ns	–
IRRIGATION SCHEME A2							
E1	–						
E2	ns	–					
E3	ns	ns	–				
E4	ns	ns	ns	–			
D2	ns	ns	ns	ns	–		
D3	ns	ns	ns	ns	ns	–	
D4	ns	ns	ns	ns	ns	ns	–

ns, non significant.

and the local population D4 the highest survival in the field (**Figure 3**). In the growth chamber, average survival was generally higher under frequent irrigation (scheme A2) than under less frequent irrigation (scheme A1). Besides E1 that demonstrated equal survival for both irrigation schemes, all populations showed lower survival percentages under A1 (**Figure 4**). A clear provenance pattern was observed in the chamber, with populations from Evros surviving better under longer drought intervals (A1 scheme) (**Figure 4** and **Table 1**). No significant differences in seedling survival were found among populations under short drought intervals (scheme A2) (**Table 1**).

When the results of both experiments were considered in total, the comparison of the HLM models showed that only the growing environment had a significant effect on seedling survival (**Tables 2**, **3**). Separately for irrigation scheme A1 in the growth chamber, provenance demonstrated a significant effect on survival, while no effect was recorded under A2. In the field experiment, population was the only significant factor affecting seedling survival. Accordingly, the partition of variance for the overall survival in both experiments was explained to a great extent by the different growing environments (73%), while provenance and population had no effect at all (**Figure 5D**). A larger proportion of variance was explained by population than by provenance under field conditions and under the irrigation scheme A1 in the growth chamber, while under A2 both provenance and population accounted for a small fraction of the overall variance (10.8 and 10.3%, respectively).

Seedling Height and Growth

In the growth chamber, seedlings from most populations reached a greater height under longer drought intervals (scheme A1), but this trend was statistically significant only for populations E1 and D3 during the first 2 years of the study (2014 and 2015) and for E3 in the first year (2014) only (**Table 4** and **Supplement Table 3**). All differences between irrigation schemes A1 and A2 observed in 2014 and 2015 disappeared in 2016. Height increment between years 2015–2016 was almost halved in comparison to years 2014–2015 (**Figures 6A,B**). Significant differences in seedling height among populations were found only under irrigation scheme A2 during years 2014 and 2015 but not in 2016 (**Table 4**).

Seedling height in the growth chamber was not influenced significantly by population or provenance, according to the HLM model comparison. A significant influence of provenance was detected for height increment between 2015 and 2016 (**Table 5**), since provenance Drama demonstrated lower height growth than Evros during the same period (**Supplement Table 3**). The irrigation scheme was a factor that significantly influenced seedling height in 2015 and growth between 2014–2015 and 2015–2016 (**Table 5**). A large proportion of the total variance in height and growth traits was explained by the irrigation schemes after the first (6.5%) and even more so after the second year (24.4%), but not after the third year of the study. Provenances and populations explained smaller proportions of the total variance in 2014 (3.0 and 5.0%, respectively), had no effect in 2015 and explained a higher proportion of the phenotypic variance in seedling height in 2016 (6.6 and 9.3%, respectively). Provenance and population had no influence on seedling height increment between 2014–2015 but accounted for a larger fraction of variance in height increment between 2015–2016 (10.1 and 15.0%, respectively) (**Figures 5A–C**).

Leaf Phenological Traits

In the growth chamber, beech seedlings demonstrated longer growing periods in all populations and during all years under frequent irrigation (scheme A2), in comparison to scheme A1, where irrigation was less frequent and drought intervals were longer. In the spring of 2014, bud burst occurred significantly earlier in the growth chamber under irrigation scheme A2, in comparison to A1, for seedlings belonging to all populations besides E1 and E4 (**Table 6**). However, in 2015, only populations E2 and E3 continued to have significant differences in bud burst between A1 and A2, while in 2016, no significant differences could be observed between the two irrigation schemes. For leaf senescence, significant differences between the irrigation schemes were observed in all populations of Drama in 2014 and in some populations in 2015 (D3 and D4), but not in 2016 (**Table 6**). In all cases for which significant differences were observed, leaf senescence under A2 occurred later than under A1. Significant differences in the length of the growing period between A1 and A2 schemes were present for all populations besides E1 and E4 in 2014 (**Table 6**). In 2015, only population E3 did not differ significantly between A1 and A2 and finally in 2016 no difference between the two schemes was detected.

Differences in leaf phenological traits were also observed among populations within each irrigation scheme. Under longer drought intervals (scheme A1), differences in bud burst among populations were recorded in all years of the experiment, while such differences were observed in 2015 and 2016 only, when

TABLE 2 | Model comparisons of the HLM models for the survival data via LRT (D: likelihood ratio statistic; P: p-value of the statistical significance of LRT test).

Dependent variables	**Model 1 (null model)**	**Model 2 (population effects)**		**Model 3 (model 2 + provenance effects)**		**Model 4 (model 3 + irrigation scheme effects)**	
	D	***D***	***P***	***D***	***P***	***D***	***P***
Survival (complete)	492.86	487.04	ns	485.17	ns	469.67	0.000
Survival under A1 scheme	182.39	178.73	ns	167.20	0.000		
Survival under A2 scheme	69.77	61.23	ns	59.41	ns		
Survival in field conditions	183.72	165.37	0.005	162.13	ns		

TABLE 3 | Parameter estimates for the best selected models (5% level of significance) for survival data.

Independent variables	**Dependent variables**			
	Survival (complete)	**Survival under A1 scheme**	**Survival under A2 scheme**	**Survival in field conditions**
Intercept (β_{0ijk})	0.76	0.96	0.88	0.23
Plot in Evros (Ref: E1)				
E2	ns	ns		ns
E3	ns	ns		0.15
E4	n.s.	ns		0.16
	Plot in Drama (Ref: D2)			**Plot in Drama** (Ref: D1)
D2	–	–		ns
D3	ns	ns		0.32
D4	ns	ns		0.26
Provenance (Ref: Drama)				
Evros	ns	0.41		
Irrigation (ref: Irrigation scheme A1)				
Irrigation scheme A2	0.14			
Irrigation G	−0.38			

ns, non significant.

irrigation was more frequent (scheme A2) (**Table 6**). In the first year of the experiment (2014), under A1, population E1 had the earliest bud burst and D4 the latest. This pattern largely changed in 2015, with E1 and E4 demonstrating the latest bud burst, while all Drama populations showed the earliest bud burst. In both 2015 and 2016, seedlings belonging to provenance Evros demonstrated a delay in bud burst in comparison to provenance Drama, under both irrigation schemes.

For leaf senescence, differences among populations in 2014 were found only under frequent irrigation (A2 scheme), in contrast to bud burst. No difference was recorded in 2015 for both schemes, while minor differences among populations were recorded in 2016 (**Table 6**). Under longer drought intervals (irrigation scheme A1), differences among populations occurred in the third year of the experiment (2016), with seedlings belonging to population E3 demonstrating a later leaf senescence than the remaining populations. Under A2, leaf senescence occurred the earliest in population E2 and the latest in D2 and D3 during 2014, while in 2016 only population D3 showed a significantly later leaf senescence.

Populations demonstrated significant differences among each other in the duration of the growing period for both irrigation schemes and for all years (**Table 6**). Under less frequent irrigation (scheme A1), populations E1 and E4, contrary to E2 and E3, had the longest vegetation period in 2014, but the shortest in 2015 and 2016. On the contrary, under the same irrigation scheme, populations D3 and D4 had the shortest growing period in 2014, which gradually increased in 2015 and 2016, as compared to D2. Populations belonging to Drama provenance had a longer growing period during all years under frequent irrigation (scheme A2), especially during 2016.

Seedlings demonstrated plastic responses between subsequent years in this study (**Figure 7**). The greatest delay in bud burst between years 2014–2015 was found for population E1 (+19 days), while seedlings belonging to D3 and D4 flushed much earlier in the second year of the study (−21 and −20 days, respectively), under A1. Under irrigation scheme A2, populations E2 and E4 had a significantly delayed bud burst in 2016 as compared to 2015. For leaf senescence, all populations showed a similar plastic response trend for both irrigation schemes A1

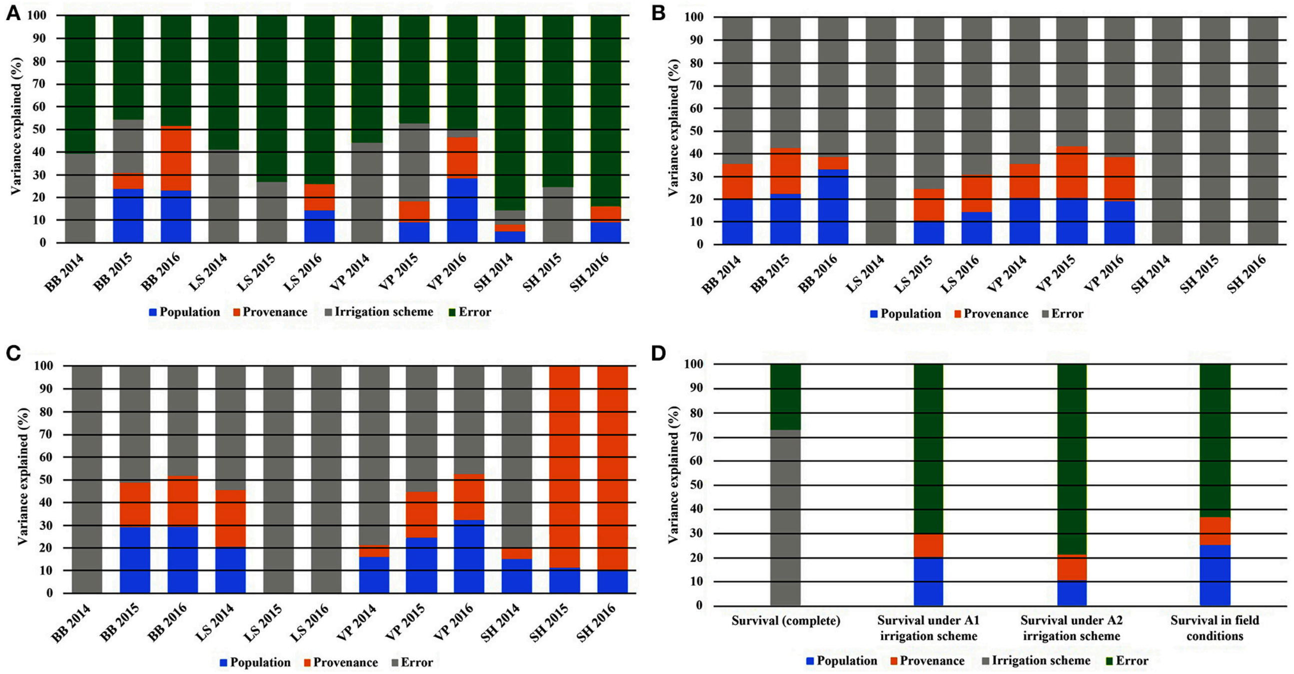


FIGURE 5 | Partitioning of variance across the years of the study for the traits of leaf phenology* and seedling height overall **(A)**, under irrigation scheme A1 **(B)**, under irrigation scheme A2 **(C)**, and for seedling survival **(D)**. *Abbreviations: BB, Bud burst; LS, Leaf senescence; VP, Growing period; SH, Seedling height.

and A2 (**Figure 7**). Seedlings presented an earlier leaf senescence in 2015 as compared to 2014, and a later leaf senescence in 2016 as compared to 2015. However, results were more pronounced under scheme A1 and the populations showing the largest response were E1, D2 and D3. Finally, the two provenances (Evros-Drama) showed different plastic response trends for the length of the growing period. The largest plastic response was observed under A1, where the populations from Evros, especially E1 and E4, had a shorter growing period in 2014–2015 than in 2015–2016, while D3 and D4 had a longer one for the same year. In 2016, all populations demonstrated an increased growing period under A1. An opposite trend was observed under irrigation scheme A2, with all populations having a shorter growing period in 2015 and a longer one in 2016 (**Figure 7**).

The comparison of the HLM models revealed a similar trend for the timing of bud burst, leaf senescence and the length of the growing period, during the 3 years of the experiment (**Table 5**). The irrigation scheme had a significant influence on all traits in the first 2 years of the experiment (2014 and 2015), but not for the last year (2016). An opposite trend was observed for population and provenance that had significant effects on bud burst and growing period in 2015 and 2016, but not in 2014. Provenance showed a significant influence on the time of leaf senescence only in the first year and population only in the last year of the study. The frequency of irrigation (schemes A1 or A2) was the only fixed effect explaining a significant proportion of variance in 2014 for all traits, while in 2016, only (or mostly) provenance and population explained part of the variance. Provenance explained a larger proportion of variance (28.6%) than population (22.8%) for bud burst in the third year of the study, while an opposite trend was observed for leaf senescence (14.5% for population and 11.3% for provenance) and the growing period (28.3% for population and 18.6% for provenance).

DISCUSSION

Seedling Survival and Height Growth

Seedling mortality in the growth chamber, for all populations and provenances, was observed exclusively during the first year of the experiment. In the environment of the growth chamber, irrigation frequency proved to be an important factor for seedling survival, since mortality increased when irrigation occurred after long intervals of drought (A1 scheme). In the field, the decisive factor for seedling survival proved to be the exposure to winter conditions. Frost events were not simulated in the growth chamber and seedlings survived better in the growth chamber than in the field, probably for this reason. This trend, where the largest differences in survival among populations occur when conditions are unfavorable, is common in provenance tests for beech (e.g., Banach et al., 2015).

Seedlings from Evros provenance generally presented higher survival in the growth chamber, under drought conditions, indicating a possible adaptation to prolonged drought intervals, since summer drought in Evros lasts longer and temperatures are generally higher than in Drama. Among the Evros populations, E4 presented significantly lower seedling survival under less frequent irrigation, indicating sensitivity to longer periods without rain. Indeed, population E4 is located in an area

TABLE 4 | Total seedling height at the end of each growth period under irrigation schemes A1 and A2 (Mean values±SE).

Population	**Seedling height (cm)***					
	2014		**2015**		**2016**	
	A1	**A2**	**A1**	**A2**	**A1**	**A2**
E1	10.71 ^{A,a} (±0.48)	9.00 ^{A,b} (±0.41)	15.52 ^{A,a} (±0.79)	12.87 ^{AB,b} (±0.68)	17.27 ^{A,a} (±0.92)	15.42 ^{A,a} (±0.62)
E2	10.27 ^{A,a} (±0.61)	9.63 ^{AB,a} (±0.72)	14.59 ^{A,a} (±0.63)	12.85 ^{AB,a} (±1.14)	15.81 ^{A,a} (±1.08)	14.32 ^{A,a} (±0.68)
E3	12.13 ^{A,a} (±0.60)	10.73 ^{AB,b} (±0.27)	15.46 ^{A,a} (±0.80)	14.75 ^{B,a} (±0.94)	17.56 ^{A,a} (±0.98)	16.92 ^{A,a} (±0.74)
E4	10.70 ^{A,a} (±0.58)	11.08 ^{B,a} (±0.53)	14.36 ^{A,a} (±0.83)	14.69 ^{B,a} (±0.76)	16.42 ^{A,a} (±0.52)	17.17 ^{A,a} (±0.73)
D2	11.19 ^{A,a} (±0.78)	10.44 ^{AB,a} (±0.72)	14.93 ^{A,a} (±1.27)	13.70 ^{AB,a} (±0.63)	17.92 ^{A,a} (±1.26)	17.08 ^{A,a} (±1.22)
D3	10.58 ^{A,a} (±0.99)	8.98 ^{A,b} (±0.94)	15.06 ^{A,a} (±1.01)	12.02 ^{A,b} (±0.85)	17.50 ^{A,a} (±2.13)	15.83 ^{A,a} (±1.56)
D4	10.92 ^{A,a} (±1.03)	10.60 ^{AB,a} (±0.55)	14.90 ^{A,a} (±1.46)	13.41 ^{AB,a} (±0.72)	16.06 ^{A,a} (±1.31)	16.55 ^{A,a} (±0.83)

**Values within columns followed by the same capital letter do not differ among populations for each irrigation scheme and year of study. Values within rows followed by the same small letter do not differ between irrigation schemes per population for each year of study separately.*

covered with beech forests characterized as an "island" of oceanic climate. This is emphasized by the frequent occurrence of *Galium rotundifolium*, a plant species acting as good indicator of oceanic climate (Ellenberg et al., 1992), which is absent in the Rodopi mountains, besides the area surrounding population E4. High variability in seedling survival and its response to irrigation frequency was also observed among populations within the Drama provenance, a possible indication of local adaptation of beech to the different microenvironments of each population.

In the field trial, survival results suggest the existence of a strong local adaptation pattern for beech seedlings, as other researchers also report (see Kreyling et al., 2012, 2014). The highest survival rate was observed for the two populations with a geographical origin closest to the location of the test site (D3 and D4). On the contrary, the more distant populations E1 and E2, that demonstrated the highest survival rates under drought conditions in the growth chamber, had the highest mortality rates under field conditions, in agreement with other beech common garden trials (Banach et al., 2015), although the opposite trend has been also reported (e.g., Sułkowska, 2004; Hofmann et al., 2015; Müller and Finkeldey, 2017). It is worth noting that adaptive differentiation to the colder conditions in the field experiment, as expressed in survival rates, was observed mainly among populations within provenances. Thus, seedlings from the distant Evros populations E3 and E4 survived as well as some populations of the local Drama provenance, indicating that environmental heterogeneity at a smaller geographical scale can create significant adaptive differentiation.

Under both irrigation schemes in the growth chamber and at the end of the third year all seedlings achieved similar heights without differences among populations and provenances. Similarly, Harter et al. (2015) did not find any differences in seedling height between two beech provenances under water deficit for 60 days. Various studies report that non-frequent irrigation leads to lower shoot height in seedlings (Arend et al., 2011; Thiel et al., 2014). However, in the current study, seedling height was generally larger under long drought intervals (irrigation scheme A1) rather than more frequent irrigation (scheme A2). In our study, the distribution pattern of water was more critical for seedling growth than the absolute amount of water received. It is possible that fewer but significant rain events may yield higher biomass increase than more frequent but minor events as it is already reported for plant communities (e.g., Bates et al., 2006; de Dios Miranda et al., 2010). This highlights the necessity for considering the rainfall patterns in terms of frequency and quantity rather than rainfall means alone as factor affecting the adaptation of plants (de Dios Miranda et al., 2009). Since this kind of irrigation pattern comparison has not yet been performed in any other common environment study for forest trees, the growth behavior of beech seedlings in this experiment needs to be further investigated using physiological and anatomical traits (Bolte et al., 2016). In any case, these responses to irrigation frequency seem to be temporary and reversible after a short time, revealing the existence of possible trade-offs between different traits as part of a complex adaptive strategy aiming at the best possible use of the available water.

Leaf Phenological Traits

In our experiment, the two irrigation frequency schemes produced different leaf phenological responses at all traits during the first 2 years of the study. In the third year, the differences in leaf phenological traits were influenced mainly by provenance and population. Considering the adaptive significance of the timing of bud burst and leaf senescence, which define the length of the growing period, we assume that seedlings probably needed a certain period of time until they were able to physiologically adapt to the growth chamber conditions and irrigation schemes.

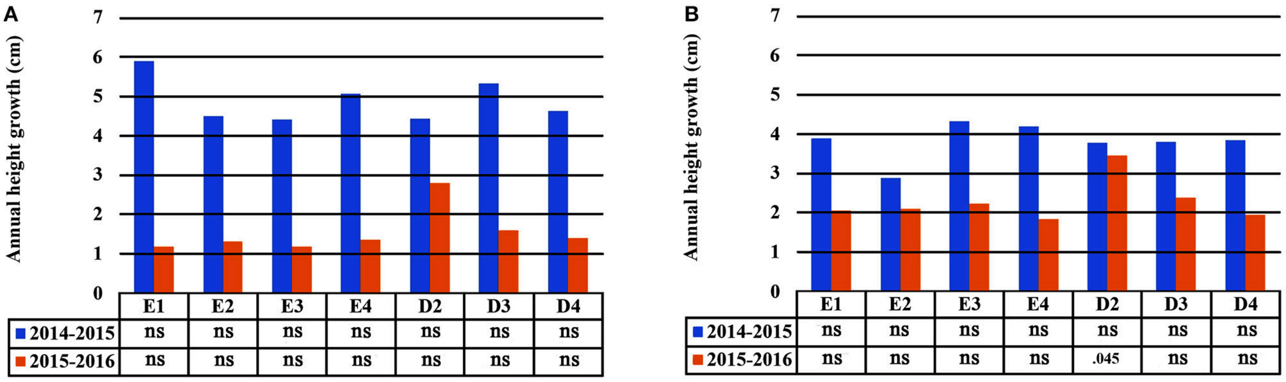


FIGURE 6 | Plastic response of populations in seedling height between consecutive years of the experiment under irrigation schemes A1 **(A)** and A2 **(B)**.

TABLE 5 | Model comparisons of the HLM models for the complete data via LRT (D: likelihood ratio statistic; P: p-value of the statistical significance of LRT test).

Dependent variables	Model 1 (null model)	Model 2 (population effects)		Model 3 (model 2 + provenance effects)		Model 4 (model 3 + irrigation scheme effects)	
	D	*D*	*P*	*D*	*P*	*D*	*P*
BB 2014	1247.2	1242.2	ns	1240.7	ns	1232.5	0.004
BB 2015	1241.8	1212.8	0.000	1191.0	0.000	1183.9	0.000
BB 2016	1267.4	1230.3	0.000	1207.3	0.000	1204.2	ns
LS 2014	923.05	912.1	ns	900.56	0.000	893.7	0.008
LS 2015	1068.8	1060.9	ns	1059.2	ns	1052.2	0.008
LS 2016	1028.3	1014.7	0.018	1014.6	ns	1014	ns
VP 2014	1267.4	1261.5	ns	1261.5	ns	1252.7	0.003
VP 2015	1281.3	1256.3	0.000	1233.7	0.000	1225.2	0.000
VP 2016	1302.3	1270.0	0.000	1251.5	0.000	1248.1	ns
SH 2014	680.42	673.39	ns	673.24	ns	672.74	ns
SH 2015	771.03	763.74	ns	763.43	ns	758.45	0.025
SH 2016	772.97	766.08	ns	765.00	ns	762.97	ns
GD 2014-20105	690.65	687.61	ns	686.32	ns	681.12	0.022
GD 2014-2015	527.75	521.75	ns	506.35	0.000	500.70	0.017

ns (non significant). Symbols: BB, Bud burst; LS, Leaf senescence; VP, Growing period; SH, Seedling height; GD, Height difference.

The results of our study imply that the duration of the growing period in beech seedlings was mostly determined by the timing of bud burst, while the differences between populations, provenances, and irrigation schemes in leaf senescence were less pronounced. Bud burst occurred earlier and leaves matured later under frequent irrigation (scheme A2) especially during the first years of the study, a trend that was consistent for all populations. Thus, the existence of longer drought intervals between irrigation events (scheme A1) has shortened the growing period of plants in the chamber, an expected response to stressful conditions.

Bud burst is considered to be under strong genetic control (Dittmar and Elling, 2006; Doi et al., 2010; Abbott et al., 2015; Gömöry et al., 2015; Müller et al., 2017) and provenances often show adaptive differences in this trait in common environment experiments, that correspond to specific environmental conditions at the sites of origin (von Wühlisch et al., 1995; Schüler et al., 2012; Kramer et al., 2017). Provenance tests all over the continent describe a general trend of populations growing in warmer and less continental climates to have an earlier bud burst than populations from colder climates (Robson et al., 2011, 2018). Extrapolating this trend to the provenances and populations of our study, we would expect provenance Evros to have an earlier bud burst than Drama. While this was indeed the case in the first year of the study in the growth chamber, the phenological trend reversed during the next 2 years. Thus, in the first year of the growth chamber experiment, provenance Evros had an earlier bud burst and a longer growing period than Drama, while in the next years, bud burst had shifted to a later date for Evros and to an earlier date for Drama. These findings show that provenances initially demonstrated the expected genetic response matching the environmental conditions at their sites of origin, with Drama having a cooler and more temperate climate than

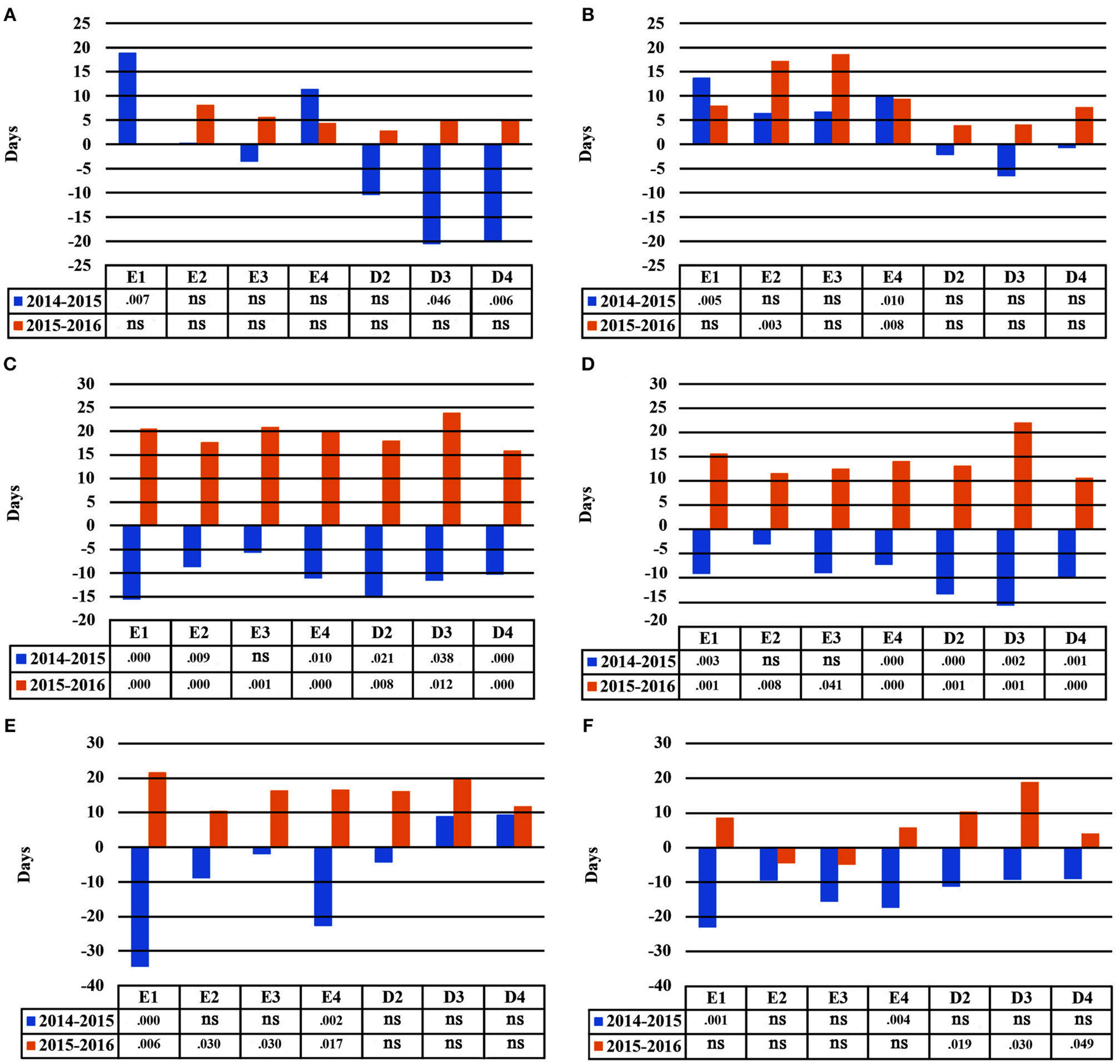
TABLE 6 | Phenology of seedlings under irrigation schemes for the years of study (Mean values±SE).

Population	2014		2015		2016	
	A1	**A2**	**A1**	**A2**	**A1**	**A2**
BUD BURST DAYS*						
E1	$101^{A,a}$ (±4.21)	$98^{A,a}$ (±3.26)	$120^{A,a}$ (±2.83)	$111^{A,a}$ (±2.57)	$120^{AC,a}$ (±2.94)	$119^{A,a}$ (±4.60)
E2	$114^{BC,a}$ (±3.90)	$97^{A,b}$ (±2.64)	$114^{AB,a}$ (±2.48)	$103^{AB,b}$ (±4.01)	$122^{A,a}$ (±2.72)	$120^{A,a}$ (±2.28)
E3	$117^{BC,a}$ (±4.71)	$94^{A,b}$ (±4.22)	$113^{AC,a}$ (±5.20)	$100^{AB,b}$ (±5.33)	$119^{AC,a}$ (±3.81)	$119^{A,a}$ (±5.38)
E4	$107^{AB,a}$ (±4.91)	$100^{A,b}$ (±3.01)	$119^{A,a}$ (±4.37)	$111^{A,a}$ (±3.01)	$123^{A,a}$ (±3.09)	$120^{A,a}$ (±2.87)
D2	$115^{ABC,a}$ (±3.27)	$95^{A,b}$ (±2.24)	$105^{BCD,a}$ (±6.07)	$93^{B,a}$ (±2.24)	$108^{BC,a}$ (±5.17)	$97^{B,a}$ (±3.27)
D3	$116^{BC,a}$ (±6.27)	$99^{A,b}$ (±3.04)	$96^{D,a}$ (±4.19)	$92^{B,a}$ (±3.04)	$101^{B,a}$ (±4.15)	$96^{B,a}$ (±6.01)
D4	$125^{C,a}$ (±1.71)	$94^{A,b}$ (±2.09)	$105^{CD,a}$ (±4.92)	$94^{B,a}$ (±2.09)	$110^{AB,a}$ (±5.82)	$101^{B,a}$ (±4.50)
LEAF SENESCENCE DAYS*						
E1	$293^{A,a}$ (±0.72)	$295^{AB,a}$ (±1.45)	$278^{A,a}$ (±1.22)	$286^{A,b}$ (±2.36)	$298^{A,a}$ (±2.44)	$302^{A,a}$ (±2.35)
E2	$294^{A,a}$ (±0.93)	$293^{A,a}$ (±0.81)	$285^{A,a}$ (±2.05)	$290^{A,a}$ (±2.23)	$303^{A,a}$ (±1.49)	$302^{A,a}$ (±2.78)
E3	$292^{A,a}$ (±0.10)	$297^{AB,a}$ (±2.69)	$286^{A,a}$ (±2.47)	$288^{A,a}$ (±3.69)	$307^{B,a}$ (±1.82)	$300^{A,a}$ (±2.20)
E4	$293^{A,a}$ (±0.70)	$296^{AB,a}$ (±0.99)	$282^{A,a}$ (±3.01)	$288^{A,a}$ (±1.54)	$302^{A,a}$ (±1.09)	$302^{A,a}$ (±1.27)
D2	$295^{A,a}$ (±2.18)	$302^{B,b}$ (±1.62)	$281^{A,a}$ (±3.00)	$288^{A,a}$ (±2.26)	$299^{A,a}$ (±1.35)	$301^{A,a}$ (±1.90)
D3	$292^{A,a}$ (±0.10)	$304^{B,b}$ (±2.13)	$280^{A,a}$ (±4.05)	$288^{A,b}$ (±0.10)	$304^{AB,a}$ (±4.30)	$310^{B,a}$ (±0.10)
D4	$294^{A,a}$ (±1.64)	$299^{AB,b}$ (±1.80)	$283^{A,a}$ (±2.26)	$289^{A,b}$ (±1.83)	$299^{A,a}$ (±1.07)	$300^{A,a}$ (±1.33)
GROWTH PERIOD DAYS*						
E1	$191^{A,a}$ (±4.24)	$197^{A,a}$ (±3.23)	$157^{A,a}$ (±3.15)	$174^{A,b}$ (±3.70)	$178^{A,a}$ (±4.25)	$182^{A,a}$ (±4.05)
E2	$179^{BC,a}$ (±3.71)	$196^{A,b}$ (±2.87)	$170^{B,a}$ (±2.88)	$186^{AC,b}$ (±4.68)	$180^{A,a}$ (±2.93)	$182^{A,a}$ (±3.66)
E3	$174^{BC,a}$ (±4.71)	$202^{AC,b}$ (±5.75)	$172^{BC,a}$ (±5.56)	$186^{AC,a}$ (±6.17)	$188^{AB,a}$ (±4.22)	$181^{A,a}$ (±6.04)
E4	$185^{AB,a}$ (±4.64)	$194^{AD,a}$ (±3.37)	$162^{AB,a}$ (±4.15)	$177^{A,b}$ (±3.45)	$179^{A,a}$ (±3.14)	$182^{A,a}$ (±3.10)
D2	$179^{ABC,a}$ (±4.70)	$205^{BC,b}$ (±4.17)	$175^{BC,a}$ (±7.49)	$194^{BC,b}$ (±3.40)	$191^{AB,a}$ (±6.11)	$205^{B,a}$ (±4.18)
D3	$175^{BC,a}$ (±6.27)	$204^{AB,b}$ (±5.88)	$184^{C,a}$ (±1.56)	$195^{BC,b}$ (±3.04)	$203^{B,a}$ (±8.10)	$214^{C,a}$ (±6.01)
D4	$168^{C,a}$ (±2.69)	$204^{BCD,b}$ (±3.81)	$177^{BC,a}$ (±6.01)	$194^{BC,b}$ (±2.99)	$189^{AB,a}$ (±5.87)	$199^{BC,a}$ (±5.02)

**Values of phenological traits within columns followed by the same capital letter do not differ among populations for each irrigation scheme and year of study. Values of phenological traits within rows that followed by the same small letter do not differ between irrigation schemes per population for each year of study separately.*

Evros. However, the projected conditions of 2050 under climate change applied in the growth chamber, probably stimulated a differentiated plastic reaction for both provenances. We assume that the same environmental signals that trigger bud flush in plants, such as temperature and humidity, had a different effect on the provenances in this study. Indeed, in a temperature manipulation experiment, Fu et al. (2012) report that artificial warming of beech seedlings significantly affected bud burst date in different provenances.

Our findings reveal two different foliar phenology patterns followed by beech seedlings in this study. These patterns seem to depend on provenance and differ mainly in bud burst timing



A

	E1	E2	E3	E4	D2	D3	D4
🟦 2014-2015	.007	ns	ns	ns	ns	.046	.006
🟧 2015-2016	ns	ns	ns	ns	ns	ns	ns

B

	E1	E2	E3	E4	D2	D3	D4
🟦 2014-2015	.005	ns	ns	.010	ns	ns	ns
🟧 2015-2016	ns	.003	ns	.008	ns	ns	ns

C

	E1	E2	E3	E4	D2	D3	D4
🟦 2014-2015	.000	.009	ns	.010	.021	.038	.000
🟧 2015-2016	.000	.000	.001	.000	.008	.012	.000

D

	E1	E2	E3	E4	D2	D3	D4
🟦 2014-2015	.003	ns	ns	.000	.000	.002	.001
🟧 2015-2016	.001	.008	.041	.000	.001	.001	.000

E

	E1	E2	E3	E4	D2	D3	D4
🟦 2014-2015	.000	ns	ns	.002	ns	ns	ns
🟧 2015-2016	.006	.030	.030	.017	ns	ns	ns

F

	E1	E2	E3	E4	D2	D3	D4
🟦 2014-2015	.001	ns	ns	.004	ns	ns	ns
🟧 2015-2016	ns	ns	ns	ns	.019	.030	.049

FIGURE 7 | Plastic response of populations between consecutive years of the experiment under irrigation schemes A1 and A2 respectively for bud burst **(A,B)**, leaf senescence **(C,D)**, growing period **(E,F)**.

in spring and less significantly in the timing of leaf senescence in autumn. Seedlings from Evros provenance showed a delay in bud burst in 2015 and 2016 in respect to 2014. This pattern is apparent under both irrigation schemes, however it is more evident under A1, suggesting that Evros provenance is better adapted to prolonged intervals between irrigation events. On the other hand, populations of Drama showed earlier budburst in the respective years under both schemes. These observations reveal a different foliar phenology pattern, where beech populations initiate their growing period sooner in order to maximize total carbon gain, as it seems to be more prone to higher temperatures during summer, under non-frequent irrigation (scheme A1). Most reports from field provenance trials for beech show that, unlike in our study, bud burst timing remains constant and the order of provenances in this regard remains unchanged during years, without a strong interaction between provenance and test site (Robson et al., 2011, 2018). We assume that stressful environmental conditions, such as the ones simulated in the growth chamber in our study, may trigger certain physiological responses that will allow trees to survive. Since phenological traits are complex in nature and in their underlying mechanisms (Vitasse et al., 2010; Fu et al., 2012; Basler and Körner, 2014), further studies of provenances and families of trees utilizing functional and anatomical traits are needed to understand these adaptation strategies better.

Adaptive and Phylogenetic Differentiation Patterns

The results of our study reveal the existence of high genetic diversity in adaptive traits in the beech forests of N.E. Greece. These adaptive differences occur at multiple spatial levels, among distant and neighboring populations. There is a clear geographical and environmental trend in adaptation to climate. On the eastern side of the study area (provenance Evros), beech populations are better adapted to dry climatic conditions with longer intervals of drought during the summer and low probability of late frosts in the spring. As a result, seedlings from Evros demonstrate higher survival and earlier bud burst in the first year of the study than seedlings from Drama, under simulated climate change conditions, especially when irrigation is not frequent. At the same time, most of the Evros populations show a specific phenotypic pattern, as a response to the simulated climate change conditions in the growth chamber, with a shortening of the growth period during the second year of the study. Respectively, populations located on the western side of the study area (provenance Drama) seem to demonstrate adaptations to more temperate conditions, characterized mainly by long and cold winters and more humid summers. Seedlings originating from Drama showed lower survival under long drought intervals during the summer in the growth chamber and a late bud burst in the first year, but then shifted their growing season earlier and flushed earlier than the Evros seedlings in the second year of the study.

Besides the different environmental conditions that may have caused adaptive genetic differentiation between the two provenances of this study, another reason for the differences in adaptive traits that exist between Evros and Drama may be the presence of different levels of a possible admixture between two beech species, *F. sylvatica* and *F. orientalis* that presumably form a contact zone in the southeastern part of Europe (e.g., Paule, 1995; Tsiripidis and Athanasiadis, 2003; Papageorgiou et al., 2008; Govaerts et al., 2013). In N.E, Greece, several authors suggest an increasing admixture trend toward the east (Moulopoulos, 1965; Tsiripidis and Athanasiadis, 2003; Papageorgiou et al., 2008; Hatziskakis et al., 2011), with provenance Evros being genetically and morphologically closer to *F. orientalis* and provenance Drama to *F. sylvatica.* Since the former species grows in warmer and drier climates than the latter, adaptive differentiation may exist between them, as suggested by earlier studies (Atalay, 1992; Tsiripidis and Athanasiadis, 2003; Papageorgiou et al., 2008).

Adaptive differences were recorded within provenances as well, indicating that beech populations that belong to the same geographic region and are located within a small distance exhibit large genetic differences in adaptive traits. Differences in altitude, aspect and topographical connectivity between populations probably define an environmental mosaic with semi-isolated patches of beech forests, where natural selection can locally cause well-adapted ecotypes that differ at a small spatial scale. In Evros, population E1 was proven to be best adapted to warmer conditions and summers with prolonged periods without rain, as shown by the high survival rate of E1 in the growth chamber, the early bud burst in the first year of the study and the defensive phenological plasticity pattern in the next years. Indeed, E1 shows adaptive differences from the neighboring population E2 (only 682 m apart), probably because the environmental conditions at these sites are critically different. Population E1 is a marginal beech stand located on a south facing slope, while E2 is a dense forest on the north facing slope of the mountain, growing under much more favorable conditions. Furthermore, population E4 showed a different adaptive pattern in seedling survival than E1, which can be attributed to the more oceanic microclimate of the specific location, as explained above.

Despite the differences in the survival rate between E1 and E4, these two populations demonstrated similarities in their phenological profile, which was much different than E2 and E3 in the Evros provenance. According to a fine scale genetic analysis of chloroplast DNA haplotypes in beech populations in the region (Manolis et al.: unpublished data), both E1 and E4 seem to derive from the postglacial expansion of the same beech population in a local refugium, where beech survived during multiple glacial cycles and probably developed effective adaptive strategies. The origin of a local glacial refugium has been suggested as a possible explanation for late bud burst of the Slovenian beech provenance Idrija-II/2 in European field trials, as a possible adaptation to long cold winters during glaciation (Brus, 2010; Robson et al., 2011, 2018). Thus, adaptive differences between distant or close-by populations may derive from selective evolutionary responses to environmental conditions of past refugia, in parallel with the ongoing processes of adaptation to current environmental conditions. This seems to be especially true in the case of disjunct "rear edge" populations of forest trees that have not received any maladaptive gene flow from the core populations of the species and maintain the ability to evolutionary adjust themselves to local climate changes (Fady et al., 2016).

Conclusions

Both hypotheses tested in our study were confirmed. Beech seedlings deriving from populations of N.E. Greece were in general able to survive well under climate change conditions, simulated and applied in the growth chamber. Plants showed adaptive differences that allowed them to avoid high levels of mortality in the growth chamber. Furthermore, beech genotypes demonstrated phenological plastic responses to different environmental conditions and precipitation frequency in particular. Beech seedlings alter the duration of their growing season as a response to environmental signals, avoiding environmental stress and high selection pressure. In our study we were able to describe different adaptation strategies, that relate to the distribution patterns of specific environmental factors, rather than the average annual or monthly values of these measures. Indeed, fluctuations in temperature and precipitation within each year seem to be crucial for survival and growth, as well as the duration of the growing season. For this reason, provenance Evros is considered to be well-adapted to a less temperate climate, due to the low rainfall during summer, despite the high annual precipitation that occurs mainly during the winter in this region. Furthermore, our study proved the adaptive significance of the distribution of precipitation at a small temporal scale, since different adaptive strategies appeared among beech seedlings when the same amount of water was distributed differently within each month. This indicates that the physiological response mechanisms of beech individuals are very complex and depend on several interacting parameters that are difficult to study in total. For this reason, conclusions about the suitability of provenances for translocation and use in afforestation or reforestation projects should consider the small scale ecotypic diversity of the species and view multiple environmental and climatic parameters in connection to each other.

Despite the existence of adaptive diversity among the populations of beech in N.E. Greece, the survival of beech and other temperate forest tree species in the future remains unknown, since the speed, the uniformity and the intensity of climate change are different in different climate models. We expect severe climate fluctuation in the near future, with an increased intensity in the forests of the Mediterranean ecoregion being most at risk. Beech populations in the rear edge of the distribution of the species have a large adaptive potential and their persistence seems to be of major importance for forests and forestry all over Europe, pressing for an adjustment of forest management and conservation policies (Mátyás et al., 2009; Lefèvre et al., 2014; Fady et al., 2016).

DATA AVAILABILITY STATEMENT

The datasets analyzed for this study can be found in ResearchGate [www.researchgate.net]. doi: 10.13140/RG.2.2.15294.13128; doi: 10.13140/RG.2.2.11099.82727.

AUTHOR CONTRIBUTIONS

GV, AP, and OG developed the original idea of the research, GV, AP, TM, and ITa planned the experiments in the chamber and the field, GV, AM, and ITs planned sampling and collected the seed, GV, TM, AM, and ITa supervised the experiment in the growth chambers, GV, AP, OG, and ITs analyzed the data, CM and AP developed the models, GV developed the first draft of the manuscript and all authors contributed in preparing the manuscript in its final form.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2018.01918/full#supplementary-material

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Diplodia Tip Blight on Its Way to the North: Drivers of Disease Emergence in Northern Europe

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Disease emergence in northern and boreal forests has been mostly due to tree-pathogen encounters lacking a co-evolutionary past. However, outbreaks involving novel interactions of the host or the pathogen with the environment have been less well documented. Following an increase of records in Northern Europe, the first large outbreak of *Diplodia sapinea* on *Pinus sylvestris* was discovered in Sweden in 2016. By reconstructing the development of the epidemic, we found that the attacks started approx. 10 years back from several isolated trees in the stand and ended up affecting almost 90% of the trees in 2016. Limited damage was observed in other plantations in the surroundings of the affected stand, pointing to a new introduced pathogen as the cause of the outbreak. Nevertheless, no genetic differences based on SSR markers were found between isolates of the outbreak area and other Swedish isolates predating the outbreak or from other populations in Europe and Asia Minor. On a temporal scale, we saw that warm May and June temperatures were associated with higher damage and low tree growth, while cold and rainy conditions seemed to favor growth and deter disease. At a spatial scale, we saw that spread occurred predominantly in the SW aspect-area of the stand. Within that area and based on tree-ring and isotope (δ^{13}C) analyses, we saw that disease occurred on trees that over the years had shown a lower water-use efficiency (WUE). Spore traps showed that highly infected trees were those producing the largest amount of inoculum. *D. sapinea* impaired latewood growth and reduced C reserves in needles and branches. *D. sapinea* attacks can cause serious economic damage by killing new shoots, disrupting the crown, and affecting the quality of stems. Our results show that *D. sapinea* has no limitations in becoming a serious pathogen in Northern Europe. Management should focus on reducing inoculum, especially since climate change may bring more favorable conditions for this pathogen. Seedlings for planting should be carefully inspected as *D. sapinea* may be present in a latent stage in asymptomatic tissues.

Keywords: carbon isotopes, dendroecology, water-use efficiency, latewood, earlywood, vascular wilt pathogen

INTRODUCTION

Globalization and climate change are driving forest pathogen invasions and disease emergence worldwide (Stenlid and Oliva, 2016). While reports on human-mediated movements of pathogens continue to cumulate (Liebhold et al., 2012; Jung et al., 2016; Redondo et al., 2018b), few cases where new outbreaks are appearing in connection with climate change have been documented (La Porta et al., 2008). Climate may limit disease by means of different, and often interacting, mechanisms, making climate-change driven outbreaks difficult to understand and predict (Sturrock et al., 2011). One possibility is that climate is limiting the distribution of the pathogen. A good example of such mechanism is the cold winters presumably limiting the northwards expansion of *Phytophthora x alni* (Brasier and S.A. Kirk) Husson, Ioos and Marçais on alder (*Alnus glutinosa* (L.) Gaertn.) (Redondo et al., 2015). The second possibility is that the pathogen is present, but climate is limiting the capacity of the pathogen to cause disease for instance by reducing its capacity to build inoculum. A good example of such mechanism was seen for *Dothistroma septosporum* (Dorogin) M. Morelet in British Columbia where large damages occurred on lodgepole pine forests (*Pinus contorta* Douglas ex Loudon) along with an increase of summer rainfall during the previous decades (Woods et al., 2005). The third and less well-documented possibility is when climate is limiting host susceptibility. In that case, outbreak etiology is complex and may involve maladaptive phenotypes (Stenlid and Oliva, 2016), changes in stress regimes (Hanso and Drenkhan, 2013; Oliva et al., 2014), changes in phenology, or the combination of both (Françoise et al., 2015).

Predicting new forest disease outbreaks also needs to consider their geographic location. Climate change for instance has been suggested to increase forest damage in northern latitudes (Sturrock et al., 2011). However, still little is known about the particular processes behind disease emergence under these conditions. For instance, short growing seasons may make trees growing in northern locations more sensitive to needle loss than their southern counterparts (Oliva et al., 2016; Stenlid and Oliva, 2016). Also, a longer daytime during summer may also extend the periods when trees photosynthesize and expose themselves to water stress. Nevertheless, more cases are needed to depict general patterns. New and, so far, innocuous pathogen encounters are increasingly reported in Northern areas (Millberg et al., 2016; Redondo et al., 2018a,b), and we lack tools to predict their potential impact.

The case of *Diplodia sapinea* (Fr.) Fuckel (syn. *Diplodia pinea* (Desm.) Kickx., *Sphaeropsis sapinea* (Fr.: Fr.) Dyko and Sutton) in Sweden represents a good example of emergence in Northern forests. The pathogen was reported for the first time in 2013 and was regarded innocuous as no associated damages were observed (Oliva et al., 2013). However, 3 years after the first observation, *D. sapinea* was found associated with an unprecedented outbreak on Scots pine (*Pinus sylvestris* L.). In August 2016, stand-level damages affected a circa (ca.) 15 ha plantation north from Stockholm, where hundreds of 20-year-old Scots pine trees appeared severely damaged. Well-developed trees had lost completely all current year's shoots, and some of them were dead. The incidence was high, with the majority of trees affected, in most cases having lost their main leader shoot. A closer look in the stems revealed that most trees were either bifurcated or displayed bushy crowns, suggesting that they had probably suffered *D. sapinea* attacks in the past.

The outbreak in Sweden not only represented a qualitative change to the previous behavior of this pathogen in Northern Europe but also at a global scale. *Diplodia sapinea* had been historically reported in Southern and Central Europe causing shoot dieback, canker, blue stain, and root disease on pines (Fabre et al., 2011; Luchi et al., 2014). Mirroring the situation in Europe, in the Southern Hemisphere, *D. sapinea* had caused outbreaks in areas with mild climates, such as South Africa and New Zealand, where mostly non-native pine plantations have been established (Burgess et al., 2004). The pathogen had also been detected in relatively cold areas, but recent detections in Estonia (Hanso and Drenkhan, 2009), Finland (Müller et al., 2018) and north-western Russia (Adamson et al., 2015) seemed to suggest an ongoing range expansion to the north. Following that pattern, pycnidia of *D. sapinea* were observed for the first time in 2013 (59°N) on a Scots pine cone in Sweden (Oliva et al., 2013). Further samplings done in that same year found pycnidia in cones all over Southern Sweden, in areas such as Uppsala, Gothenburg, Malmö, and Visby, on both non-native *Pinus nigra* Arnold and Scots pine. In spring 2014, a small group of trees displaying shoot blight damages from previous year (2013) were found north from Gothenburg, though as in previous cases, symptoms corresponded to isolated branches or trees.

In this research, we aimed at understanding the causes of the *D. sapinea* outbreak in Sweden, as a way to improve our capacity to predict disease emergence in northern conifer forests. We did so by testing five explicit hypotheses on disease emergence. The first hypothesis concerned the origin of the pathogen. Until 2016, the steady increase of reports in Northern Europe fitted well in the picture of a pathogen slowly expanding its range. However, the isolated nature of the outbreak and the high severity of damage over several hectares raised the question of whether the observed damages could be the result of introduction of a new aggressive strain or a cryptic species of the pathogen (Hypothesis 1). We tested hypothesis 1, by studying the population structure of the pathogen in the outbreak area in comparison with that of isolates previously discovered in Sweden in areas with no apparent damage and with isolates of other parts in Europe, such as Estonia, Spain, and Italy, as well as from Turkey.

A regional survey of other attacks was also attempted in order to gain insights on the origin of the outbreak. Confirming an isolated nature of the outbreak (Hypothesis 2) could support the idea that infected planting stock was perhaps used in that particular stand. In forest nurseries in Wisconsin (USA), up to 27% of red pine seedlings were found to carry latent infections (Stanosz et al., 1997), and we know that in the 1950s, *D. sapinea* was present in Swedish forest nurseries (Molin et al., 1961). Thus, in order to test hypothesis 2, we surveyed the surroundings of the outbreak area for stands also affected by *D. sapinea*.

The role of weather on disease development was also explored. *D. sapinea* enters the host through stomata of elongating needles at expanding shoots and injured tissue (Brookhouser and Peterson, 1971). *D. sapinea* remains in a latent stage in asymptomatic trees (Smith et al., 1996), while disease is often induced by stress factors such as drought (Bachi and Peterson, 1985; Stanosz et al., 2001), hail (Zwolinski et al., 1990), or mechanical wounding (Chou, 1987). At regional scale, severity of damages has been associated with higher temperatures in Italy (Bosso et al., 2017) and France (Fabre et al., 2011); however, the role of weather in northern latitudes is unknown. We hypothesized that weather conditions played a role on the development of the *D. sapinea* outbreak (Hypothesis 3). In order to test hypothesis 3, we reconstructed the *D. sapinea* epidemic in the stand by inferring the time when trees had lost the leader shoot. We modeled the development of the epidemic and correlated departures from the expected disease progression curve with tree growth and monthly weather conditions.

Damages were not homogeneous across the stand, pointing to disease development being modulated by spatial features. Across the outbreak, there was also variation in terms of damage among trees. Within the same plot, highly damaged trees were found nearby asymptomatic trees, also pointing to the role of some tree features in the development of the outbreak. Disease in northern latitudes has been sometimes associated with maladaptive phenotypes (Stenlid and Oliva, 2016), such as pine trees planted in fertile sites that would normally correspond to Norway spruce (*Picea abies* (L.) Karst.) (Witzell and Karlman, 2000). Given the link between drought and Diplodia shoot blight, we hypothesized that Scots pine phenotypes with lower water use efficiency (WUE hereafter) would be more susceptible to *D. sapinea* (Hypothesis 4). In order to test hypothesis 4, we reconstructed radial growth (earlywood and latewood widths), quantified non-structural carbohydrate (NSC hereafter) concentrations in several tissues (branch, needles), and measured wood C isotope discrimination (δ^{13}C) of trees severely attacked by *D. sapinea* in comparison with asymptomatic trees growing nearby.

Understanding the spatiotemporal development of the outbreak was also attempted by focusing on the spread of the pathogen. *Diplodia sapinea* has large spores that are released from pycnidia by raindrop splashes (Brookhouser and Peterson, 1971; Swart et al., 1987), thus we expected that *D. sapinea* would disperse in short distances in the studied stand (Hypothesis 5). We tested hypothesis 5 by comparing spore captures underneath diseased trees along a range of severity of symptoms, and between wet and dry periods. We also explored the use of qPCR instead of time-consuming spore counting measures for future monitoring purposes.

MATERIALS AND METHODS

Population Structure Studies

The affected stand was located next to a highway (E4), northwest from the Stockholm Arlanda Airport (59°40′51.2″N, 17°52′20.8″E), and it corresponded to a ca.15 ha Scots pine plantation established in 2000 around some older Norway spruce forest patches.

In order to confirm the identity of the pathogen causing the outbreak, three attacked shoots per tree from 40 trees were collected (**Figure 1**). Five wood pieces were taken from the margins of necrotic tissues of each shoot samples and were placed in a plate containing 2% malt extract agar. After a two-day incubation at 21°C, fungal colonies were randomly picked up from three of the wood pieces, and they were transferred to a new plate. After two weeks, one of the three isolates was chosen by its morphological similarity with the rest and recultured a second time for DNA extraction. After 5 days at 21°C, two pieces (~0.5 cm^2) of mycelium were harvested and stored at −20°C. DNA from all 40 isolates was extracted with a NaOH/Tris-HCl-based method described in Wang et al. (1993).

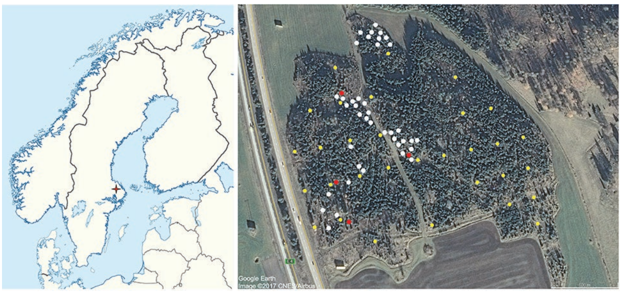

FIGURE 1 | Location of 40 trees used for isolation and spore trapping (white), 31 plots used for damage assessment and dendrochronology (yellow), and location of the four plots where the 24 trees used for physiological measurements were located within the affected stand (red). Image on the left modified from NordNordWest - own work, using World Data Base II data, CC BY-SA 3.0. Image on the right modified from Google Earth Image © 2017 CNES/Airbus.

We searched for signs of genetic differentiation between *D. sapinea* isolates in the outbreak area and isolates from Sweden, Europe, and Asia Minor (Turkey). *D. sapinea* population from Sweden consisted of 92 isolates. The collection was obtained in an isolation campaign carried out in 2013, when single-spore isolates were obtained from infected pine cones from five different locations: Visby (four *P. nigra* cones), Lomma (four *P. nigra* cones), Gothenburg (four *P. mugo* Turra cones), Uppsala (one *P. sylvestris* cone), and Fjällnora (one *P. sylvestris* cone). Single-spore isolates were taken by collecting pycnidia from cones and dissolving them in water and plating them in water agar. Individual germlings were retransferred to malt extract agar. For comparison with the rest of Europe and Asia Minor, a total of 65 isolates from Estonia (two locations, 11 isolates), Spain (five locations, 23 isolates), Italy (two locations, 15 isolates), and Turkey (two locations, 16 isolates) were included. The isolates in Spain, Italy, and Turkey were obtained in a similar scheme as Swedish ones from 2013, that is, single-spore isolates from cones. Estonian isolates were collected by direct isolation from symptomatic shoots and cones.

Isolates used for population structure were confirmed to be *D. sapinea* by being positive based on the specific PCR essay from Smith and Stanosz (2006). No major morphological differences were observed among the isolates of the outbreak area; therefore, a subsample of seven out of 40 was sequenced. Sequencing targeted the internal transcribed spacer (ITS) 1 and 2 and the 5.8S ribosomal RNA gene with the ITS1f and ITS4 primers (Gardes and Bruns, 1993). The purified PCR products were sequenced by Macrogen (Macrogen Inc., Seoul, Korea), edited in SeqMan pro (DNAStar, Inc., Madison, WI, USA; version 12.0.0), and blasted in GenBank (Genbank accession numbers: MK120100 - MK120106).

We searched for genetic differentiation among populations by looking for allelic variation in 10 simple sequence repeat (SSR) markers (Burgess et al., 2001; Bihon et al., 2011). The original panel of 16 markers was shortlisted to the ones that showed any variability. SSRs were amplified following Burgess et al. (2001). Amplification products were diluted 10- to 200-fold and send to Uppsala Genome Centre for fragment size analysis. SSR alleles were discriminated with the GeneMarker software (Softgenetics, State College, PA). Population structure analyses were carried out with the package POPPR (Kamvar et al., 2014) for R version 3.0.3 (The R Foundation for Statistical Computing). Combining the data of the 10 SSR markers, a multilocus genotype (MLG) was determined for each isolate. Swedish populations collected in 2013 and 2016 and the rest of European populations were compared in terms of rarefied numbers of MLGs and Simpson's index of diversity. Geographical differentiation among European countries was tested by means of a MANOVA analysis, calculated based on D-Jost distance, and visualized with a minimum spanning network. D-Jost values and statistical significance were calculated by bootstrapping with and without clone correction in the R package DEMEtics v.0.7-8.

In order to further support species identification, a sporulation test was conducted for two isolates from the outbreak area belonging to two different haplotypes. Isolates were cultivated on autoclaved pine needle extract (*P. sylvestris* needles ground on liquid nitrogen, 2% Agar) (Luchi et al., 2007). Cultures were kept under constant fluorescent light at 28°C. Pycnidia formed after one week on the surface of the agar and pine needle debris at the ground of the culture. After two weeks, five mature pycnidia per isolate were harvested. Conidial length and width, color, septation, and wall texture of 62 spores were measured from digital images recorded with a Leica DFC285 camera (Leica Microsystems, Switzerland) attached to a microscope (Axioplan, Carl Zeiss AG, Germany) and processed with the NIH imageJ software (version 1.52b, http://rsb.info.nih.gov/ij/). Morphological and morphotype identification followed the observations and identification key of Palmer et al. (1987) and Phillips et al. (2013).

In October 2016, thirteen Scots pine stands situated in a radius of 5 km from the outbreak area were surveyed. A second survey was undertaken in May 2017 including 28 Scots stands with a similar age as the plantation of the initial outbreak in a radius of 30 km. Shoots displaying symptoms were examined for characteristic pycnidia and microscopically screened for spores of *D. sapinea*, but no isolations were undertaken. Isolates from surveys around the infected area in 2016 were not included in the population structure analysis.

Tree Measures and Assessment of Damage

In October 2016, a systematic tree assessment was carried out across the outbreak area. A total of 264 pines within 31 plots were measured (**Figure 1**). Plots were circular and had a 2 m radius (12.6 m^2). In each plot, stem girth, height, and infection level of all trees with a girth larger than 3 cm at breast height were measured. Signs of bifurcation in the stem as well as the infection of the leader shoot were recorded. Stem girth was measured at breast height. The infection level per tree was estimated as the percentage of infected shoots proportional to all shoots from the upper third of the living crown. Signs of previous bifurcations were present all over the stand, thus timing of former attacks was inferred by recording the number of internodes (years) after the bifurcation, that is, after the leader shoot was lost. Additionally, a measure of exposure of the crown to wind and rain was taken in a scale from 0 to 4; according to which, a value of 4 indicated that the tree had a fully exposed crown, while a value of 0 indicated that the tree was completely surrounded by other trees. We extracted two increment wood cores from each tree at breast height using a Pressler increment borer. Wood cores were air-dried and their surface was carefully sanded until tree-ring boundaries were clearly visible. Then, tree rings were visually cross-dated and earlywood and latewood widths were separately measured with precision of 0.001 mm using a binocular microscope and the LINTAB package (Rinntech, Heidelberg, Germany). Earlywood and latewood widths were visually distinguished by experienced dendrochronologists based on the change in wood color and tracheid transversal dimensions. Tree-ring widths were transformed into basal area increments (BAI), assuming a circular shape of the stem. The COFECHA program (Holmes, 1983) was used to evaluate the visual cross-dating of tree-ring series.

Physiological Comparison Between *D. sapinea* Defoliated and Non-defoliated Trees

In February 2017, six pairs of defoliated and non-defoliated trees were selected in four different plots within the outbreak (n = 24 trees). In each plot, defoliated/non-defoliated tree pairs were stratified by size, so two big-diameter trees, two medium-sized, and two small trees were measured. Trees were chosen to be as close as possible (max. distance of 5 m). Trees were felled and discs were cut from the base and below the living crown. Tree growth was obtained from wood discs as done from increment wood cores. To couple growth measures with water-use efficiency, we compared carbon isotope ratios ($^{13}C/^{12}C$, $\delta^{13}C$) in wood formed in the years 2012, 2013, 2014, and 2016 between healthy and infected trees yielding a total of 96 wood samples. The years 2012 and 2014 corresponded to years with colder-than-normal spring temperatures, while spring temperatures in 2013 and 2016 were warmer-than-normal. The wood samples for $\delta^{13}C$ analyses were dried in the oven at 70°C for 48 h, then whole annual tree rings were separated using scalpels, and the resulting wood samples were ground to a fine powder. Wood aliquots (0.001 g) were weighed on a balance (AX205 Mettler Toledo, OH, USA) into tin foil capsules and combusted using a Flash EA-1112 elemental analyzer interfaced with a C isotope ratio mass spectrometer (Thermo Fisher Scientific Inc., MA, USA). Isotope analyses were conducted at the UC Davis Stable Isotope Facility (Davis, USA). Stable isotope ratios were expressed relative to Vienna Pee Dee Belemnite (VPDB). The standard deviation was better than 0.1‰.

Recently formed (one-year old) needles, branch sapwood, and root samples were also taken in order to assess whether NSC concentrations differed between damaged and undamaged trees. From damaged trees, samples were taken from needles and branches proximal to the damaged area, so comparison with the effects of *D. sapinea* defoliation with distal tissues, such as roots, could be possible. Samples were transported to the laboratory in a portable cooler, where they were frozen and stored at −20°C until freeze-dried. Samples were weighed and milled to a fine and homogeneous powder using a ball mill (Retsch Mixer MM301, Leeds, UK). Soluble sugars were extracted with 80% (v/v) ethanol, and their concentration was colorimetrically determined using the phenol-sulfuric method (Buysse and Merckx, 1993). Starch and complex sugars remaining after ethanol extraction were enzymatically digested with an enzyme mixture containing amyloglucosidase to reduce glucose as described in Palacio et al. (2007). NSC measured after ethanol extractions were regarded as soluble sugars, whereas carbohydrates measured after enzymatic digestion were considered to be mostly starch. The NSC concentration was calculated as the sum of soluble sugars and starch concentration.

Quantification of Spore Production Under Infected Trees

Forty spore traps were placed directly underneath forty trees with varying levels of damage (**Figure 1**). Spore traps had a height of about 50 cm and consisted of one horizontally fixed filter paper (Munktell, Ahlstrom; Ø90 mm) and one microscopy slide covered with two stripes of tape coated with permanent adhesive on both sides (Scotch® Double Sided Office Tape). The first set of traps was placed in 2016, from 30th of September to the 10th of October during a period with little rain (total precipitation 3.6 mm, 3 days of rain, average temperature 8.0°C); the second set was placed from the 21st of October to the 31st, during a rainy period (total precipitation 24.4 mm, 7 days of rain, average temperature 5.4°C). For spore counting, microscopy slides were divided into 22 rectangles of 0.5 cm × 1.3 cm each. Half of those 22 rectangles were screened under the microscope, where *D. sapinea* spores were distinguished by morphological characteristics (Cheng-guo et al., 1985). In order to evaluate the possibilities of monitoring *D. sapinea* by qPCR, we compared the number of spores counted in the microscope slide with the quantity obtained in terms of copy numbers from qPCR. For that, DNA was extracted from filter papers, by placing the entire filter in a 50-ml falcon tube under sterile conditions. After addition of 20 ml SDS buffer, filters were incubated for 90 min at 65°C. Next, the filter paper was removed and 20 ml of isopropanol was added and incubated overnight. On the following day, the sample was centrifuged at 7000 rpm for 10 min and the supernatant removed. From there and on, DNA extraction was continued with the NucleoSpin® Macherey-Nagel Soil Kit, following manufacturer's instructions. qPCR was done following the TaqMan™ setup designed by Luchi et al. (2005). Each 20 µl reaction consisted of a final concentration of 1× SsoAdvanced™ Universal Probes Supermix (BioRad), 250 nM of each primer, 200 nM probe, and 1 µl DNA extract/1 µl sterile water as non-template control. The qPCR program consisted of 2 min at 95°C, followed by 40 cycles of 10 s at 95°C and 15 s at 60°C. Copy numbers were obtained by averaging three technical replicates of each sample.

Data Analysis

The spatial association between disease and growth was examined by correlating the number of attacked trees and the relative X and Y coordinates of the plots ($n = 31$). A composite variable representing a gradient from SW to NE was calculated as the product of the coordinates. The development of the disease in each plot in time was modeled as linear function ($n = 9$ years), and the significance of that correlation was calculated. The correlation was used as a measure of disease increment and was also regressed against the SW-NE location of the plot. When modeling tree features associated with damage, we ran a stepwise selection to reduce the number of variables, always including the plot as a random factor.

We performed an epidemiological analysis in relation to tree growth and weather. We reconstructed the epidemic by pooling the number of records of attacks among all trees in the stand ($n = 264$). Development with time was modeled with a linear function ($n = 9$ years). BAI measures were integrated for all trees in the stand ($n = 264$) and were also regressed against time with a linear function ($n = 9$ years). Departures from a linear increase in both the epidemiological and the growth model were transformed into studentized residuals, which were then correlated with month's daily average temperature and precipitation sum for the period 2007 to 2016. Weather data for the outbreak stand were obtained by the interpolation tool provided by the Swedish Meteorological Institute and Hydrological Institute and available on the web.[1]

Earlywood and latewood widths were compared between pairs of trees defoliated by *D. sapinea* ($n = 12$) and non-defoliated trees ($n = 12$) separately for every year from 2007 to 2016. $\delta^{13}C$ comparisons were done similarly, but in that case all years were considered a factor in the analysis and the particular tree was included as a random factor in a mixed model. The degrees of freedom were adjusted by a Kenward-Roger approximation. Comparisons of NSCs and their different fractions between defoliated ($n = 12$) and non-defoliated trees ($n = 12$) were done by an ANOVA analysis including the plot as a blocking factor in the model.

The association between spore captures and tree features was done by regression ($n = 40$ and 39 traps in wet period and dry period respectively). Inclusion of variables in the model was done by stepwise selection. The association between spore captures and location was calculated by correlating the number of spores with the coordinates of each particular spore trap obtained by GPS *in situ*. Correlation between qPCR values and spore counts was done on data of 77 traps as some paper filters were lost during the rainy week. We tested whether the correlation between gene copies number (qPCR of filter paper trap) and spores (sticky traps) was different during the rainy and the dry week by including "week" as a factor in the model, both in the intercept and the slope "week × spore number." All analyses were carried out in Minitab® 18.1 for Windows.

RESULTS

Genetic Background of the Isolates in the Outbreak Area

Diplodia-like colonies represented a 76% of the isolates obtained from symptomatic tissues in the outbreak area. Isolates showed first a fast-growing white mycelia which turned to gray/black as cultures became old. Isolates obtained from the outbreak area corresponded to *D. sapinea* with a 100 % match to the sequence of *Sphaeropsis sapinea* 18S ribosomal RNA gene (GenBank: JF440618.1). Those, and the rest of isolates from Europe, included in the population structure analysis were positive for *D. sapinea* based on specific primers.

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[1] luftweb.smhi.se

Based on 10 SSR markers, the European population of *D. sapinea* was highly clonal, as only 28 MLG were found among 197 isolates. Rarefied numbers of MLG showed between 3 and 5 MLG (per 10 isolates) among all studied countries with the exception of Turkey, whose isolates displayed a distinct and much higher diversity than the other areas (9 MLG per 10 isolates) (**Table 1**). We found signs of geographic differentiation between European populations, accounting for 24% of the genetic variance ($p < 0.001$) (**Figure 1**). The Estonian population was the least differentiated from the rest (average D-distance of 0.05), while Swedish populations was the most dissimilar from the others (average distance 0.15), in particular from southern Spanish and Turkish populations (**Table 1**). Minimum spanning network showed a cluster of Turkish isolates highly differentiated from the rest (**Figure 1**). When using clone correction, significant differentiation was only found between Sweden and Turkey (D-Jost = 0.14, $p = 0.003$). No differentiation was found among Swedish populations (**Table 1**). The isolates of the outbreak were not genetically different from isolates previously obtained in Sweden (**Figure 2**), although they appeared to be slightly less diverse. The most abundant MLG in the outbreak, arbitrarily named G28, was also relatively abundant in 2013. That same haplotype was not found anywhere else in Europe. Spore size of G28 was similar to that of the haplotype G19, the most abundant in Sweden before the outbreak (length: 40.0 vs. 40.9 µm, $p = 0.08$; 15.4 vs. 15.5 µm, $p = 0.85$, respectively). In both cases, conidia produced *in vitro* were non-septated and showed a rough wall texture. Spore size, shape, and number of septa matched those of morphotype A.

Spatiotemporal Spread of the Epidemic

Symptomatic trees with similar symptoms were found in 7 of 13 stands in the vicinity of the outbreak area (<5 km). Further away (<30 km), incidence was much lower, with 3 of 28 stands with signs of *D. sapinea*. In any of the surveyed stands, damage severity resembled the one observed in the outbreak area. Across the outbreak, 85% of the trees were infected, while 53 % showed damage on the leader shoot. Among attacked trees, almost a third of the shoots in the upper third of the crown were affected (28%). There was a clear spatial pattern across the stand, where severity appeared to be higher in the southwest area of the stand (**Figure 3**). Based on the number of previous loses in the leader shoot, we reconstructed the epidemic back to 2007. Three plots situated in the north/north-western area of the stand had the oldest attacks (**Figure 3**). From 2007 onwards, we observed a significant ($p < 0.05$) increase of the number of attacks over the years in ca. half of the plots (48%). The largest increments were observed in the southwest area of the stand ($R^2 = 0.13$, $p = 0.043$). At plot level, no association between disease progression or damage and diameter, height, exposure, or tree growth was found. At tree level, the percentage of dead shoots correlated with both the number of previous attacks ($p < 0.001$) and tree height ($p = 0.006$).

Weather Conditions Associated With Tree Growth and Disease Increase

The number of putative *D. sapinea* attacks increased linearly over time (**Figure 4A**). In 2013 or 2016, the number of attacks was higher-than-expected, while in 2012, 2014, and 2015 the epidemic declined. In the years with largest attacks, trees grew less than expected (**Figure 4B**), and there was a strong negative correlation ($p < 0.001$) between disease increment and radial growth over time (**Figure 4C**). The same negative association between growth and *D. sapinea* was found in relation to weather conditions. Warm temperatures in May were associated with a lower tree growth ($p = 0.004$) and higher disease levels ($p = 0.042$) (**Figure 4D**). A nearly significant association was also found between low June temperature and growth ($p = 0.054$). No significant association between tree growth, disease, and precipitation was found (**Figure 4E**).

TABLE 1 | Population structure among *Diplodia sapinea* isolates from five different locations in Sweden collected in 2013 and from the location of the outbreak, and genetic distance among Sweden's *D. sapinea* population and other four populations in Europe.

Population	*n*	MLG	eMLG	Simpson's index corrected	Jost's D genetic distance				
Sweden					**Arlanda**	**Fjällnora**	**Gothenburg**	**Gula Stigen**	**Lomma**
Arlanda, 2016	40	4	2.62	0.387					
Fjällnora, 2013	7	2	2	0.286	−0.05				
Gothenburg, 2013	9	2	2	0.223	**0.03**	0.00			
Gula Stigen, 2013	31	2	1.93	0.322	0.02	−0.01	−0.04		
Lomma, 2013	20	3	2.98	0.679	0.00	−0.02	0.00	−0.02	
Visby, 2013	25	4	2.99	0.510	**0.04**	0.02	−0.01	-0.03	−0.02
Europe					**Sweden**	**Estonia**	**Spain**	**Turkey**	
Sweden	132	6	3.19	0.620					
Estonia	11	5	5.00	0.782	**0.12**				
Spain	23	6	5.01	0.810	**0.14**	0.01			
Turkey	16	12	9.17	0.966	**0.19**	**0.07**	**0.07**		
Italy	15	4	3.47	0.657	**0.16**	0.02	**0.06**	**0.07**	

Data based on 10 SSR loci. The number of isolates (n), multilocus genotypes (MLG), multilocus genotypes rarefied to 10 isolates (eMLG), and Simpson index corrected for different sample size are shown. Jost's D genetic distance calculated without clone correction. Jost's D-values significantly different from 0 are shown in boldface.

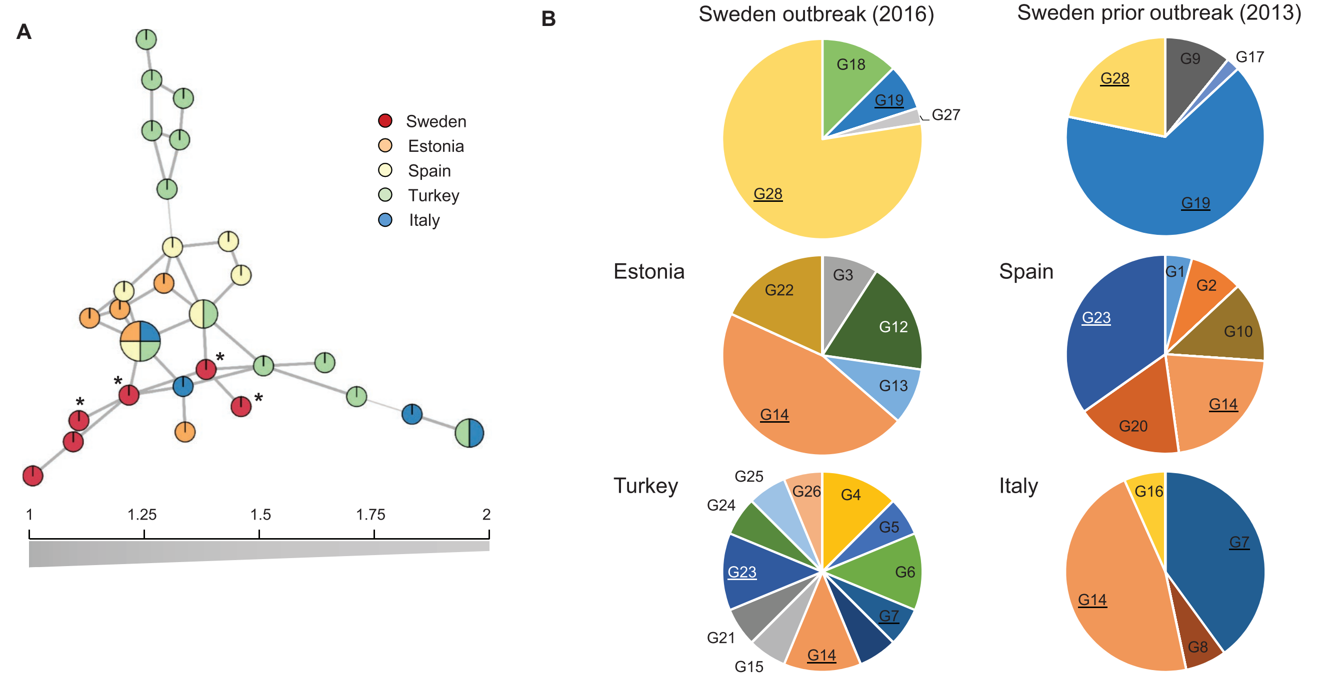

FIGURE 2 | Minimum spanning network among *D. sapinea* haplotypes in Europe **(A)**. Haplotypes from the outbreak area are marked with an (*). Pie charts with relative proportion of haplotypes among populations **(B)**. Underlined haplotypes are found in more than one location.

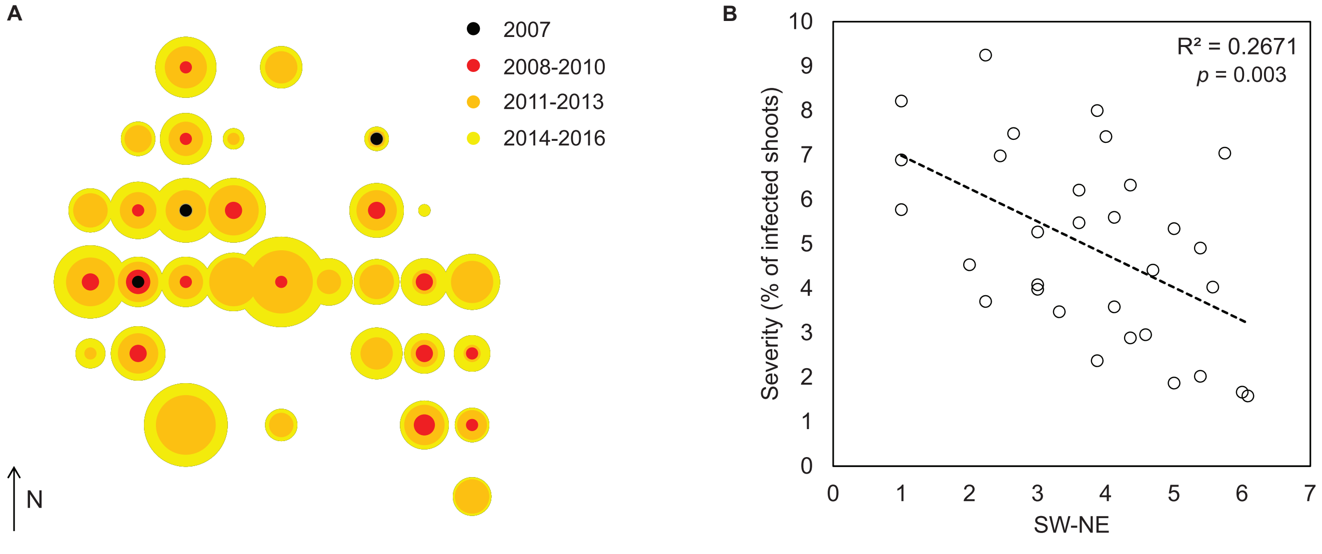

FIGURE 3 | Spatial and temporal spread of *D. sapinea* within the affected Scots pine stand **(A)** and association between severity in 2016 and location ($n = 31$ stands) **(B)**. SW-NE was calculated as the product of the relative X and Y coordinates of the sampling plots; higher values indicate NE locations, while low values indicate SW locations. Both severity and SW-NE location are plotted following square-root transformation.

Physiology of Attacked Trees

Comparing pairs of trees defoliated by *D. sapinea* and non-defoliated trees revealed that *D. sapinea* attacks affected mainly latewood production (**Figure 5A**). The year of the outbreak (2016) latewood was halved (0.74 vs. 0.35, $p = 0.011$), while no differences in terms of earlywood production were observed (**Figure 5B**). Heavily defoliated trees were characterized by displaying consistently lower $\delta^{13}C$ values irrespective of whether the year had a warm or cold spring (**Figure 5C**). Defoliated trees had lower NSC concentrations in needles and branch sapwood during the winter after the outbreak (**Figure 5D**), but no differences were found in root NSCs.

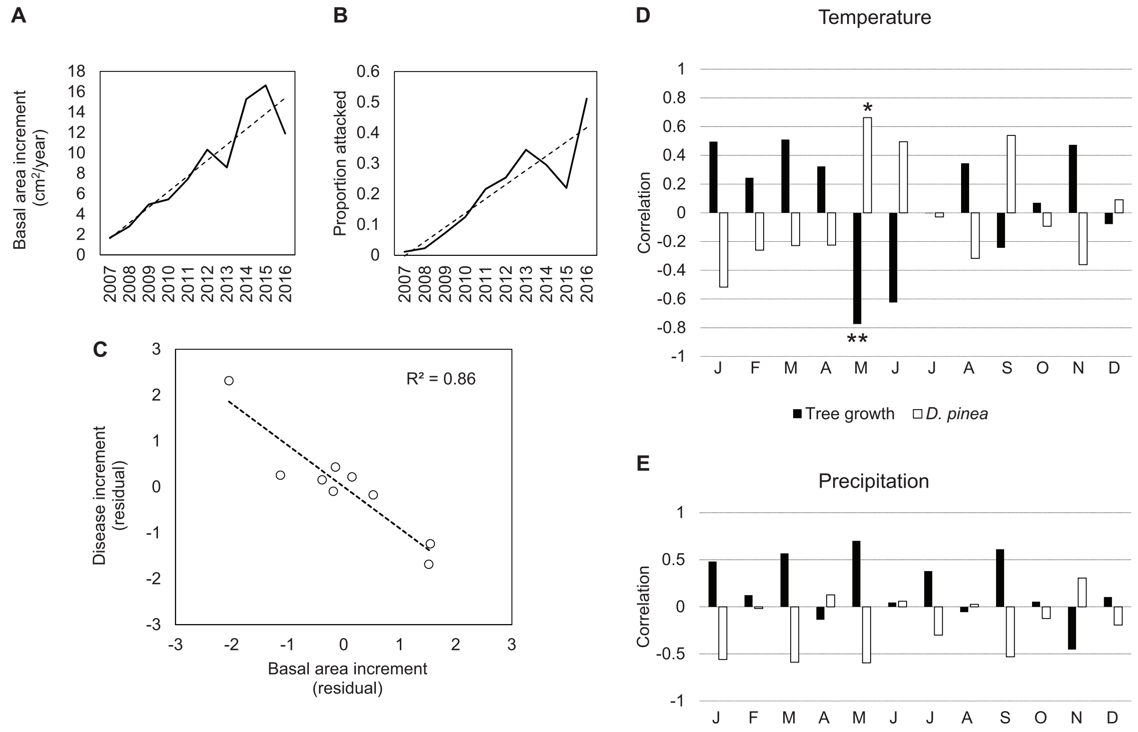

FIGURE 4 | Temporal association between tree growth, *D. sapinea* epidemic, and weather conditions. **(A)** Tree radial growth based on basal area increments (BAI) and development of *D. sapinea* in the stand from 2007 to 2016 (n = 264 trees) **(B)**, correlation between standardized residuals from *D. sapinea* increments and BAI of 264 trees over 9 years **(C)**, correlation between average monthly temperatures **(D)** and precipitation **(E)** from 2007 to 2016 (n = 9 years) and standardized residuals from *D. sapinea* increments and BAI from 264 trees. Dashed lines in a, b, and c show adjusted linear regression. Significance levels in bar plots: $^{**}p < 0.01$; $^{*}p < 0.05$.

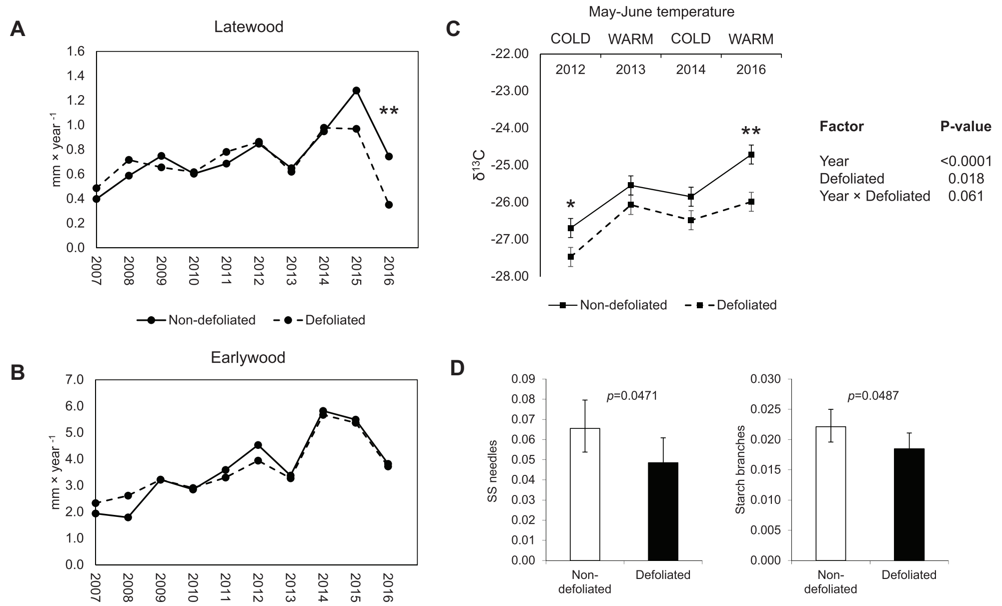

FIGURE 5 | Comparative physiological performance between trees defoliated by *D. sapinea* (n = 12 trees) and non-defoliated trees (n = 12 trees). Latewood **(A)** and earlywood **(B)** widths in the period 2007–2016. **(C)** Carbon isotope ratio (δ^{13}C) comparison between defoliated and non-defoliated trees across years when tree growth was high and spring conditions were cold (2012 to 2014) and years when spring was warm and growth was low (2013 and 2016). **(D)** Soluble sugar (SS) and starch concentration (% of dry weight) differences between defoliated and non-defoliated trees in needles and branch sapwood. Significant levels: $^{**}p < 0.01$, $^{*}p < 0.05$.

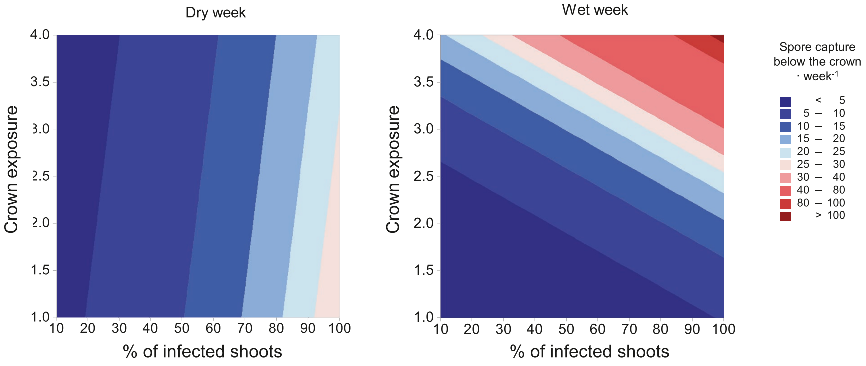

FIGURE 6 | Weekly spore captures depending on the rain conditions, tree exposure, and percentage of infected shoots. Spores were trapped under each tree (n = 40 trees/traps during dry period, and n = 39 during wet period). Tree exposure ranges from 0 to 4 and measures how exposed to rain and wind was the crown of the tree: 0, tree surrounded by other trees; 4, tree growing in open space. No spore trap was placed under a tree with less than 10% defoliation or with a lower exposure than 1.

Spore Dynamics Within the Outbreak

Spores collected during a wet period correlated with the amount of spores collected during dry conditions indicating a spatial consistency (R^2 = 0.59, p < 0.001). Spore captures tended to be larger in the southern part of the stand both under wet ($R^2 = 0.25$, $p = 0.001$) and dry weather conditions ($R^2 = 0.59$, $p = 0.013$). When considering the association between the amount of captured spores and the characteristics of the tree above the trap, we found a positive correlation with the percentage of dead shoots ($p = 0.006$) (**Figure 6**). During the wet week, also the exposure was associated with spore captures (p = 0.031 and p = 0.033, respectively for dead shoots and exposure), indicating that for a given level of damage, larger captures were obtained under crowns more exposed to wind and rain. Copy numbers obtained by qPCR on filter traps correlated significantly with spore captures on sticky slides ($R^2 = 0.24$, $p < 0.001$). However, the association tended to be lower ("week × spore number," $p = 0.060$) during wet periods (4115 copies detected by qPCR/spore detected in the sticky trap) than during dry periods (1904 copies/spore).

DISCUSSION

Disease emergence has been traditionally associated with the lack of co-evolution between host and pathogen. However, the Swedish outbreak of *D. sapinea* was illustrative of how a pathogen can establish in a new area and emerge as a new endemic disease. Isolates found in the outbreak area were not genetically different from isolates obtained in previous surveys on asymptomatic forests in Sweden. The most dominant clone found in 2016 was already present in Sweden in 2013, initially discarding our first hypothesis of the outbreak being caused by a novel and more aggressive strain. The Swedish *D. sapinea* outbreak did not seem to fit either into the traditional picture of an introduced pathogen having problems to adapt to novel environmental conditions. Rather than causing intermittent boom-and-bust outbreaks, *D. sapinea* showed a good adaptation to a new environment, and its population displayed a steady increase over several years in the studied stand. Warm May and June temperatures apparently favored disease, which became more severe in the SW area of the stand, more exposed to sun and presumably warmer. Within that area, defoliation appeared on trees with a lower WUE, as indicated by their δ^{13}C values. Symptomatic trees were the ones contributing more to the spread of the pathogen. Spores were captured during both wet and dry conditions; however, spore discharge seemed to be larger when affected crowns were more exposed to rain and wind. Our main finding is that northern conditions pose no apparent limitation for Diplodia tip blight. Nevertheless, further investigations should focus on understanding the origin of the inoculum of the outbreak.

We can only speculate with the origin of the inoculum of the outbreak area. One possible scenario is that *D. sapinea* was introduced in the stand with planting material bearing latent infections. We know that *D. sapinea* was present in Swedish nurseries during the 1960s (Molin et al., 1961) although the general prevalence in planting material is unknown. Work in Wisconsin (USA) has shown that incidence of *D. sapinea* could be as high as 30% of asymptomatic nursery stock (Stanosz et al., 2007) and that disease would only appear after seedlings would be subjected to water stress once out-planted. The absence of symptoms in the vicinity of the damaged stand fits well in the picture of a possible localized introduction from infected material in the stand of the outbreak; however, further investigations must be carried out to confirm that. Isolated trees showing severe symptoms were found in the immediacy of the outbreak, but those seem to fit better as part of the natural spread from the main stand, than as part of a widespread introduction. Recent surveys on spruce and pine seedlings in Sweden have not detected the pathogen (Menkis et al., 2016), so it seems that the current risk of *D. sapinea* being spread all over Sweden is rather low, although further surveys on the areas where seedlings from the same batch used in the outbreak area were planted should be undertaken.

D. sapinea seems to find highly favorable conditions to cause disease in Sweden, which is at odds with the absence of symptoms in the past. One possibility is that the disease has been misidentified, or perhaps confused with damages caused by *Melampsora* sp. Castagne, *Gremmeniella abietina* (Lagerb.) M. Morelet, or *Lophodermium seditiosum* Minter, Staley and Millar. In fact, and based on the reconstruction of damages, severity should have been high in previous years, and no one reported them. Overlooking severe damages in the forest may be not so unlikely after all. The outbreak in 2016 affected trees that were next to the highway, and no one noticed the damages even severity was ca. 90%. Another possibility is that *D. sapinea* outbreak in 2016 responded to very special conditions that precluded an explosion of damages. However, 2016 did not seem to be extreme regarding any of the weather variables considered. The possibility of a local haze storm or other phenomena cannot be discarded. However, the reconstruction of the epidemic based on tree bifurcations seems to bring the origin of the outbreak way back in time.

It is unclear which could be the underlying mechanism behind the association between *D. sapinea* attacks and warm conditions during May and June. In May and June, shoots start to develop, but completion ends more towards end of June, when correlation was no longer significant. Warm temperatures may enable the pathogen to develop endophytically in the bud immediately before sprouting (Brookhouser and Peterson, 1970). Alternatively, warm temperatures could increase drought stress in the new shoots, a condition that has been found associated with *D. sapinea* damages in ours and in previous reports (Bachi and Peterson, 1985; Stanosz et al., 2001). We observed that warm conditions in spring seemed to be detrimental for tree growth, pointing to the fact that high temperatures during shoot elongation may pose some stress to the tree. The importance of thermal conditions of May and June could also be associated with day length and photosynthetic activity. During summer, in northern locations, trees may be active for longer hours every day than in southern locations, extending the period of time when shoots may become susceptible due to lower water potentials caused by photosynthesis. As an example, the nearby city of Stockholm has an average of 18 hours of daylight on 1st June. A lack of lignification during early shoot development could also be associated with a higher susceptibility (Jalkanen and Kurkela, 1984; Petäistö and Repo, 1988; Petäistö, 1999).

The clonal structure found across Europe supports the idea of a predominantly asexual reproduction in *D. sapinea* in the continent. The low variability observed among our populations may be due to the low resolution of the markers. However, genotypic diversity in our study was similar to that found in other studies in South Africa (Burgess et al., 2001; Bihon et al., 2011). That and the fact that markers were able to detect a diversity hotspot in Turkey seems to reject resolution limitations. A certain level of adaptation cannot be fully discarded since there seems to be a geographical pattern across Europe, as observed in more localized studies (Luchi et al., 2014). However, without phenotypic data on the isolates, it is not possible to link the genetic structure with some sort of adaptation to northern latitudes, as done for other pathogens such as *Heterobasidion parviporum* Niemelä and Korhonen (Müller et al., 2015). Further investigations on survival and pathogenicity of northern *D. sapinea* isolates should be carried out.

A more liberal use of water seemed to a key phenotype associated with susceptibility. Even though there was some variation in terms of δ^{13}C across years, no association with high or low temperatures was found, and rather it seemed that the differences in terms of WUE were consistent between symptomatic and asymptomatic trees irrespective of growing under favorable/unfavorable conditions. Previous studies have brought up the role of tree phenotype increasing susceptibility (Witzell and Karlman, 2000; Oliva et al., 2014, 2016), which, in northern conditions, seems to be relating fast growth with disease (Stenlid and Oliva, 2016). This issue is particularly important in northern areas where forest regeneration is mostly done by planting and where growth expectations in high site index areas may carry a higher susceptibility. In Scandinavia, Scots pine and Norway spruce may be alternatively planted in the same stand (as in the case of the outbreak area), and therefore, provenances carrying more resistant phenotypes could be used as a prevention strategy in areas with high inoculum pressure.

Spore captures showed that spread is possible under wet or dry conditions, in contrast with previous studies where a more seasonal dispersal was found (Brookhouser and Peterson, 1971; Swart et al., 1987). We only sampled two weeks in autumn; thus, further experiments should be carried out to find whether these findings hold during summer or spring. Nevertheless, our results pointed to a different spore dynamic during rainy and dry weather. In both, captures were larger under more damaged trees. Spore deposition within the same tree may be an important component of the epidemic, as shown by the fact that highly damaged trees were also those with more attacks in the past. However under rainy conditions, crown exposure also favored dispersal. One possibility is that under an open crown the spore trap captured not only spores from the immediate tree but also from the surroundings. Also, a more open crown may facilitate a better wetting of the crown and a higher number of pycnidia being hydrated and releasing spores. Combining spore trapping on filter papers with qPCR seemed to be a powerful tool to monitor *D. sapinea*, although the lower efficiency under rainy conditions needs to be considered.

D. sapinea proved to be a damaging pathogen in economic terms, not so much because of its impact on growth, as losses were mainly seen on the latewood production (representing a small fraction of the ring), but because of its capacity to kill the leader shoot(s), disrupt the shape of the growing crown, and decrease the quality of the stem. Impact on NSC reserves was localized in proximal tissues to the attack area (needles and shoots), but no overall effect was detected in the root system where a large amount of NSCs are stored during winter. *D. sapinea* seemed to be able to survive well under nearly boreal conditions; therefore, disease management should focus on reducing inoculum. Inoculum build-up seems to be favored by warm conditions in spring, which may have implications under future climate projections. In Sweden, spring temperatures have shown a steady increase over the last decades, while summer, autumn, and winter temperatures seem more stable.[2] Seedlings and also seeds should be carefully inspected as *D. sapinea* may be present in a latent stage in asymptomatic tissues.

AUTHOR CONTRIBUTIONS

JO discovered the outbreak, coordinated the project, performed field work, analyzed the data, and drafted a first version of the manuscript. LB performed field measures, isolations, and molecular work, including population structure analyses. JC and ÁS-M did the dendrochronological, NSC, and isotopic measures. KA, CC,

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RD, NL, DM, AL, RD, and ŞÖ sampled *D. sapinea* in their respective countries and provided isolates or DNA. JS contributed designing the experiment. JO, JS, and RD funded the project. All authors contributed to the manuscript writing.

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OPINION
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Pathogens—The Hidden Face of Forest Invasions by Wood-Boring Insect Pests

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INTRODUCTION

Forests are one of the most important global vegetation types, serving functions from supporting healthy watershed, to wildlife habitat, and economic industries through their harvesting and tourism value. Understanding and mitigating threats to these resources is therefore of great societal importance. Forest pests are among the most pertinent and obvious threats to forest health, with their impacts amplified by contemporary issues such as climate change and global trade (Allen et al., 2010; Wingfield et al., 2016). In particular, recent increases in the frequency and severity of insect outbreaks have led to the loss of significant forest areas from across the globe (Roy et al., 2014) and highlighted the need for rigorous research to understand the underlying basis of their impacts.

Introduced wood-boring beetles have been particularly damaging, which are often released from competitors and predators in their new environments, and target healthy trees without evolved resistance. In their native range, they typically colonize only dead or dying trees and are, therefore, not recognized as harmful. Recent examples of invasive pests include the emerald ash borer (*Agrilus planipennis*), which primarily attacks ash trees, the redbay ambrosia beetle (*Xyleborus glabratus*) infesting members of the laurel family (*Lauraceae*), and the Asian longhorned beetle (*Anoplophora glabripennis*) and polyphagous shot hole borer (PSHB), which are capable of infesting a wide range of tree species (Baranchikov et al., 2008; Fraedrich et al., 2008; Haack et al., 2010; Eskalen et al., 2013; Paap et al., 2018). Since their accidental introductions from Asia, these aggressive pests have been implicated in the mortality of millions of trees in Europe and North America, and the potential for the further spread of these and other pest species poses a major threat to the health of global forests.

While forest insect pests have understandably attracted strong research attention, especially in countries where forestry is a major economic industry, far less understood are the pathogens they carry and how these contribute to forest damage, especially under climate change. For instance, the redbay ambrosia beetle, PSHB and longhorned beetles described above have each been found to carry assemblages of fungi, including pathogenic species (Fraedrich et al., 2008; Linnakoski et al., 2018; Paap et al., 2018). Here we focus on fungal pathogens vectored by wood-boring insect pests, and argue that they can amplify the negative effects of these pests and cause significant forest damage in their own right (Fraedrich et al., 2008; Ploetz et al., 2013). The purpose of this opinion article is to shed light on these implications, discuss the mechanisms underlying their interactions with host trees, and highlight the research required to resolve gaps in knowledge and progress understanding of this topic.

WOOD-BORING INSECTS AS FUNGAL VECTORS

Interactions between fungi and wood-boring bark and ambrosia beetles (**Figure 1**) are the most intensively studied pathogen-insect relationships in forest ecosystems. The natures of these interactions are diverse, ranging from incidental associations in shared habitats to co-evolved obligate nutritional mutualisms (Farrell et al., 2001; Roe et al., 2011; Hulcr and Stelinski, 2017).

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Fungal partners benefit from these associations through enhanced transmission, via transport to new trees and habitats.

An increase in the global trade of wood products has enhanced the risk of exotic insect and fungi introductions (Sikes et al., 2018), which may be present in poorly treated timber and wood packaging materials. Scolytine beetles are the most common group of invasive insects detected at border inspections (Lee et al., 2007; Lawson et al., 2018). Their niches overlap with native forest insects in many regions, and as a result, novel insect-fungal interactions are likely to arise. Under this scenario, introduced invasive pathogens may be passed to native insect species to vector, and invasive insect pests may become vectors for native or already established invasive pathogens (Haack, 2006; Wingfield et al., 2016). In both cases, novel fungi-host tree interactions are likely to arise, with unpredictable implications, and indeed, such relationships are increasingly recognized as a concern for forest ecosystems (Lu et al., 2011; Ploetz et al., 2013; Wingfield et al., 2016). Less well acknowledged is the possibility that the same type of novel interactions could result from distributional changes of endemic insect and tree species under conditions of climate change.

Novel beetle-fungus interactions include some of the most important invasive species affecting forest ecosystems, such as the Dutch elm disease and beech bark disease (Ploetz et al., 2013; Santini and Faccoli, 2013; Cale et al., 2017). However, an understanding of the associated fungal assemblages such as these is rare. Indeed, novel and unexpected vectors and host trees have even recently been detected for the extensively studied Dutch elm disease (Jankowiak et al., in press). Most insect and fungal species are not recognized as harmful in their native ranges and have therefore received little research attention. Such fundamental research is further complicated by the need for accurate identification of insects and fungi—these are two of the most species rich groups of organisms, the majority of which are undescribed (Stork et al., 2015; Hawksworth and Lücking, 2017)—but aided by the continual efficacy and affordability of genotyping methods. Establishing baseline information on insect-fungal associations is important for understanding pathogenic potential when beetles expand into new areas, and a lack of such information could hinder the timeliness of risk assessments and mitigation strategies following insect pest range expansions.

TREE DAMAGE

Tree damage by fungi is inherently linked to the beetle lifecycle (Raffa et al., 2015; Hulcr and Stelinski, 2017). Beetles play important roles in natural (undisturbed by human activity) forest ecosystems, where they typically infest dead or weakened trees and thus participate in forest succession/renewal through the breakdown of biomatter (Raffa et al., 2015). However, occasionally beetles aggregate, especially after natural disturbance events, and mass attack healthy trees in densities sufficient to cause significant damage (Raffa et al., 2015). As part of this process, fungal spores carried on the beetle exoskeleton, in specialized structures called mycangia or via other associated organisms (such as phoretic mites), are inoculated into the tree sapwood (Linnakoski et al., 2016; Hulcr and Stelinski, 2017).

It is not well-known why some beetles that usually colonize dead or stressed hosts in their native range attack healthy trees when arriving in a new area (Hulcr and Dunn, 2011). Recent examples of this process include both insects capable attacking a wide range of host trees (*A. glabripennis* and PSHB), and those infesting a narrow range of hosts (such as *A. planipennis* and *X. glabratus*) (Baranchikov et al., 2008; Fraedrich et al., 2008; Haack et al., 2010; Eskalen et al., 2013; Paap et al., 2018).

Although connections between insect damage and fungal activity in wood were recognized as early as the 19th century (Hartig, 1878), it has been difficult to elucidate the relative roles of each in tree damage as they typically occur in multipartite associations in "noisy" natural systems. However, recent studies have shed light on the potential for pathogens to amplify insect damage (and vice versa), indicating that at least some fungal associates have the ability to catabolize conifer defense compounds and improve beetle tunneling behavior (Wadke et al., 2016; Zhao et al., 2018). Indeed, Zhao et al. (2018) found that bark beetles preferred substrate colonized by fungi and avoided phenolics (plant defense compounds). Although tree damage is often attributed to insects, the role of fungal pathogens in lowering host defenses is likely to be important in many cases: partitioning damage caused by fungi and beetles may be important for targeting mitigating strategies and should be a priority for future research.

Clearly tree resistance is a critical parameter when considering the negative impacts of insect pests and pathogens. Environmental perturbations, especially those associated with climate change, such as droughts, floods, storms, and elevated temperatures, are particularly concerning. These events can cause physical damage to trees (Allen et al., 2010), enhancing the ease of colonization by insects and their accompanying fungal pathogens, while prolonged stress may also impair the ability of trees to direct resources toward defense and repair (Bolton, 2009). For example, inoculation experiments with Norway spruce seedlings have demonstrated that temperature and CO_2 level increases based on future climate predictions, as well as reduced water availability, can amplify the damage caused by certain fungal species (Linnakoski et al., 2017a,b); although the extent to which these findings are caused by changes in fungal virulence as opposed to host resistance, remains to be determined.

CONCURRENT INFECTIONS

As part of their lifecycle, beetles can acquire and deposit multiple fungi (both co-evolved symbiotic and opportunistic species) at the same time. For example, of 298 spruce bark beetles sampled during an outbreak in Finland, more than two fungal species were concurrently found on over 40% of individuals (Linnakoski et al., 2016). Indeed, it is very likely that most trees are simultaneously infected by multiple pathogens (co-infections). However, up until recently the prevailing paradigm of infection biology was based on a single pathogen causing a single disease (shown using Koch's

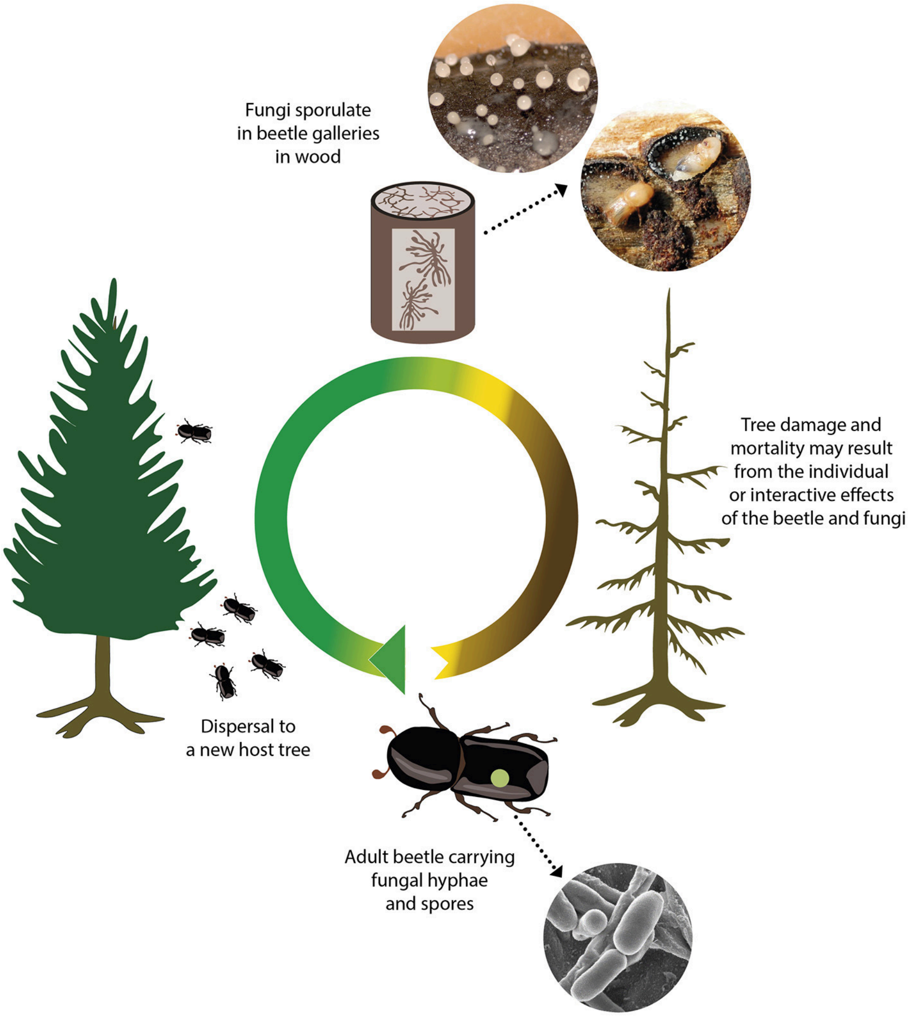

FIGURE 1 | The beetle lifecycle and role in fungi transmission. Tree damage caused by fungi associated with wood-boring insects is inherently linked to the beetle lifecycle. The beetle life cycle begins following hatching from an egg in a maternal gallery (tunnel) under the tree bark. The larvae remains in the tunnels, feeds on the phloem and gnaws tunnels. During this stage, fungi grow and sporulate and can serve as a source of nutrition for beetle larvae. Larvae pupate and develop into adults under the bark, before flying in search of new host trees and transporting fungi with them. Fungal spores carried on their exoskeleton, in specialized structures called mycangia or via other associated organisms, are then inoculated into a new host tree.

postulates), and this out-dated model remains dominant in forest pathology (Tollenaere et al., 2016).

Most research focused on the potential role of co-infections in plant diseases comes from agricultural systems (Lamichhane and Venturi, 2015; Tollenaere et al., 2016). Here studies have demonstrated cases in which severe plant disease can result from co-infections but not from single infections (Rochow and Ross, 1955)—but also that co-infections can reduce the negative impacts of a severe pathogen (Round and Wheeler, 1978). In forestry, a recent study demonstrated the role of co-infections as a cause of Acute Oak Decline (Denman et al., 2018). Fungal viruses (mycoviruses) may also cause changes in fungus aggressiveness (Pearson et al., 2009; Vainio et al., 2018) and therefore alter tree disease outcomes. It is established from forest pathosystems that fungal species and strains interact with each other and differ in their pathogenic potential (Krokene and Solheim, 1998; Repe et al., 2015; Linnakoski et al., 2017a,b). As a result, secondary infections with less virulent species or strains may provide mitigation tools against disease outbreaks.

CONTROL OF FUNGAL FOREST INFECTIONS

The most efficient strategy to protect trees is to prevent the introduction of wood-boring insects and their associated pathogens. Unfortunately, current regulatory efforts are inadequate to detect unknown species (Roy et al., 2014), and in several cases, damaging invasive forest pests and pathogens have been novel to science, or poorly understood in their native environment.

What then can be done? As the transfer of fungal pathogens is intimately connected to wood-boring insects, control efforts targeted at insect vectors are likely to be most effective. Innovative biocontrol tools, such as volatile compounds (VOCs), can form part of an integrated strategy. These regulate insect communication and can be utilized as potential repellents against beetles at outbound transport points or increase the efficacy of luring traps (Kandasamy et al., 2016; Hughes et al., 2017). Following prevention, early detection and range minimization are important, and should occur in urban areas that serve as entry points of invasions (Colunga-Garcia et al., 2010; Paap et al., 2017), and by forest monitoring and removal of infested trees by sanitation cuttings. More widespread strategies include the use of other pathogens as biocontrol agents to enhance the natural defense mechanism of trees and interfere with infections (Postma and Goossen-van de Geijn, 2016). For example, entomopathogens can be used as biocontrol agents against wood-boring beetles (Hajek and van Frankenhuyzen, 2017), and mycoviruses can be utilized in the control of fungal forest pathogens (Pearson et al., 2009; Vainio et al., 2018). However, the development of any biological control tool is a long process, which first requires sound knowledge of each organism involved.

CONCLUSIONS

Managing increasingly disturbed forests will be an important global challenge moving forward. While significant research effort has been devoted to understanding and mitigating the effects of insect pests on forest health (Vega and Hofstetter, 2015), in this article we highlight the comparatively neglected role of the fungal pathogens they carry. These are able to inflict significant forest damage (Wingfield et al., 2016), and it is likely that their negative impacts will be amplified over future decades due to environmental perturbations associated with climate change.

Fundamental baseline information on the diversity and frequency of most insect-fungal associations is currently lacking. Such information is important for recognizing novel vector-fungi associations, which have the potential to cause serious forest damage (Ploetz et al., 2013; Wingfield et al., 2016), and may aid in the timeliness of risk assessments and mitigation strategies following distributional changes of insect pests. The frequency and extent to which fungal associates facilitate and amplify insect damage is an intriguing line of enquiry and another area in need of research attention. Although evidence is currently limited to a small number of studies and systems, such as Dutch elm disease (Ploetz et al., 2013; Santini and Faccoli, 2013), it may be a common phenomenon associated with insect damage and provide a potential means to limit their impact.

While important advances have been made in understanding the impacts of fungal pathogens associated with forest insect pests, many pertinent questions remain and we hope this article will help stimulate research to investigate them.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Long-Term Studies Reveal Differential Responses to Climate Change for Trees Under Soil- or Herbivore-Related Stress

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Worldwide, trees are confronting increased temperature and aridity, exacerbating susceptibility to herbivory. Long-term studies comparing patterns of plant performance through drought can help identify variation among and within populations in vulnerability to climate change and herbivory. We use long-term monitoring data to examine our overarching hypothesis that the negative impacts of poor soil and herbivore susceptibility would be compounded by severe drought. We studied pinyon pine, *Pinus edulis*, a widespread southwestern tree species that has suffered extensive climate-change related mortality. We analyzed data on mortality, growth, male reproduction, and herbivory collected for 14–32 years in three areas with distinct soil-types. We used standardized precipitation-evapotranspiration index (SPEI) as a climate proxy that summarizes the impacts of drought due to precipitation and temperature variation on semi-arid forests. Several key findings emerged: (1) Plant performance measurements did not support our hypothesis that trees growing in stressful, coarse-textured soils would suffer more than trees growing in finer-textured soils. Stem growth at the area with coarse, young cinder soils (area one) responded only weakly to drought, while stem growth on more developed soils with sedimentary (area two) and volcanic (area three) substrates, was strongly negatively affected by drought. Male reproduction declined less with drought at area one and more at areas two and three. Overall mortality was 30% on coarse cinder soils (area one) and averaged 55% on finer soil types (areas two and three). (2) Although moth herbivore susceptible trees were hypothesized to suffer more with drought than moth resistant trees, the opposite occurred. Annual stem growth was negatively affected by drought for moth resistant trees, but much less strongly for moth susceptible trees. (3) In contrast to our hypothesis, moths declined with drought. Overall, chronically water-stressed and herbivore-susceptible trees had smaller declines in performance relative to less-stressed trees during drought years. These long-term findings support the idea that stressed trees might be more resistant to drought since they may have adapted or acclimated to resist drought-related mortality.

Keywords: climate change, drought, growth, herbivory, long-term, reproduction, tree

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INTRODUCTION

Heightened drought severity and warming temperatures due to climate change have contributed to world-wide increases in tree mortality (Allen et al., 2010; McDowell et al., 2018). Physiological drivers of mortality include hydraulic failure and carbon starvation (McDowell et al., 2011; Adams et al., 2017) with drought leading to mismatches between the water demand of aboveground tree biomass and water availability in the soil (Jump et al., 2017). Herbivorous insects and fungal pathogens can exacerbate drought impacts on trees (Allen et al., 2010; Anderegg et al., 2015a). Drought affected forests consist of areas that differ significantly in mortality (Mueller et al., 2005a; Gitlin et al., 2006; Linares et al., 2011; Olano et al., 2015), and trees that live and die following severe drought often grow side by side (Ogle et al., 2000; Sthultz et al., 2009a). Given that the incidence of severe drought is projected to increase (Seager et al., 2007; Garfin et al., 2013; IPCC, 2014; United States Global Change Research Program [USGCRP], 2017), and that tree mortality leaves a legacy that alters carbon cycling (Anderegg et al., 2015b), understory community composition (Kane et al., 2011), and community interactions (Gilman et al., 2010), it is critical to understand the factors that contribute to variation in tree mortality. It is equally important to understand how surviving trees and their pests respond following drought to better predict the future of forested ecosystems.

The impacts of drought on trees may differ across the landscape due to differences among sites in soil texture and water holding capacity. Mortality of trees was associated with low water holding capacity due to a combination of soil depth and texture in the semi-arid southwestern United States (Peterman et al., 2013). Stands with hotter, drier climates and low soil available water capacity showed limited regeneration following drought-related tree mortality (Redmond and Barger, 2013; Redmond et al., 2015). However, soil texture did not influence tree mortality following severe drought in the Amazon Basin (Williamson et al., 2000). Also, the effects of soil properties may vary temporally as the precipitation regime changes. The inverse texture hypothesis (Noy-Meir, 1973) proposes that plants growing in coarse-textured soil experience less water stress than plants growing in fine-textured soil and Sala et al. (1989) suggested this soil texture effect differed with annual precipitation. Longer-term data on tree performance at sites that differ in soil properties and that include drought and non-drought years could help reconcile conflicting patterns.

Intraspecific variation in drought tolerance can be significant in trees (Goodrich et al., 2016; Trujillo-Moya et al., 2018) and associated with differences in mortality during extreme drought (Sthultz et al., 2009a; Gehring et al., 2017) and growth recovery following drought (George et al., 2017). Common garden studies conducted across drought periods in *Larix decidua* (George et al., 2017) and *Pinus sylvestris* (Taeger et al., 2013) revealed significant differences among provenances in a tree's capacity to both withstand drought and to reach pre-drought growth levels after drought. Similarly, intraspecific variation in Norway spruce (*Picea abies*) explained up to 44% of the phenotypic variation in drought response (Trujillo-Moya et al., 2018). Many studies demonstrating the importance of intraspecific genetic variation in drought response compare individuals of widely distributed plant species that occupy markedly different environments. It is less clear how intraspecific genetic differences contribute to the variable levels of mortality observed among trees occupying the same or similar sites.

Differential herbivory also can contribute to differences in tree performance under drought conditions. Drought interacts with insect herbivory by altering plant defenses, influencing water and nutrient content of plant tissues, and altering chemical cues used by insects to identify hosts (Kolb et al., 2016a). While these interactions can lead to herbivore outbreaks that increase tree mortality (Ayres and Lombardero, 2000; Breshears et al., 2005; Raffa et al., 2008; Allen et al., 2010), drought can also reduce resource quality for herbivores, resulting in herbivore population declines (Kolb et al., 2016a). Insect herbivores also can be affected directly by the high temperatures often associated with summer drought. For example, warm temperatures combine with drought-stressed susceptible hosts increase bark beetle populations to epidemic levels (Raffa et al., 2008). Studies that examine both tree and herbivore performance over time can help us understand the contribution of herbivory to declines in tree performance with drought.

While differences in soil properties, intraspecific trait variation, and insect herbivory all influence susceptibility to, and recovery of trees from drought, these factors are rarely studied simultaneously. Because trees are long-lived and integrate climatic variation across multiple years, it is especially important to evaluate these complex interactions over the long term as climate changes (Linares et al., 2011; Anderegg et al., 2015b; George et al., 2017). Long-term studies are well suited for exploration of changes in relationships over time. One example is the change in responses of C_3 plants, which initially responded positively to elevated atmospheric CO_2, versus C_4 grasses which responded positively at 20 years (Reich et al., 2018). Furthermore, by comparing precipitation manipulation experiments to studies of productivity responses to annual precipitation variation across space versus across time, Estiarte et al. (2016) found that studies across time were more accurate predictors of future responses. Thus long-term studies across time and space that encompass strong variation in precipitation are critical to understanding responses to drought.

In this study we use a model tree species, pinyon pine (*P. edulis*) to examine how soil type, intraspecific variation in drought tolerance and herbivore susceptibility interact to influence tree performance before and during long-term drought. Warming temperatures combined with extreme drought and herbivory resulted in significant *P. edulis* mortality across 12,000 km^2 of the southwestern United States in 2002–2003 (Breshears et al., 2005). Several studies have examined the physiological basis and environmental drivers (Adams et al., 2009, 2015, 2017; Peterman et al., 2013; Grossiord et al., 2017; Redmond et al., 2017; Guérin et al., 2018) of this large-scale mortality. A synthesis of this research indicated significant differences among studies in the importance of tree density, site elevation and soil type to mortality (Meddens et al., 2015). Also, while the contribution of bark beetle herbivory to tree mortality has been demonstrated in *P. edulis* (Santos and Whitham, 2010; Gaylord et al., 2013; Anderegg et al., 2015a), other insect herbivores affect *P. edulis* performance and may influence its future distribution. Larvae of the pinyon stem-boring moth (*Dioryctria albovittella*) feed preferentially on the terminal shoots and female cones of mature trees. In studies conducted before long-term drought, chronic herbivory by this moth reduced trunk growth and female cone production, and altered *P. edulis* architecture to a shrub-like form (Whitham and Mopper, 1985). Despite their poor growth prior to drought, moth susceptible trees had three-fold higher survival during drought than moth resistant trees (Sthultz et al., 2009a), a trait also observed in their offspring (Sthultz et al., 2009b; Gehring et al., 2017).

In their synthesis of the drivers of *P. edulis* mortality, Meddens et al. (2015) developed a conceptual framework describing how soil, climate, abiotic agents, and tree attributes relate to *P. edulis* mortality via the physiological mechanisms that contribute to mortality. We follow that framework in the development of our hypotheses. Our overarching hypothesis is similar to that of Meddens et al. (2015) in that we propose that the negative impacts on *P. edulis* of growing in poor soil (an abiotic stressor), and experiencing chronic herbivory (a biotic stressor), would be compounded by severe drought. The hypotheses we describe were developed when our monitoring program began in the 1980's and 1990's, before some contradictory observations had been made (Sthultz et al., 2009a; Meddens et al., 2015; Gehring et al., 2017). We use long-term data on mortality, stem growth, male reproduction, and moth herbivory of *P. edulis* trees at areas in northern Arizona to test the following hypotheses: (H1) trees growing in shallow, nutrient-poor, coarse-textured soil with low water holding capacity will suffer greater declines in performance than trees in areas with deeper, higher nutrient, fine-textured soil with higher water holding capacity under drought conditions; (H2) trees experiencing chronic moth herbivory would suffer compounded declines in performance due to drought compared to herbivore resistant trees. We also examine long-term trends in levels of moth herbivory to test the hypothesis that moth herbivory would increase as trees experienced greater drought (H3). We expected moth herbivory to increase with drought stress because *D. albovittella* is more abundant in stressful sites with shallow, coarse-textured, nutrient-poor soils (Gehring and Whitham, 1994; Cobb et al., 1997).

These hypotheses are evaluated in context of the slope of the relationship of tree and herbivory measures regressed on the standardized precipitation-evapotranspiration index (SPEI) (Vicente-Serrano et al., 2010; Beguería et al., 2014). SPEI integrates precipitation, temperature, and evapotranspiration and is a proxy for drought stress impacts on plants that can be tailored to the timing of the system (Huang et al., 2015; George et al., 2017). SPEI is a relative measure of drought stress within a particular location with a mean of zero over time, where relatively warm and dry conditions are indicated by negative values. SPEI does not capture differences among our areas in water availability due to soil differences, rather it allows us to track the effects of drought through time within an area. Based on previous results in southwestern United States tree species, we chose to use SPEI over the year preceding a performance measure (Williams et al., 2013; Huang et al., 2015). Significant positive relationships of performance measures with SPEI indicate decreased performance under drought stress, while no significant relationship indicates that performance remains relatively constant with increasing drought stress. Our hypotheses would be supported by slopes of regression of tree performance measures with SPEI that are greater for the coarse soil (H1) and herbivore susceptible (H2) groups. For moth herbivory, we predict a negative slope with SPEI, which would indicate that the number of moth killed stems increases with drought stress (H3). Tests of these hypotheses are important to identify the long-term responses of individual trees and an associated herbivore to drought stress. Our findings may help land managers mitigate the trajectory of *P. edulis*, which is projected to be extirpated from Arizona this century due to climate change (Rehfeldt et al., 2006).

MATERIALS AND METHODS

Characteristics and Soils of the Three Areas

All three study areas are woodlands dominated by *P. edulis* and *Juniperus monosperma*. Study area one is near Sunset Crater National Monument on the cinder field associated with an ~1000 years old volcanic eruption (**Table 1**). This area has coarse-textured soils with low water holding capacity and is represented by six sub-sites while study areas two and three have finer-textured soils with greater water holding capacity and are represented by three sub-sites each (Gehring and Whitham, 1994; Miller et al., 1995; Selmants and Hart, 2008, **Table 1**). Cinder soils of area one have lower nutrient levels of nitrogen, phosphorus, potassium, magnesium, calcium, sodium, copper, and manganese compared to soils developed on limestone and sandstone substrates (area two) (Cobb et al., 1997; Swaty et al., 1998). Other studies have also shown the young, cinder soils to be lower in phosphate, soil moisture, NO_3 mineralization, and NH_4 mineralization (Gehring and Whitham, 1994, 1995). Additional studies comparing area one to area three, which is a much older volcanic-substrate soil, also found area one to be lower in clay, nitrogen, carbon, and soil water (Selmants and Hart, 2008; Selmants and Hart, 2010; Looney et al., 2012). Area three is lower than area one in plant available forms of phosphorus, as would be predicted for an older, versus younger, volcanic soil (Selmants and Hart, 2010). Because of generally lower nutrient status and low water holding capacity, trees growing in the cinder soils of area one experience more stressful abiotic conditions. The trees at the young cinder soil sites c,d (see **Table 1**) of area one exhibit higher incidence of susceptibility to the stem-boring moth (*D. albovittella*) and studies of moth susceptible and resistant trees in these areas began in the 1980's (see references under H2 and H3 sections below) hence hypotheses H2 and H3 will be addressed with trees from area one only. Annual stem growth measures are available starting with year 1986, strobili counts

TABLE 1 | This design table for the cross soil-type area comparisons in H1 shows the locations and number of trees sampled (N) for each the sampling area and the sub-sites within them.

Area	Sub-site	Latitude	Longitude	*N*	Soil characteristics (from Miller et al., 1995)
1	a	−111.39	35.336	63	Deep, extremely cindery sandy loam, excessively drained
1	b	−111.41	35.39	60	Deep, extremely cindery, coarse sand, excessively drained
1*	c,d	−111.43	35.39	122	Deep, extremely cindery, coarse sand, excessively drained
1	e	−111.46	35.418	64	Deep, extremely cindery, coarse sand, excessively drained
1	f	−111.48	35.44	64	Deep, extremely cindery, coarse sand, excessively drained
2	a	−111.41	35.15	64	Shallow, fine sandy loam
2	b	−111.42	35.18	65	Shallow, fine sandy loam
2	c	−111.4	35.13	62	Deep, fine sandy loam
3	a	−111.84	35.52	65	Moderately deep, very cobbly, clay loam
3	b	−111.84	35.54	64	Moderately deep, very cobbly, clay loam
3	C	−111.86	35.55	64	Deep, very cindery loam

Sub-sites consist of a set of sampled trees that are contiguous and distinct from other sub-sites within an area. Areas one and three are on volcanic substrates and area two is sedimentary. The asterisk denotes sub-sites that where merged for analyses because they were spatially contiguous. Trees for H2 and H3 are in area one sub-site "c,d".

TABLE 2 | Model selection table used to choose the form of correlations in time for the analysis of stem growth and drought across areas/soil types.

Y	Significant fixed factors and covariates	MA, *p*=	AR, *q*=	AIC
Stem growth	Area, SPEI, Area*SPEI, height, btd	0	0	90253.4
Stem growth	Area, SPEI, Area*SPEI, height, btd	0	1	88466.9
Stem growth	Area, SPEI, Area*SPEI, height, btd	0	2	87363
Stem growth	Area, SPEI, Area*SPEI, height, btd	1	0	87164.7
Stem growth	NA	1	1	Singular
Stem growth	SPEI, Area*SPEI, height, btd	1	2	86533.8
Stem growth	Area, SPEI, Area*SPEI, height, btd	2	0	86693.3
Stem growth	SPEI, Area*SPEI, height, btd	2	1	86530.1
Stem growth	SPEI, Area*SPEI, height, btd	2	2	86528.6

*ARMA analysis in the nmle package in R allowed us to test MA, moving average windows; AR, autoregressive time lags of 0, 1, and 2 years each. The fixed factors and covariates included in each model are the same and sub-site is nested within area for all models. Model selection was based on having an AIC at least "2.0" less than the next best model. The model chosen is highlighted in gray and has a MA = 2 and AR = 1. Model output details for the selected model is in **Table 3A**. btd, basal trunk diameter.*

starting in year 1995, and moth killed stems (H3 only) starting in year 1982.

Trait Measures

When trees were selected for the monitoring program, their basal trunk diameters and heights were measured. Stem growth (length added in a year) was measured after the conclusion of growth in late summer or fall on eight haphazardly selected stems from around the entire tree. The stem growth record was extended back in time by using bud scars as indicators of growth in years prior to the first measurement date (Ewers and Schmid, 1981). When possible, this method was also used to fill in years that measures were missed over the course of the 14–32 years of monitoring. Male strobili clusters (pollen cones) were counted in the spring; each tree was counted twice and the two counts were averaged. Moth killed stems show a characteristic browning and wilting that was used for visual identification (Whitham and Mopper, 1985; Gehring and Whitham, 1994). Moth killed stems were counted in the late summer and early fall after the moth larvae had completed feeding. Ladders were used to view the tree canopy as needed. Moth killed stems were counted twice on each tree and the two counts were averaged. Tree mortality was monitored beginning in the extreme drought year of 2002 and was rare prior to 2002.

Hypothesis 1: Tree Performance Across Three Areas/Soil Types

Thirty-two medium and 32 small trees in each of the replicate sites (**Table 1**) within an area/soil-type were selected haphazardly by finding interspersed trees meeting height and basal trunk diameter requirements for small and medium categories of trees. Most trees were selected in 1995, but a few more trees were added through 1999 to account for tree mortality if there were suitably sized trees available within the sub-site. Small trees had mean basal trunk diameter (±1 SE) of 6.42 cm (±0.08) and medium trees had basal trunk diameter of 15.25 cm (±0.17) at the start of the study. **Table 1** shows the locations of replicate sites within areas that make up the nested geographic design. This design increased the generality of the comparison across areas with contrasting soils type by increasing spatial representation. Total sample sizes were: area one = 373, area two = 191, area three = 193 (**Table 1**). Stem growth was measured for years

1986–2007. We counted male strobili clusters on each tree from year 1995–2008.

Hypothesis 2: Relative Performance of Moth Resistant and Susceptible Trees Within Area One

At the main cinder soil study area, 25 moth resistant and 25 susceptible trees were chosen for long-term monitoring and have been reported on previously (Brown et al., 2001; Cobb et al., 2002; Gehring et al., 2017). The trees are closely matched in age and basal trunk diameter, and the height difference reflects the tree versus shrub architecture that results from moth herbivory (Brown et al., 2001; Cobb et al., 2002). Linear models found significant differences among tree groups only for height and not for basal trunk diameter or age. Moth resistant trees had basal trunk diameters of mean = 20.3 cm (*SE* = 0.73) and heights of mean = 3.8m (*SE* = 0.13) at the start of monitoring. Moth susceptible trees had basal trunk diameters of mean = 18.9 cm (*SE* = 0.62) and heights of mean = 2.2 m (*SE* = 0.087) at the start of monitoring. Stem growth and strobili production were monitored annually as described above, except during the extreme drought year of 2002 when new growth was not produced on most trees.

Hypothesis 3: Moth Relative Abundance Monitoring Within Area One

The number of moth killed stems was monitored from 1981 to 2009 on large moth susceptible ($N = 20$) and resistant trees ($N = 19$) in the main cinder soil study area. These trees have been reported on previously (Whitham and Mopper, 1985; Gehring and Whitham, 1991; Mopper et al., 1991; Brown et al., 2001). Resistant trees had basal trunk diameters of mean = 44.5 cm (*SE* = 2.2) and heights of mean = 6.55 m (*SE* = 0.21) at the start of monitoring. Susceptible trees had basal trunk diameters of mean = 42.4 cm (*SE* = 1.9) and heights of mean = 3.8 m (*SE* = 0.13) at the start of monitoring. The analysis of moth herbivory will focus on these large trees because of the power available in the longer record that includes more non-drought years.

Climate Proxy and Analyses

We chose 12-month periods for annual calculations of the Standardized Precipitation Evapotranspiration Index, SPEI, as our climate proxy based on: (1) the known sensitivities of pinyon pine to precipitation, summer heat, and vapor pressure deficit (Adams et al., 2009, 2015, 2017; Grossiord et al., 2017; Guérin et al., 2018), (2) the success of SPEI and similar measures for assessing drought effects on trees in southwestern forests (Williams et al., 2013; Anderegg et al., 2015b; Huang et al., 2015; Redmond et al., 2017), and (3) the annual time increment inherent in our data. This measure allowed us to assess the basic patterns of growth, male reproduction, and moth herbivory as correlated with the important climate drivers incorporated into SPEI (precipitation and estimated evapotranspiration as influenced by temperature), while acknowledging that climate data sparseness and lack of inclusion of soil information means this metric will not fully represent drought stress variation in time and space for our individual study sites (Kolb, 2015). SPEI, was calculated and downloaded from the Desert Research Institute website for each of the 11 sites at which trees were monitored (Vicente-Serrano et al., 2010; Abatzoglou et al., 2017). We used the SPEI calculated based on the 12 months prior to September of the measurement year for stem growth and moth killed stem relationships. For the male strobili count measures, we use SPEI for the 12 months prior to May of the measurement year since strobili appear in May. In some cases, sub-sites within areas 1–3 were close enough that the values for interpolated SPEI were identical.

For stem growth area comparisons, we used the lme procedure in the NLME package in R to conduct autoregressive, moving average, mixed-model analysis that allows us to account for time lags (Crawley, 2012; Pinheiro et al., 2018). Tree was always included as a random factor in time (repeated measure) in these analyses. The repeated measures analyses allowed us to accommodate individuals that were added after the initial start of sampling and individuals that died after the start of data collection. We used the time series functionality for lme to test for autoregressive and moving average effects across time lags of 1 and 2 years. The Akaike Information Criterion (AIC) was used for selecting among these models with different correlation structures in time (Burnham and Anderson, 2004). We compared autoregressive (AR) and moving average (MA) effects for $t = 0$, 1, or 2 years in an ARMA (p,q) model where p = the AR lag and q = the MA window, because previous work suggested the likelihood of lag effects (Anderegg et al., 2015b).

Our stem growth and strobili count models for the comparison of patterns with drought (SPEI) across areas with contrasting soil types (H1) included an area fixed effect for the three areas with distinct soil types, SPEI and its interaction with areas, height and basal trunk diameter as well as random effects for sites with areas and for individual trees across years. We included the initial size measures as covariates instead of small and medium size categories to increase the power of the analysis. The results were similar when the size groups were analyzed separately. The correlation coefficient for the basal trunk diameter and height is 0.64, which is low enough to justify including both measures of size. For male strobili counts, we used a Poisson link function in a mixed-model, repeated measures analysis in glmmTMB (Brooks et al., 2017). Mortality for the area comparisons was analyzed in a binomial model of cumulative mortality between 2002 and 2007 since this data gave us enough information content for a meaningful analysis.

The data sets for H2 and H3 were not large enough to meaningfully test for autocorrelation in time. These monitoring data sets include trees at just one site. Because these trees are more uniform in size than the trees in the area comparisons, and the sample size was smaller, we did not include size-related covariates in the analyses for H2 and H3. For stem growth, we again used the lme procedure and for strobili and moth-killed stem counts we used glmmTMB with a Poisson link function.

RESULTS

Model Selection and Multi-Year Effects

The results of model selection for choosing the time period and type of lags included in the final model of stem growth by area are in **Table 2**. In addition to correlations in time, our stem growth models for the comparison of patterns with drought across areas with contrasting soil types included an area fixed effect for the three areas with distinct soil types, SPEI and its interaction with area, height and basal trunk diameter and random effects for sub-sites within area and for individual trees across years. We choose the ARMA (2,1) model with a moving average window, MA = 2 and autoregressive lag, AR = 1. The model choice was based on the criteria of having an AIC more than two lower than any other model (the difference in AIC was 3.3), as suggested by Burnham and Anderson (2004). The next best model was MA = 1, AR = 2 and gave similar significances and parameter estimates for the fixed effects in the model. Based on these results, there are time lag effects for growth in this system, but their exact form is less certain since some models are similar in AIC. These lag effects indicate that multiple years of drought had greater effects together than when drought years were isolated. Variation in the ARMA time lags included in the model does not alter the basic patterns observed in significance of the fixed factors or covariates, and only makes slight modifications to the magnitude of the coefficients for these parameters.

Hypothesis 1: Performance of Trees in Areas With Different Soil-Types

We hypothesized that trees growing at areas determined to be abiotically stressful due to coarse soil texture and low water holding capability before drought would suffer the greatest declines in performance as measured by stem growth, male strobili production, and mortality, but instead observed the opposite patterns. Trees in the coarse soil area (area one), did not suffer greater loss of performance during drought than the other areas (**Figure 1** and **Table 3**). While **Figure 1** shows the actual data and a rough correspondence between SPEI and performance measures, it does not show the full extent of the relationship (slope) between drought and stem growth because it does not account for the time series aspects of the data that are incorporated in the model. For area one, there was a significant, but small, positive slope for the relationship between stem growth and SPEI (slope = 0.66, SE = 0.10, slope different from 0: $p < 0.001$) so stem growth decreased slightly with increased drought. The other two areas had significantly greater slopes of the relationship between stem growth and SPEI when compared to area one (area two slope = 3.13, $p < 0.001$ and area three slope = 2.18, $p < 0.001$, **Table 3A**). These regression results indicate a lower responsiveness of growth to drought in area one (**Figure 1** and **Table 3A**). Taken together, these results show that trees in area one respond less to drought than expected.

Data on male strobili counts also contradicted our hypothesis for the performance of trees growing in the coarse soils of area one relative to areas two and three. Male strobili counts were positively correlated with SPEI (**Figure 1** and **Table 3B**) in all areas, indicating that strobili production was negatively affected by drought stress. The SPEI in **Figure 1** is shifted a few months from the one used for the modeling of male strobili counts because strobili are produced months before growth is completed for the year. Although strobili production declined

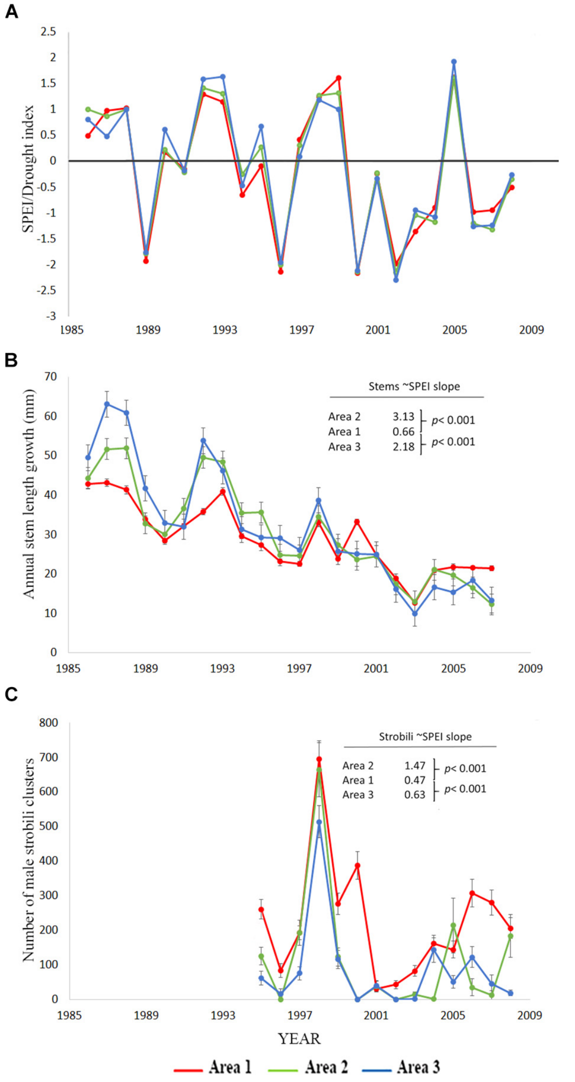

FIGURE 1 | Data for the cross soil-type/area comparisons testing H1. **(A)** Standardized precipitation-evapotranspiration index, SPEI from the DRI website for the 12 months preceding the completion of annual stem growth (Abatzoglou et al., 2017) for which negative numbers indicate greater drought stress. SPEI for the strobili count analysis is different from the one used for stem measures as it is based on the 12 months prior to pollen production in May. Area one is the stressful, coarse soil site. **(B)** Annual stem length growth mean ± SE for the three areas. The coefficients provided are the slopes of the stem growth relationship to SPEI from the mixed model ARMA analysis in **Table 3A** and the *p*-values are tests of significant difference between the area one slope versus areas two and three. **(C)** Number of male strobili clusters mean ± SE for the three areas. The coefficients provided are the slopes of the male strobili count relationship to SPEI from the repeated measures Poisson link function analysis in **Table 3B** and the *p*-values are tests of significant difference between the area one slope versus areas two and three.

TABLE 3 | Statistical analysis for comparisons of areas response to drought (H1).

(A) Annual stem length growth: ARMA model.

	Value	Std. error	DF	*t*-value	*p*-value
Intercept (=area 1)	14.49	1.48	11516	9.786	0.0000
Area 2	0.73	1.64	8	0.443	0.6693
Area 3	1.73	1.64	8	1.054	0.3229
SPEI (=area 1 slope)	0.66	0.10	11516	6.954	0.0000
Basal trunk diameter	0.35	0.09	728	3.955	0.0000
Height	5.99	0.84	728	7.134	0.0001
Area 2*SPEI	2.47	0.17	11516	14.148	0.0000
Area 3*SPEI	1.52	0.16	11516	9.223	0.0000

(B) Number of male strobili clusters: poisson link function.

	Estimate	Std. error	*z*-value	Pr(>| *z*|)
Intercept (=area 1)	−3.33	0.873	−3.8	0.0001
Area 2	−1.14	0.641	−1.8	0.0752
Area 3	−0.75	0.603	−1.2	0.2163
SPEI (=area 1 slope)	0.47	0.001	467.2	0.0000
Basal trunk diameter	0.38	0.022	17.2	0.0000
Height	1.45	0.200	7.3	0.0000
Area 2*SPEI	1.00	0.004	269.0	0.0000
Area 3*SPEI	0.16	0.003	58.1	0.0000

*(A) Stem length growth was modeled in an autoregressive-moving average framework with fixed factors and covariates of area/soil type, drought index (SPEI), the interaction of SPEI and area, initial tree basal trunk diameter and initial tree height. See **Table 2** for model selection resulting in a moving average window = 2 years and an autoregressive parameter = 1 year for the model below. **(B)** Number of male strobili clusters were modeled repeated measures mixed-model with a Poisson link function with fixed factors and covariates of area/soil type, drought index (SPEI), the interaction of SPEI and area, initial tree basal trunk diameter and initial tree height. Random factors in both analyses included sub-sites within areas and individual trees across years. The comparisons of interest are primarily the slopes of the SPEI covariate and how it varies across areas. The intercept and slope for SPEI represent area one and coefficients for areas two and three and their interaction with SPEI represent the degree to which they differ from area one (testing H1).*

in all three areas, the slope of the relationship between SPEI and strobili production was low in area one (slope = 0.47, $p < 0.001$, Poisson link function) relative to areas two and three, which had greater decreases in strobili with drought (slope area two = 1.47, $p < 0.001$ for the contrast with area one and slope area three = 0.63; $p < 0.001$ for the contrast with area one, **Table 3B**).

Also in contrast to hypothesis 1, mortality was not greater for area one (cinder soils) in comparison to the other areas (**Figure 2** and **Table 4**), but instead was either significantly lower or similar. The binomial model of mortality shows that area one had a significantly lower proportion dead (0.30) than area two (0.63, $p = 0.00186$). We were not able to distinguish the proportion dead at area three (0.48) from area one ($p = 0.14036$). Lack of statistical power for this test limits interpretation for area three relative to area one, but it is unlikely that the true mortality is higher for area one.

Hypothesis 2: Performance of Moth Susceptible Versus Moth Resistant Trees Within Area One

We hypothesized that moth susceptible trees suffering chronic herbivory before drought would suffer the greatest declines in performance during drought. However, two lines of evidence show the opposite result or are inconsistent with this hypothesis. First, the stem growth of moth resistant trees was more negatively affected by drought than the stem growth of moth susceptible trees (**Figure 3**). Moth resistant trees had a significant positive relationship between stem growth and SPEI (slope = 3.34, $p < 0.001$; **Table 5A**) meaning that stem growth declined with increasing drought stress. The slope of the SPEI regression for moth susceptible trees is much less (slope = 1.13, $p = 0.0002$ for contrast with resistant tree slope, **Table 5A**). This analysis supports the finding of a smaller decline in stem growth for moth susceptible trees with drought (**Figure 3**) than moth resistant trees. Moth resistant trees had a significant positive relationship between male strobili counts and SPEI (slope = 0.551, $p < 0.001$; Poisson link function, **Table 5B** and **Figure 3C**) meaning that male strobili counts declined with increasing drought stress. Moth susceptible trees, however, had a smaller slope for the relationship with SPEI (slope = 0.225, $p < 0.001$; Poisson link function, **Table 5B** and **Figure 3C**), suggesting that male function in susceptible trees was less affected by drought than resistant trees.

Hypothesis 3: Moth Killed Stems on Susceptible and Resistant Trees Within Area One

We hypothesized that the number of moth killed stems on trees would increase with drought-induced stress. However, in contrast with the hypothesis, moth herbivory declined substantially with

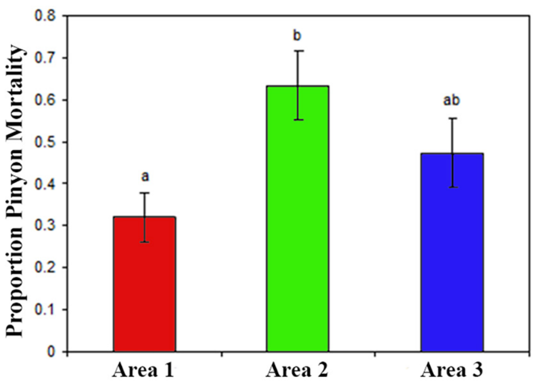

FIGURE 2 | For the cross soil-type/area comparison (H1) these data are the cumulative mortality from 2002 to 2007 across the three areas. Significant differences as indicated by different letters above the bars come from the binomial mixed model in **Table 4**.

drought for moth susceptible trees as indicated by the significant positive relationship slope with SPEI (slope = 0.014, $p < 0.001$; Poisson link function, **Table 6**). The decline through time as SPEI decreases is seen in the moth killed stem count data for susceptible trees (**Figure 4**). According to our model, resistant trees saw a slight increase in herbivory with increasing drought (slope = −0.012, $p < 0.001$; Poisson link function, **Table 6**).

DISCUSSION

The Importance of Long-Term Data to Detect Differential Responses

Long-term data can yield unexpected results (Jackson et al., 2018), non-linear or non-additive responses (Rudgers et al., 2018), and distinct answers from predictions based on spatial variation (Estiarte et al., 2016). In long-term studies up to 32 years in duration, we found variation in the response to climate change as measured by stem growth, mortality, and male strobili production across spatial variation in soil type and between herbivore susceptible and resistant trees interspersed on a fine spatial scale. A landscape level study of *P. edulis* showing greater mortality in coarse-textured soils led us to hypothesize that the coarse, cinder soil sites where pinyon grew more slowly before drought (Cobb et al., 2002) should also suffer greater declines in performance during drought (Peterman et al., 2013), however, we found the opposite. Similarly, poor performance of herbivore susceptible trees before drought and positive responses of the herbivore to tree stress led us to hypothesize greater effects of drought on susceptible trees, however, we again found the opposite. Instead moth attack rates declined and susceptible trees showed very little effect of drought on tree performance. These changes in tree performance and impacts of herbivory across years would not have been detected without long-term data collection that spanned drought and non-drought years. Because of the cumulative multi-year effects of drought on plant growth, a single drought year or even a decade of study in a more constant environment does not reflect the shifts in fundamental patterns that are revealed with changing environmental conditions over many years.

Spatial Variation in Relative Performance Through Time

Before a series of drought years, most indicators suggested that trees growing in the cinder soil areas were under higher abiotic stress as measured by water availability, soil nutrients, plant growth, reproduction, conelet mortality, and insect attack (Christensen and Whitham, 1991; Gehring and Whitham, 1994; Cobb et al., 1997; Swaty et al., 1998; Looney et al., 2012). Based on these findings, we hypothesized (H1) that *P. edulis* growing in the coarse soils (area one) would suffer greater declines with drought in fitness-related traits including survival, stem growth, and male strobili production. This hypothesis was not supported by the data. The cinder soil area (area one), had equal or lesser mortality than other areas and had minor declines in growth and male strobili production with increasing drought stress through time relative to areas two and three. We suggest two explanations for these observations, which may act together and support the hypothesis that most tree species are relatively close to their hydraulic limits (Choat et al., 2012).

First, pinyons in area one that survive in the stressful water and nutrient poor cinder soil environment are adapted and/or acclimated to low water conditions. The relatively strong performance of trees in the cinder soil area through

TABLE 4 | Mortality across area/soil type comparisons was tested (H1) with a mixed, binomial model of cumulative mortality from 2002 to 2007, a time of extreme drought with ongoing mortality.

	Estimate	Std. error	*z*-value	Pr(>| *z*|)
Intercept (=area 1)	−0.66	0.38	−1.714	0.0866
Area 2 difference from intercept	1.50	0.49	3.112	0.0019
Area 3 differences from intercept	0.71	0.48	−1.474	0.1404
Basal trunk diameter	−0.04	0.02	−1.764	0.0778
Height	0.15	0.20	0.784	0.4333

*Sub-sites were random factors nested within areas. Area one is represented by the intercept and area two and three coefficients represent differences from that intercept. Area two has significantly higher mortality than area one (**Figure 2**).*

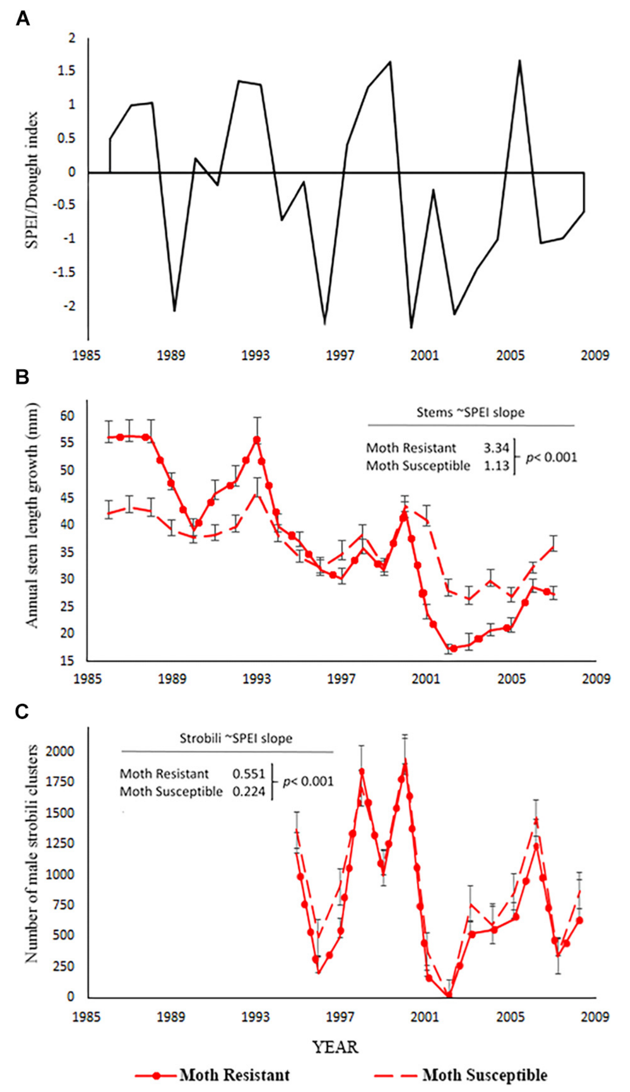

FIGURE 3 | Data for the moth resistant and susceptible tree comparisons (H2). **(A)** Standardized precipitation-evapotranspiration index, SPEI from the DRI website for the 12 months preceding the completion of annual stem growth (Abatzoglou et al., 2017) for which negative numbers indicate greater drought stress. SPEI for the strobili count analysis is different from the one used for stem measures as it is based on the 12 months prior to pollen production in May. **(B)** Annual stem growth mean ± SE for resistant and susceptible trees. The coefficients provided are the slopes of the stem growth relationship to SPEI from the mixed model analysis in **Table 5A** and the *p*-value is the test of significant difference between slopes for moth resistant versus moth susceptible trees. **(C)** Number of male strobili clusters mean ± SE for the resistant and susceptible trees. The coefficients provides are the slopes of the male strobili count relationship with SPEI from the mixed-model analysis in **Table 5B** and the *p*-value is the test of significant difference between slopes for moth resistant versus moth susceptible trees.

severe and repeated drought years suggests that they may have previously adapted and/or acclimated to chronic water stress due to the low water holding capacity of cinder soils. There may have been strong natural selection since trees established in this area in the 1000 years since the volcanic eruptions that established these cinder fields. Development of root and hydraulic characteristics via acclimation to dry soils may lead to trees with improved acclimation to low water conditions that occur during drought (Brunner et al., 2015; Lübbe et al., 2016). For example, *P. edulis* seedlings grown in cinder soils from area one had 1.5-fold more root biomass and 47% higher ectomycorrhizal fungal colonization than seedlings grown in sandy-loam soils from area two (Gehring and Whitham, 1994). Greater investment in roots and ectomycorrhizal fungi could be beneficial during drought when soil resources are further limited. In support of this hypothesis, nitrogen fertilization of *P. edulis* reduced investment belowground and contributed to high mortality during drought in New Mexico (Allen et al., 2010). Greater investment in roots has been associated with resistance to extreme drought in other pines (Matías et al., 2014; Kolb et al., 2016b), but is not always associated with greater drought survival (Kolb et al., 2016b). Thus, acclimation and adaptation of pinyons to chronically stressful cinder soils may have resulted in their higher performance during current droughts than pinyons growing on nearby soils that were less stressful under non-drought conditions. Studies on other plant species suggest that both adaptation and within and across generation acclimation via plasticity are likely to contribute to the drought tolerance of cinder areas trees (Khalil and Grace, 1992; Franks et al., 2007; Aitken et al., 2008; Yakovlev et al., 2012; Latzel, 2015; Trujillo-Moya et al., 2018). A definitive test of the importance of acclimation and adaptation to these patterns in *P. edulis* requires common garden or greenhouse experiments.

Second, abiotic and biotic differences among areas also may contribute to differential performance under drought. In particular, smaller amounts of precipitation in drought years may be more available in coarse cinder soils where infiltration is rapid (Looney et al., 2012; Peterman et al., 2013; Kolb, 2015). Supplemental watering during drought had a larger positive impact on *P. edulis* growing in young, coarse volcanic soil than in older soils derived from the same parent material (Looney et al., 2012). These data, and ours, are consistent with the "inverse texture hypothesis" of Noy-Meir (1973) which proposes that plants growing in coarse-textured soil experience less water stress than plants growing in fine-textured soil because precipitation is more readily lost from the surface of fine-textured soils via evaporation and/or runoff. Sala et al. (1989) suggested a cut-off for reversal of the soil texture effect at 370 mm of precipitation per year that suggests the potential for year-to-year changes in the effects of soil texture on performance. Interactions with soil microbes also vary with soils, drought, and stress in *P. edulis* (Gehring and Whitham, 1994; Gehring et al., 1998, 2014; Swaty et al., 1998; Gordon and Gehring, 2011) and can have important effects on tree performance (Gehring et al., 2014, 2017). Ectomycorrhizal fungi of the genus *Geopora* promoted drought tolerance in *P. edulis* (Gehring et al., 2014, 2017) and dominated on cinder soils both prior to and during drought (Gehring et al., 1998; Gordon and Gehring, 2011), but were uncommon in non-cinder soils, including area two (Gehring et al., 1998). Thus, variation across the landscape in mycorrhizal symbiont interactions could contribute to variation

TABLE 5 | Repeated measures, mixed models were used to test for fixed effects of resistant versus susceptible tree groups, the climate proxy, SPEI, and their interaction on the performance measures of **(A)** stem length growth and **(B)** number of male strobili clusters.

(A) Annual stem length growth: linear model.

	Value	**Std. error**	**DF**	***t*-value**	***p*-value**
Intercept (=resistant)	37.70	1.62	965	23.271	0.0000
Susceptible	−0.79	2.29	48	−0.348	0.7295
SPEI (=resistant slope)	3.34	0.422	965	7.918	0.0000
Susceptible: SPEI	−2.21	0.584	965	−3.791	0.0002

(B) Number of male strobili clusters: generalized linear model: poisson link function.

	Estimate	**Std. error**	***z*-value**	**Pr(> |*z*|)**
Intercept (=resistant)	6.798	0.153	44.56	0.0000
Susceptible	0.356	0.242	1.47	0.1420
SPEI (=resistant slope)	0.551	0.002	230.59	0.0000
Susceptible*SPEI	−0.326	0.004	−75.53	0.0000

*Coefficients and significance testing for fixed factors for testing H2 are in the table below. The comparisons of interest are primarily the slopes of the climate proxy, SPEI, covariate and how it varies across resistant and susceptible tree categories. Both models include individual trees as random factors. The intercept represents resistant trees and SPEI is the slope with SPEI for resistant trees. Susceptible and susceptible*SPEI are the degree to which the main effect and slope differ from the resistant trees (test of H2).*

TABLE 6 | The relationship between moth killed stems model as a Poisson response variable and the climate proxy, SPEI, for large resistant and susceptible trees (as assessed at the beginning of monitoring) was tested with a repeated measures, mixed model analysis with SPEI as a covariate (H3).

	Estimate	**Std. error**	***z*-value**	**Pr(> |*z*|)**
Intercept (=resistant)	6.102	0.028	217.08	0.0000
Susceptible	0.073	0.040	1.86	0.0636
SPEI (=resistant slope)	−0.012	0.002	−6.06	0.0000
Susceptible*SPEI	0.026	0.003	9.29	0.0000

*Individual trees were modeled as random factors in time. The intercept represents the resistant category of trees and the slope coefficient for SPEI represent the relationship between moth killed stems and SPEI for resistant trees. The coefficient for susceptible trees and susceptible*SPEI represent the degree to which susceptible trees differ from resistant trees in intercept and slope, respectively (**Figure 4**).*

in drought tolerance, alone or in conjunction with pinyon genetic variation and soil moisture properties.

Our findings that male reproduction was negatively affected by drought supports other research documenting the potential sensitivity of pinyon pine reproduction to climate change. Cone production has declined broadly across the landscape (Redmond et al., 2012) and pollen germination is impaired by the temperatures that *P. edulis* is predicted to experience as climate changes (Flores-Renteria et al., 2018). In this long-term study, we also saw declines in the production of male strobili with drought. While sporadic reproduction and repeated measures on trees experiencing mortality obscure these trends in the raw data (**Figure 1C**) the analysis in **Table 3B** supports male strobili declines with drought. Areas one and two show differential responses in strobili production to drought with area one responding less to drought stress and area two responding more (**Figure 1C** and **Table 3B**). Strobili production was of intermediate responsiveness to drought at area three which means strobili did not follow exactly the same pattern as stem growth. Area three has older, volcanic substrate soil that is relatively low in phosphorus (Selmants and Hart, 2010). Because lower phosphorus has been associated with lower allocation to male reproduction (Lau and Stephenson, 1994; Fujita et al., 2014) this might be an area to pursue in future studies of pinyon allocation to growth versus male reproduction.

Decline in Moth Resistant Tree Performance and Moth Attack During Drought

Previous work on the stem-boring moth and its interactions with pinyon pine has shown that this herbivore decreased trunk and branch growth of pinyon pine by ∼45% and shifted reproductive allocation away from female cones and toward male strobili (Whitham and Mopper, 1985; Cobb et al., 2002; Mueller et al., 2005b). This led to the hypothesis that susceptible trees would not fare well with the added stress of drought (H2). Other evidence, such as poor survival of moth resistant trees relative to susceptible trees during drought (Sthultz et al., 2009a) and poor performance of the offspring of moth resistant trees relative to susceptible trees in a common garden (Gehring et al., 2017), has been accumulating since the start of the monitoring program described here that contradicts H2. Our long-term observations

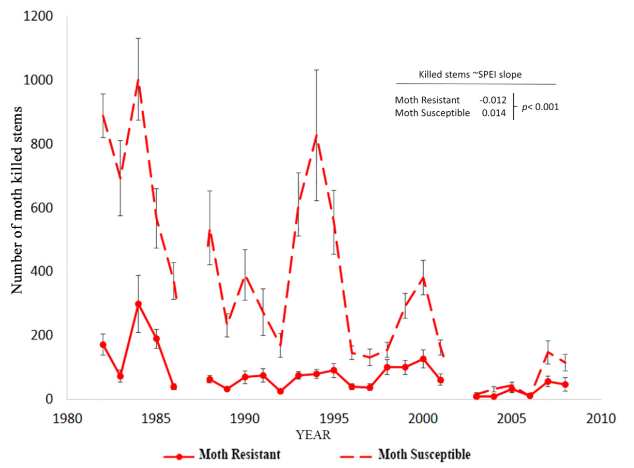

FIGURE 4 | The decline in moth killed stem counts on large susceptible trees through time in comparison to resistant trees. There is a significant positive relationship between moth attack and the climate proxy, SPEI, for susceptible trees (**Table 6**).

of tree growth through time also indicates that moth resistant trees are less tolerant of long-term drought than moth susceptible trees suggesting tradeoffs in which moth resistant trees perform best under normal conditions, but susceptible trees perform best during drought conditions.

Intraspecific variation in drought tolerance has been observed within populations of other species of pine (Gaspar et al., 2013; Kolb et al., 2016b). In ponderosa pine (*P. ponderosa)*, most of the variation in drought-adaptive traits of southern populations occurred within populations (Kolb et al., 2016b). While there is abundant evidence that drought tolerance varies among conifer populations (Moran et al., 2017), the variation among moth resistant and susceptible trees within area one suggests that within population variation is also significant and important to evaluate for reforestation programs where warmer, drier climates are projected.

Our results from male strobili counts demonstrate greater declines under drought for moth resistant trees. In a study conducted before the 2002 mortality event, Cobb et al. (2002) showed that greater strobili production on moth susceptible trees resulted in 1.5-fold greater pollen production from moth susceptible than moth resistant trees. Our data indicate that drought is likely to increase this difference; declining strobili production following drought suggest that resistant trees are even less likely to serve as pollen donors under drought. Research on pollen viability conducted on cinder soils showed that *P. edulis* pollen was highly sensitive to the temperatures predicted with climate change during both the dispersal and germination stages of development (Flores-Renteria et al., 2018). Further research is necessary to determine if resistant and susceptible trees now differ in pollen viability, potentially further shifting male function to moth susceptible, drought tolerant trees. We acknowledge that female cone production, which was not described in this study, may be a more important limitation to reproduction. Female cone production has declined precipitously in the southwest with drought (Redmond et al., 2012).

The decline in moth attack rates with drought was unexpected because earlier studies found that trees growing under favorable, non-drought conditions, moth resistant trees produced two–three times more wound resin than susceptible trees, which is important in resisting damage from stem-boring moths (Mopper et al., 1991; Cobb et al., 1997). Hence, we expected that the moth would continue to increase in numbers on trees further stressed by drought (H3), consistent with the plant stress hypothesis (White, 1969, 1976). Instead, we found that with further increased drought stress, moth attack rates declined. One interpretation of this decline would be that there is a direct impact of drought conditions on moth overwintering, survival, and growth. The alternate interpretation would be an intermediate stress hypothesis in which low and high stress trees are not suitable as hosts for stem-borers because of a tradeoff in which low stress results in vigorous growth with too much resin production that kills larvae, while high stress results in little resin, but too little growth to accommodate tunneling stem-borers. In this scenario, only intermediate stress results in high enough stem growth for stem-borers to thrive and a reduction in resin production to a level that moth larvae can tolerate. This second hypothesis is supported, in part, by experimental water and fertilizer treatments in which experimentally reduced-stress trees suffered significantly less moth herbivory than control trees (Cobb et al., 1997).

There are complex interactions between plant growth and herbivory in this system. Resistant trees grow more slowly than susceptible trees before insect attack begins on mature trees as demonstrated by tree ring studies (Ruel and Whitham, 2002) and common garden studies on maternal families (Gehring et al., 2017). Once herbivory begins, susceptible trees experience lower growth rate and low cone production. This study, and other accumulating evidence, suggests that the relatively good performance of insect susceptible trees during drought is due to a combination of drought resistance and release from herbivore pressure. Thus, periodic drought may help maintain susceptible trees in this system. While cone and seed production is very low during drought, it is likely to shift toward favoring susceptible trees which contribute more pollen during dry times, as well as potentially producing more female cones due to reduced losses of cone-bearing shoots to moth herbivory.

AUTHOR CONTRIBUTIONS

AW contributed to data collection, data analysis, and led the writing of the manuscript. NC contributed to the design of the work and the collection of data. LF-R contributed to analysis and figures and revised the manuscript. CG helped design the study, collect the data, and write the manuscript. SM helped collect the data and revise the manuscript. TW helped design the study, collect the data, and revise the manuscript.

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The Potential of Liming to Improve Drought Tolerance of Norway Spruce [*Picea abies* (L.) Karst.]

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In response to a wide-spread decline in forest vitality associated with acid rain in the 1980s, liming of soils has been implemented in many federal states in Germany to buffer further acid deposition and improve availability of nutrients such as calcium and magnesium. As a consequence, it may also increase vitality and depth of fine-root systems and hence improve the drought tolerance of species such as Norway spruce [*Picea abies* (L.) Karst.], which occurs mostly on acidic forest soils. However, the influence of repeated liming on drought tolerance of trees has never been studied. Here we compared the resistance, recovery and resilience of radial growth in *P. abies* in relation to drought in limed and control stands and assessed how the dosage and interval between lime application and drought year influences the radial growth response of *P. abies*. We analyzed radial growth in 198 *P. abies* trees of six experimental sites in south–west Germany. An analysis of the radial increment over the last 30 years allowed the analysis of drought events shortly after the first liming (short-term effect) as well as posterior drought events (mid- to long-term effects). Generalized linear models were developed to assess the influence of drought intensity, site and period since first liming on the drought tolerance of Norway spruce. Regardless of drought intensity, there was no general increase in drought resistance of Norway spruce in response to liming. However, drought resistance of radial growth improved on a loamy site that was additionally treated with wood ash 30 years after the first lime application. Furthermore, recovery and resilience of radial growth after severe drought events were generally better in spruce trees of limed treatments. This indicates a shorter stress period in spruce trees growing on limed soil, which may reduce their susceptibility to secondary, drought-related pests and pathogens.

Keywords: Norway spruce, liming, drought tolerance, resistance, resilience

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INTRODUCTION

The adaptation of forests to climate change is currently a major challenge of silviculture. In Central Europe, this applies in particular to forests dominated by Norway spruce [*Picea abies* (L.) Karst.] which has been cultivated on many sites, where it will no longer be suitable or associated with high production risks under future climate conditions (Hanewinkel et al., 2013). On the other hand, Norway spruce is still the economically most important tree species in Germany. According to the results of the last German Forest Inventory from 2012 (BWI III), Norway spruce has a share of 25% of the total forest area and will dominate the German raw timber supply until the middle of this century (BMELV, 2018).

To improve the tolerance of Norway spruce stands against climate change impacts, different silvicultural strategies can be pursued. In the long-term, more drought tolerant tree species may be admixed or used to replace Norway spruce (Vitali et al., 2017). In the short-term, stability and vitality of existing trees and stands may be increased through silvicultural manipulations such as thinning that provide more growing space to trees (Kohler et al., 2010; Sohn et al., 2012).

Likewise, liming is assumed to promote the vitality of forest stands on acidic soils. In many federal states of Germany, liming has been practiced since the early 1980s in response to a wide-spread decline in forest vitality associated with acid rain. For this purpose, mostly magnesium-containing limestone powder (dolomite) has been applied in dosages of ca. 3 t ha^{-1} every 10 years. The main goal of these lime applications has been to increase the buffering of the acidity input from atmospheric deposition to counteract the man-made soil acidification and loss of soil quality (Ulrich, 1986; Huettl, 1989; Wilpert et al., 2013). Furthermore, lime application may increase the availability of Ca, Mg, and possibly other nutrients to trees, stimulate decomposition of forest floor material and its incorporation in the mineral soil, and increase fine-root vitality and proliferation in deeper soil layers (Schäffer, 2006; Wilpert et al., 2013).

Based on these effects of liming, it has been proposed that liming should be continued to be applied on acidic soils to increase the drought tolerance of forests (Landesanstalt für Umwelt Messungen und Naturschutz Baden-Württemberg [LUBW], 2013). However, there has been no study that has explicitly analyzed the effects of liming on the drought tolerance of forests. Mostly, existing liming experiments addressed short to medium-term effects on soil properties and forest nutrition (Ulrich, 1986; Huettl, 1989; Kreutzer, 1995; Ponette et al., 1997; Schaaf and Hüttl, 2006). Few studies considered long-term effects on tree growth. Following a once-only lime application of 2 t ha^{-1}, the growth of Norway spruce in the Northern Black Forest increased for more than 25 years (Spiecker, 1991). In that study, also interannual weather effects on the growth response after liming have been described, however, resistance and resilience of tree growth in relation to particular drought events were not evaluated quantitatively. In Norway spruce stands limed once with 2.5 t ha^{-1}, a two-phase growth response with a strong increase (plus 54%) in the first 8 years after liming, and a subsequent decrease to a growth level which remained considerably higher than in the control stand (plus 20%) was observed (van der Perre et al., 2012). Particularly in the second phase (>8 years after liming), the interannual variability of the "growth gain associated to liming" (van der Perre et al., 2012) increased significantly and could be partially explained by interannual variation in weather conditions. This increase in "weather-sensitivity" of radial growth after liming was explained through the Norway spruce-specific, shallow root system as well as the limited "depth effect" of only one liming measure (van der Perre et al., 2012). Corresponding to these results, there are several studies that found even more shallow fine-root systems of Norway spruce after liming (Kreutzer, 1995; Helmisaari and Hallbäcken, 1999). This would mean that liming may rather increase the risk for drought stress. In contrast, Schäffer (2006) found for Norway spruce a deeper and more homogeneous root penetration as well as higher fine root densities in the mineral soil, even more than 50 years after lime application. However, the lime dosage in that experiment was extremely high (10 t ha^{-1}).

To what extent repeated applications of lime actually improve drought tolerance of single trees or forest stands remains unclear. There have been no studies on the growth response during (resistance) and following drought years (recovery and resilience) in relation to the dosage and number of lime applications as well as the interval between lime application and the drought event. Therefore, the hypotheses addressed in this study were:

(1) Resistance, recovery and resilience of radial growth in *P. abies* in relation to drought are higher in limed than in control stands.
(2) Effects of liming on radial growth resistance, recovery and resilience of *P. abies* are influenced by site, the number of lime applications, as well as the period between lime application and drought event.

MATERIALS AND METHODS

Study Sites

The study sites are part of a long-term liming experiment in the German federal state of Baden-Württemberg, which was initiated in the beginning of the 1980s. We selected six sites (**Table 1**) which are located in different climatic regions and representing typical forest soils in those regions. The sites are distributed over an area extending 270 km in north–south (Heidelberg to Bad Waldsee) and about 120 km in west-east (Herzogenweiler to Hospital) directions. They represent a broad and typical variety of Norway spruce stands in South–West Germany. Sites are characterized by a homogeneous topography to exclude expositional effects on tree growth.

All sites received the first application of dolomite of 3 t ha^{-1} in 1985 and contained an untreated control. In 2003, all sites were limed again with 6 t ha^{-1} of pure dolomite. The higher dosage was chosen to trigger more pronounced effects of liming on soils and forest stands than could be observed until then. At sites Heidelberg, Horb and Weithard, the third lime application 2015 was done with a 3.85 t ha^{-1} dolomite – wood ash mixture in a ratio of 3:1 whereas at sites Bad Waldsee, Herzogenweiler and Hospital common dolomite (3.85 t ha^{-1}) was applied also in 2015. The wood ash was taken only from the combustion of untreated woods and subject to thorough quality control (Wilpert et al., 2016).

Soils were sampled at five plots per treatment down to a depth of 60 cm in May 2015 using root corers with a diameter of 8 cm. In the field, fine-root tips were counted at the core surfaces in 5 cm depth increments. Soil chemical variables were determined according to the protocol of the Gutachterausschuss forstliche Analytik (2009). The pH was measured in 1 M KCl and cation

TABLE 1 | Summary information on main characteristics of the experimental sites.

	Bad Waldsee	Heidelberg	Herzogenweiler	Horb	Hospital	Weithard
Latitude [°]	47°50′	49°30′	48°01′	48°28′	48°07′	47°58′
Longitude [°]	9°41′	8°47′	8°20′	8°32′	9°41′	9°17′
Mean annual temperature [°C] 1971–2013	8.7	9.4	6.8	8.4	8.2	7.9
Annual precipitation [mm] 1971–2013	970	1130	1190	1160	840	830
Altitude [m asl]	580	490	950	630	650	630
Main tree species	*P. abies*	*P. abies*	*P. abies*	*P. abies*	*P. abies*	*P. abies*
Mixed tree species	*F. sylv., L. dec.*		*A. alba*	*A. alba*	*F. sylv., L. dec.*	
Soil types	Cambisols	Podsols	Cambisols (stagnosols, luvisols)	Cambisols (stagnosols, luvisols)	Stagnosols (luvisols)	Stagnosols (cambiosols, luvisols)
Parent material	Glacial till (Würm stage)	Sand stone (middle lower Trias)	Sand stone (upper lower Trias)	Sand stone (upper lower Trias)	Glacial till (Riss stage)	Glacial till (Würm stage)
Soil texture class	Loam	Sandy loam	Loam	Clay loam	Loam – silty loam	Loam
Humus types	Mull to moder mull	Mull	Mull to moder mull	Mull	Mull to mor moder	Mull to mor moder

Mixed tree species are abbreviated as F. sylv. (Fagus sylvatica L.), L. dec. (Larix decidua MILL.), and A. alba (Abies alba MILL.). Meteorological data derived from Dietrich et al. (2018).

TABLE 2 | Mean pH values and base saturation (BS) in upper (0–20 cm) and lower (21–60 cm) mineral soil layers at study sites (measured before liming campaign 2015).

Site	Bad Waldsee		Heidelberg		Herzogenweiler		Horb		Hopsital		Weithard	
Treatment	**Liming**	**Control**	**Liming**	**Control**	**Liming**	**Control**	**Liming**	**Control**	**Liming**	**Control**	**Liming**	**Control**
pH (KCl) 0–20 cm	3,8	3,4	3,8	3,0	3,7	3,4	3,7	3,4	3,6	3,4	3,7	3,5
pH (KCl) 21–60 cm	4,1	3,9	4,0	4,1	3,9	3,9	3,8	3,7	3,7	3,7	3,8	4,0
BS [%] 0–20 cm	33,0	9,0	82,0	8,0	30,0	4,0	41,0	20,0	29,0	8,0	37,0	8,0
BS [%] 21–60 cm	10,0	6,0	26,0	5,0	9,0	4,0	8,0	13,0	14,0	18,0	14,0	7,0

exchange capacity in 1M NH_4Cl-solution. Base saturation was calculated as the percentage ratio of the sum of the ion equivalents of Ca, K, Mg, and Na to the cation exchange capacity. All soil chemical properties of the limed sites represent soil properties before liming in 2015. As indicated by pH values between 3 and 4 (**Table 2**), soils at all study sites were very acidic. Previous liming had either no (soil depth 21–60 cm) or only a moderate effect (soil depth 0–20 cm) on pH. In contrast, liming had considerably increased base saturation, especially in the top mineral soil at 0–20 cm depth meaning that the risk for deficiencies in basic nutrients – which may occur at these low soil pH levels – had been reduced after liming.

Also, liming lead to higher numbers of fine roots, mainly in the topsoil. This effect was strongest at the Herzogenweiler site and weakest at Hospital (**Figure 1**).

Sampling of Tree Cores and Measurement and Processing of Tree-Ring Series

Trees selected for coring showed no visible signs of pathogens, needle abscission, dieback or other damages. Additionally, diameter at breast height (dbh, 1.3 m), and height were measured for all sample trees. The ratio of height over dbh was used as an indicator for single tree stability and competition pressure (Albrecht et al., 2012). Using height and age data of trees, site indices according to yield tables of Schober (1995) (mean annual increment for 100 years = MAI_{100}) were derived. Except for the site Horb, no distinct differences in tree and stand variables (e.g., height, diameter in breast height, stand age) could be observed between the two treatments. At Horb, differences in tree variables between the two treatments could be attributed to the younger age of the control (by 35 years) when compared to the limed stand (**Table 3**). Overall, growth properties observed in trees from both treatments have been within the normal range being typical for Norway spruce stands in Baden-Württemberg (BWI III).

Increment cores were taken in the summers of 2016 (Heidelberg, Horb, Weithard) and 2017 (Bad Waldsee, Herzogenweiler, Hospital) from 198 *P. abies* trees of (co-) dominant canopy status. Sample trees were evenly distributed among both treatments at each site. From each tree, two increment cores were taken at breast height in northern and western cardinal directions. Cores were dried and subsequently sanded to obtain clean surfaces for the analysis of radial increments. To determine the radial increment, tree-ring widths were measured to the nearest 0.01mm using a Lintab digital positioning table (Rinntech, Heidelberg, Germany) in conjunction with the time series analysis program TSAP

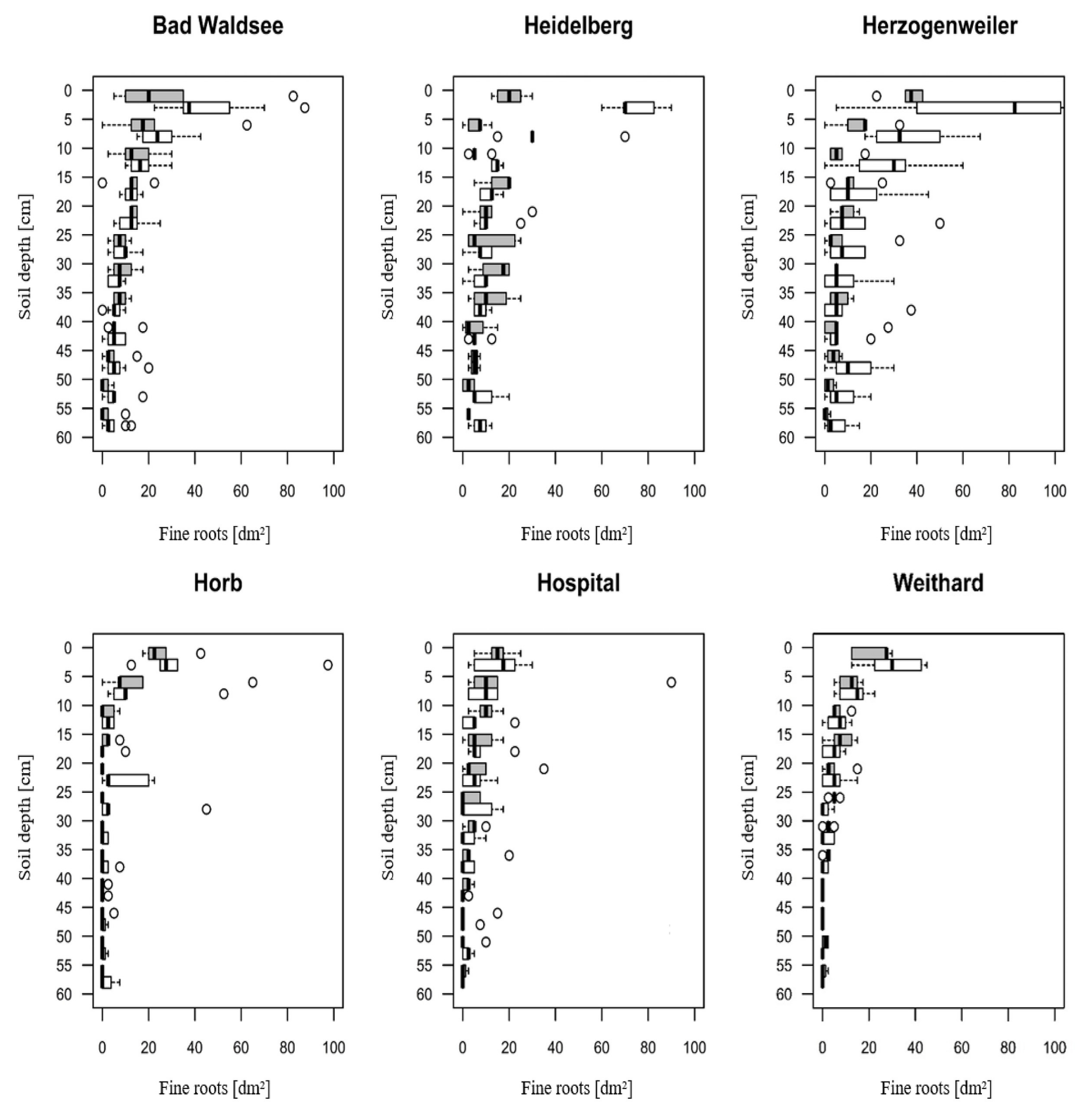

FIGURE 1 | Fine-root densities (tips per dm^2 soil) in 0 to 60 cm soil depth at study sites (measured before liming campaign 2015). Gray box plots indicate the control group; white box plots denote the liming treatment. Circles indicate outliers.

TABLE 3 | Mean tree height, diameter at breast height (dbh), height to diameter ratio (h/d), stand age and site index of sampled *P. abies* trees.

Site	**Bad Waldsee**		**Heidelberg**		**Herzogenweiler**		**Horb**		**Hospital**		**Weithard**	
Treatment	**Liming**	**Control**	**Liming**	**Control**	**Liming**	**Control**	**Liming**	**Control**	**Liming**	**Control**	**Liming**	**Control**
Tree height [m]	33.3	32.7	33.5	34.2	31.2	31.9	33.6	30.1	32.8	32.8	35.5	34.9
dbh [cm]	48.4	47.0	48.0	47.1	43.2	46.8	45.5	34.4	47.2	46.5	55.2	50.1
h/d ratio	69.7	70.3	71.1	73.5	72.3	68.8	74.3	88.2	70.7	71.0	65.3	69.9
Stand age [yr]	70	70	75	75	85	95	95	60	80	65	90	75
Site index [m^3yr^{-1}]	15.3	15.0	14.7	15.0	11.3	10.8	11.8	14.7	13.4	15.8	14.0	15.7
N sample trees	16	16	18	18	16	16	17	17	16	16	16	16

TABLE 4 | Dendrochronological indices (mean sensitivity (ms), first-order autocorrelation (ar1), cross-correlation between single series (rbar), and the expressed population signal (EPS) for *P. abies* trees from all study sites and treatments.

Site	Bad Waldsee		Heidelberg		Herzogenweiler		Horb		Hospital		Weithard	
Treatment	**Liming**	**Control**	**Liming**	**Control**	**Liming**	**Control**	**Liming**	**Control**	**Liming**	**Control**	**Liming**	**Control**
ms	0.263	0.260	0.267	0.287	0.222	0.211	0.260	0.257	0.268	0.255	0.281	0.263
ar1	0.774	0.746	0.812	0.746	0.858	0.859	0.876	0.892	0.732	0.794	0.876	0.863
rbar	0.533	0.519	0.754	0.673	0.795	0.670	0.830	0.835	0.508	0.623	0.784	0.706
EPS	0.939	0.928	0.975	0.957	0.983	0.966	0.981	0.981	0.938	0.958	0.975	0.959

(Rinntech, Heidelberg, Germany). Individual tree-ring series were cross-dated visually using the TSAP software, also an interval sign test (Gleichläufigkeit) was applied (Schweingruber, 1983). Ring series that could not be synchronized were removed from further analyses. Tree-ring series from the two cores of one tree were averaged and subsequently, series were transformed to basal area increment (BAI) which is less biased by stem geometry or age trends (West, 1980) and hence expected to provide better dendrochronological estimates (LeBlanc, 1990). Afterwards, BAI series were detrended by applying a smoothing spline function with a 50% frequency cutoff of 2/3 of the series length to remove long-term trends owing to age, size and stand dynamics (Fritts, 1976). Calculation of basal area increment, detrending and the quality control of the tree-ring data were conducted using the software *R* 3.4.4[1] and the packages *dplR* (Bunn, 2008), and *detrendeR* (Campelo et al., 2012).

Dendrostatistical indices were used to assess the accuracy and quality of tree-ring data. We calculated mean sensitivity (ms), first-order autocorrelation (ar1), cross-correlation between single series (rbar), and the expressed population signal (EPS) (Fritts, 1976; Wigley et al., 1984). The computed dendrochronological indices (**Table 4**) indicated an overall good accuracy of the used tree-ring data. Mean sensitivity ranged between 0.21 and 0.28, which is common for trees of temperate forests (Fritts and Shatz, 1975). Also, the values for ar1 and rbar were within a favorable range (Lebourgeois et al., 2005). Although the validity of the expressed population signal itself is highly discussed (Buras, 2017), the EPS values for all sites and treatments were clearly above 0.85, which is commonly used as an accuracy threshold for studies of tree populations (Wigley et al., 1984).

Identification of Drought Years

To identify and classify retrospectively drought events that occurred at the study sites, the non-dimensional, multi-scalar Standardized Precipitation Index (SPI) was used (McKee et al., 1993). To calculate the index, site specific data of monthly precipitation sum provided by the Forest Research Institute of Baden-Wuerttemberg (FVA) were used. Meteorological data were derived from regionalized data according to Dietrich et al. (2018). Data gaps were filled with time series of monthly precipitation sums from nearby meteorological stations of the German National Meteorological Service (DWD). All meteorological stations were located less than 15 km from the study sites and at a comparable altitude (Kunz et al., 2018). In case that more than one station was available, the data of the additional stations were used to control quality of the meteorological time series (Mayer et al., 2005). Following the recommendations by Hayes et al. (1999), SPI-3 (June to August precipitation of the current year) time series were calculated from 1961 to 2016. The calculation of the SPI was conducted using the software *R* 3.4.4 and the package *spi* (Neves, 2012).

For an accurate interpretation of the index, objective drought thresholds have to be defined (Quiring, 2009). According to McKee et al. (1993), SPI values up to -1 define normal conditions, SPI values between -1 and -1.49 represent 'moderate drought' conditions, SPI values between -1.5 and -1.99 indicate 'severe drought', and SPI values ≤ -2 indicate 'extreme drought'. In our study, years in which at least moderately dry periods were present (SPI values below -1), were selected as 'drought years' and further studied. The most common and distinct drought years were 1962, 1983, and 2003, which were detectable at all six study sites (**Table 5**). The years 1964, 1998, and 2015 were detectable as drought years at four study sites. The most extreme single drought event was detected 1983 in Weithard, where a SPI value of -3.48 was reached. After the start of the liming experiment in 1985, only two extreme drought events have been detected with SPI-3 value of -2.35 in Bad Waldsee in year 2003 and SPI-3 value of -2.13 in Horb in year 2015.

Responses of Radial Growth to Climatic Variations

We conducted retrospective analyses of radial growth before, during, and after drought years. For this purpose, indices for resistance (*Rt*), recovery (*Rc*), and resilience (*Rs*) of radial growth in relation to moderate, severe, and extreme drought years were calculated following the approach of Lloret et al. (2011). In general, resistance, recovery and resilience can be seen as components of a species' ability to tolerate stress (Pimm, 1984), where tolerance is defined as the potential of a single species or the whole ecosystems to withstand stress and/or swing back into a stable state following stress exposure without changing into a new system (Peterson et al., 1998). Against this background, we calculated these drought tolerance indices as follows:

$$Rt = \frac{\text{BAI DY}}{\text{BAI PreDY}} \tag{1}$$

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[1] www.r-project.org

TABLE 5 | Summary of the SPI-3 values calculated for the months June to August of the current year.

Bad Waldsee		Heidelberg		Herzogenweiler		Horb		Hospital		Weithard	
Year	**SPI-3**	**Year**	**SPI-3**	**Year**	**SPI-3**	**Year**	**SPI-3**	**Year**	**SPI-3**	**Year**	**SPI-3**
2003	−2.35	**1983**	−2.85	**1983**	−2.30	**1983**	−2.74	**1983**	−2.71	**1983**	−3.48
1983	−2.27	1976	−2.36	1991	−1.73	2015	−2.13	**1962**	−2.45	**2003**	−1.70
1998	−1.92	**2003**	−1.91	1998	−1.73	1981	−1.75	1998	−1.92	1964	−1.68
1962	−1.85	1964	−1.66	1985	−1.71	**1962**	−1.65	**2003**	−1.62	2015	−1.37
2015	−1.64	**1962**	−1.58	1988	−1.43	1976	−1.58	1964	−1.57	1981	−1.35
1981	−1.38			1994	−1.22	**2003**	−1.55	2015	−1.21	1984	−1.22
2004	−1.35			**1962**	−1.20	1989	−1.49	2006	−1.06	**1962**	−1.11
2006	−1.18			1984	−1.14	1964	−1.44			1972	−1.02
				1974	−1.12					2013	−1.02
				2003	−1.12					1998	−1.01
				2005	−1.01						

Only years with at least 'moderate drought' are shown (SPI-3 values below −1). Drought years are ordered by increasing SPI-3 values. Years in bold characters indicate droughts year that were detectable at all six study sites. SPI-3 values in light gray cells indicate 'moderate drought years,' mid gray cells mark 'severe drought years,' and dark gray cells represent 'extreme drought years.'

$$Rc = \frac{\text{BAI PostDY}}{\text{BAI DY}} \tag{2}$$

$$Rs = \frac{\text{BAI PostDY}}{\text{BAI PreDY}} \tag{3}$$

where BAI DY is the detrended annual basal area increment (BAI) during the drought year, BAI PreDY is the BAI during the years before the drought, and BAI PostDY is the BAI during the years after the drought of individual trees (Lloret et al., 2011). Based on Kunz et al. (2018) who found that neither extended, nor shortened reference periods between 3 and 5 years changed results in the calculation of resistance, recovery, and resilience significantly, we used a period of 5 years before and after the drought event as reference periods for the calculation of these indices. No indices of recovery and resilience could be calculated for the drought year 2015 as increment cores were taken in summer 2016 and 2017. The calculation of the drought tolerance indices was conducted using the software *R* 3.4.4 and the package *pointRes* (van der Maaten-Theunissen et al., 2015).

Statistics

We used generalized linear models (GLMs) to analyze to what extent factorial variables such as "treatment" (limed, control) or "site" and continuous variables such as "stand age at drought event," "site index," and "time period between first liming and drought year" (Δt) explained variation in Rt, Rc, and Rs. Originally, we intended also to test the "time period between the last liming and drought year" (Δt_{last}) and the "total dosage of lime applied at drought event." However, as a result of the temporal distribution of the three lime applications and the identified drought years we had finally too few data to establish models including these latter two variables. For non-limed control plots, we also assigned corresponding Δt values so that we could test in joint models whether possible Δt effects could exclusively be attributed to liming or occurred also in non-limed control plots.

We used GLMs as our target variables were mainly not normally distributed and could also not be consistently normalized through common transformation methods. We fitted separate models for each of the three drought intensity types (moderate, severe, and extreme droughts). The model parametrization was done stepwise considering all available continuous and factorial variables. Non-significant variables were excluded from the model if this led to a simpler model with a more favorable AIC (Akaike's information criterion). We also considered interactions between variables, especially between site and treatment to check for site-specific liming responses. In cases, where we found models with a positive response to liming, we additionally tested the influence of time period between first liming and drought year by adding the variable Δt to the best model of the corresponding target variable.

All data analysis was done with the software R 3.4.4. Tests for normal distribution were conducted using the package "nortest." Generalized linear models were fitted using the "gls" function from the package "nlme." Model selection was done using the package "MuMIn" based on AIC. Model visualization was carried out using the package "visreg."

RESULTS

Radial Increment Series of Sample Trees

When comparing the mean radial increment series of Norway spruce trees between treatment and control groups, no positive effect of liming on diameter growth was evident except for the Herzogenweiler site (**Figure 2**). This was in accordance to the positive effects of liming on fine root distribution (**Figure 1**) which was strongest at Herzogenweiler. At Hospital, radial increments of trees in the control were continuously – before and after the liming experiment had started – higher than in the limed treatment. This may be explained with differences in age-related growth dynamics at this site (**Table 3**). Similarly, at Weithard, the limed site had initially lower radial increments than the control

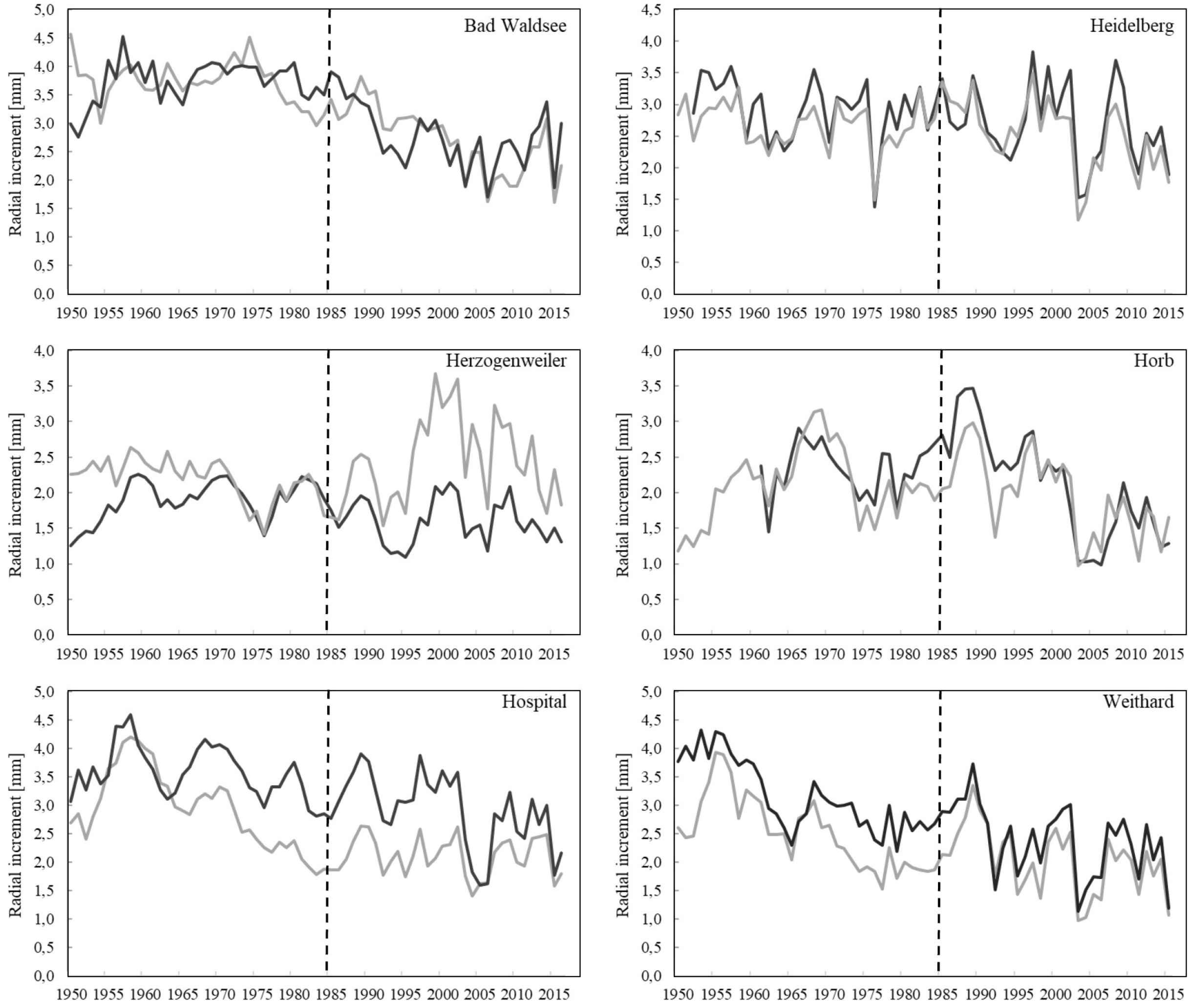

FIGURE 2 | Mean radial increment of *P. abies* trees in limed treatments (gray lines) and controls (black lines) of the different study sites. Lime application started in 1985 (dashed line).

but within 5 years after the lime application it reached the growth level of the control. This can be interpreted as liming effect. At all study sites, the long-term mean radial increment ranged between 2 and 3 mm year^{-1}.

Drought Resistance of BAI (Rt)

Model results showed that site was the predictor variable that explained most variation in resistance of *P. abies* across all drought intensities (**Table 6**).

In moderate drought years, the BAI in spruce trees at Horb was not reduced and at all other sites only slightly reduced, respectively (**Figure 3A**). The strongest growth depression of 15% compared to the pre-drought level was detected at Hospital. In the second best model, we also found for the factor treatment a significant effect. However, the effect size was almost negligible as the resistance (Rt) was only 3% better at limed than at control sites (**Table 6**). We also tested models considering potential interactions between site and treatment, but no site-specific treatment effect could be detected.

In years classified as severe droughts, we found no treatment effect at all but the site-specific resistance to drought (Rt) varied considerably. At Heidelberg, Horb and Weithard, radial growth was reduced to less than 70% of the pre-drought level, whereas only a slight reduction at Bad Waldsee (−6%) and Hospital (−5%) and almost no change of Rt at Herzogenweiler could be observed (**Figure 3B**).

Owing to the low number of extreme drought years in the 30-year period after first lime application in 1985, only the two study sites Bad Waldsee and Horb and the two drought years 2003 and 2015 could be used for model analyses. The three best models with comparable AICs between −46 and −45 (**Table 6**) identified a better resistance (Rt) at site Horb (+25%), a negative influence of site index on Rt (with a slope of −0.053 per unit site index) and a site-specific treatment effect at site Horb where Rt of limed Norway spruce trees were distinctly better (+20%)

TABLE 6 | Model results (glm) for the BAI resistance (Rt) to drought of *P. abies*.

	Predictors for Rt								
Drought type	**Treatm. control**	**Site**	**mai$_{100}$**	**Stand age at drought**	**Elevation**	**Site × Treatment**	**AIC**	**Delta**	**AIC weight**
Moderate		**X**					−318	0	0.66
(*N* = 348)	**X (−3.3%)**	**X**		x			−316	2	0.30
	x	**X**					−312	6	0.03
				x			−279	39	0.00
	X (−3.5%)			x			−277	41	0.00
Severe		**X**					−213	0	0.97
(*N* = 300)	x	**X**					−206	7	0.03
	x	**X**		x			−195	18	0.00
					0.0005		−122	91	0.00
	x				**0.0005**		−116	97	0.00
Extreme			**−0.0529**				−46.1	0	0.39
(*N* = 58)		**X**					−45.5	0.6	0.28
	x	**X**				**X**	−44.9	1.2	0.21
	X (−9.1%)	**X**					−43.3	2.8	0.09
	x		**−0.0502**				−39.9	6.2	0.02

Only the best fitting five models according to the AIC values are listed for moderate droughts (SPI −1 to −1.49), severe droughts (SPI −1.5 to −1.99) and extreme droughts (SPI ≤ −2), respectively. X indicates significant factorial predictors, x indicates non-significant factorial predictors and bold figures provide the slope of significant continuous predictors. For the factor "Treatment" also the relative intercept change from control to limed treatment is listed.

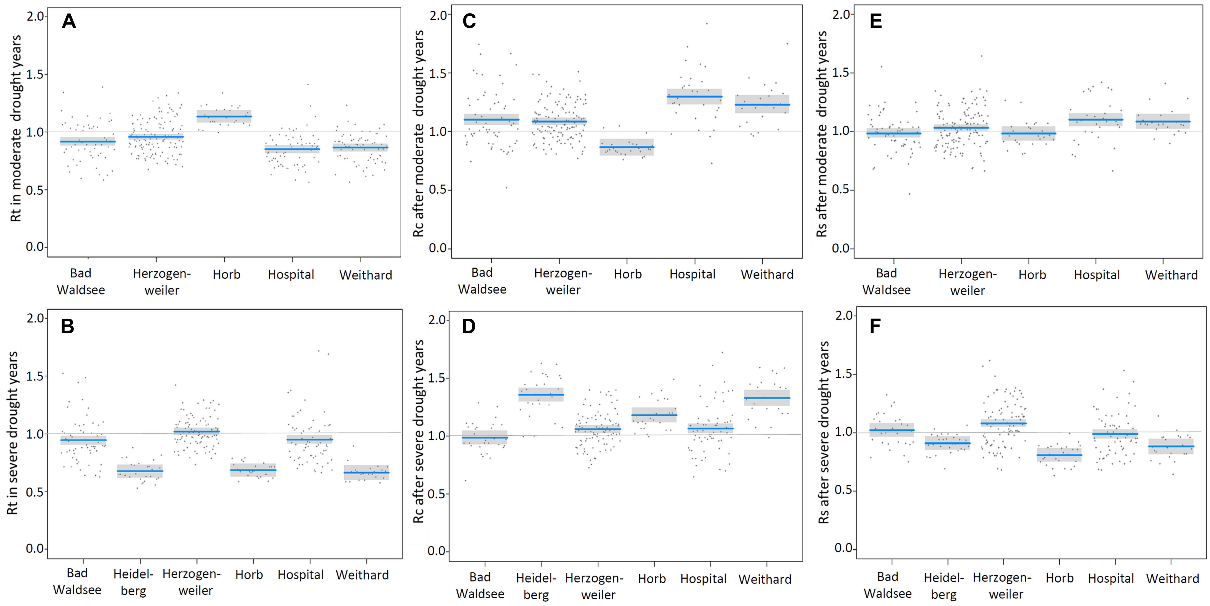

FIGURE 3 | Resistance "Rt" **(A,B)**, recovery "Rc" **(C,D)** and resilience "Rs" **(E,F)** of detrended annual basal area increment in relation to moderate (upper panels) and severe drought years (lower panels) at the different sites (blue lines: modeled values of Rt, Rc and Rs; gray bands: confidence interval for the expected value; dark gray dots: residuals).

compared to control trees while at Bad Waldsee no treatment effect could be detected (**Figure 4**).

Growth Recovery After Drought (Rc)

For Rc and likewise for Rs (chap. 3.4), modeling included only moderate and severe droughts as for the second of the two detected extreme drought events (2003 at Bad Waldsee and 2015 at Horb) post drought year tree-ring data were not available (sampling at Horb in summer 2016) and the remaining sample size was not sufficient to establish valid models.

Also for recovery (Rc), site was the most distinct factor to predict radial growth recovery of Norway spruce, regardless of drought intensity. Considering the best models for moderate drought years, no treatment effect on Rc could be detected

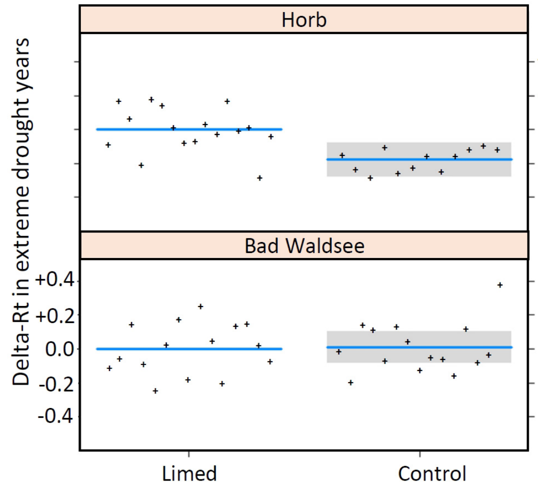

FIGURE 4 | Interaction model (Site × Treatment) for the resistance of radial growth (rt) in extreme drought years expressed as the difference (ΔRt) between control and limed treatment. Extreme drought events (SPI ≤ -2) occurred only in 2015 at Horb and 2003 at Bad Waldsee (plots created by R function "visreg, type = contrast," therefore confidence bands are not given for the reference "Limed").

(**Table 7**). The recovery of BAI after moderate droughts compensated at least growth depressions during previous drought years at all sites with the exception that at Horb the index of Rc indicated a growth reduction of about 15% in the 5 post drought years (**Figure 3C**). However, this Rc reduction corresponded exactly to the amount of additional growth in the dry year (Rt), indicating that after the dry year growth rate returned to the "normal growth" before moderate drought years (**Figure 3A**).

For severe droughts, models showed clearly that site and treatment were the most important factors to predict Rc. Based on the best model (AIC = −161), the growth recovery after severe drought was approximately 8% better in trees from limed sites compared to controls (**Table 7**). However, Rc varied to a much greater extent between sites (**Figure 3D**). At Bad Waldsee, Herzogenweiler and Hospital, Rc indices were close to 1.0 which was already observed for Rt, meaning that radial growth was only slightly affected during and after severe droughts at these sites. In contrast, the growth recovery at sites which showed highest growth depressions (Rt) during the drought, ranged from ca. 20% at Horb to almost 40% at Heidelberg and Weithard. We also found significant models for Rc considering the elevation as negative predictor variable or testing the interaction between site and treatment. However, these models had poor AIC values (AIC-Delta between +24 and +73) and were therefore rejected (**Table 7**).

Growth Resilience After Drought (Rs)

Also for the growth resilience after drought (Rs), site was the most important predictor in models for both drought intensities.

For moderate droughts, the model with best AIC (=−192) clearly indicated no treatment effect for Rs ($p = 0.46$). The only model with a significant predictor included the factor site but had already a slightly poorer AIC (Delta +6; see **Table 8**). The two sites with highest growth depression during and best recovery after moderate drought years, Hospital and Weithard, also had the highest resilience of radial growth (Rs) (>1.1). However, also the remaining sites showed Rs indices of at least 1.0 which indicated that there were no medium-term growth reductions following moderate droughts (**Figure 3E**).

For severe droughts, the two best models with AICs of −172 and −169 (**Table 8**) identified relatively wide, site-specific variations of Rs and a general (i.e., not site-specific) treatment effect which indicated a 6% better resilience of BAI in trees on limed sites compared to control trees. At Horb (Rs = 0.8), Weithard (Rs = 0.9), and Heidelberg (Rs = 0.9), the mean radial growth in the 5 years after severe droughts did not completely return to the pre-drought growth levels, in spite of the high recovery rates described above (**Figures 3D,F**). In contrast,

TABLE 7 | Model results (glm) for the BAI recovery (Rc) of *P. abies* after drought.

	Predictor for Rc								
Drought type	**Treatm. control**	**Site**	**mai$_{100}$**	**Stand age at drought**	**Elevation**	**Site × Treatment**	**AIC**	**Delta**	**AIC weight**
Moderate		**X**					−99	0	0.98
(*N* = 270)	x	**X**					−91	8	0.02
	x	**X**		0.0011			−79	20	0.00
	x	**X**				**X**	−77	22	0.00
			0.0142				−45	54	0.00
Severe	**X (−7.8%)**	**X**					−161	0	0.99
(*N* = 139)		**X**					−152	9	0.01
	X (−8.1%)	**X**		0.0007			−148	13	0.00
	x	**X**				**X**	−137	24	0.00
	X (−6.4%)				**−0.0003**		−88	73	0.00

Only the best fitting five models according to the AIC values are listed for moderate droughts (SPI −1 to −1.49) and severe droughts (SPI −1.5 to −1.99) respectively. X indicates significant factorial predictors, x indicates non-significant factorial predictors and bold figures provide the slope of significant continuous predictors. For the factor "Treatment" also the relative intercept change from control to limed treatment is listed.

TABLE 8 | Model results (glm) for the BAI resilience (Rs) of *P. abies* after drought.

	Predictor for Rs							
Drought type	**Treatm. control**	**Site**	**mai_{100}**	**Stand age at drought**	**Elevation**	**AIC**	**Delta**	**AIC weight**
Moderate	x					−192	0	0.75
(*N* = 270)			−0.0014			−188	4	0.13
				−0.0011		−186	6	0.06
		X				−186	6	0.05
	x		−0.0012			−181	11	0.00
Severe		**X**				−172	0	0.85
(*N* = 270)	**X (−5.8%)**	**X**				−169	3	0.15
	x	**X**		0.0012		−156	16	0.00
					0.0004	−153	19	0.00
	X (−5.2%)				**0.0004**	−149	23	0.00

Only the best fitting five models according to the AIC values are listed for moderate droughts (SPI −1 to −1.49) and severe droughts (SPI −1.5 to −1.99), respectively. X indicates significant factorial predictors, x indicates non-significant factorial predictors and bold figures provide the slope of significant continuous predictors. For the factor "Treatment" also the relative intercept change from control to limed treatment is listed.

all sites with high growth resistance to severe droughts (Bad Waldsee, Herzogenweiler, Hospital) also showed favorable Rs indices ≥ 1.0.

Influence of Time Period After First Lime Application on Rc and Rs

To further study the observed treatment effect for recovery and resilience of radial growth after severe droughts, Δt – the time period between first lime application and the drought year – was added to the best models described above. For Rc, neither a general nor a treatment-specific influence of Δt could be detected. In contrast, a significantly positive effect of Δt was found for the resilience of radial growth (Rs). However, this effect of Δt could not be assigned to liming as there was no interaction between Δt and treatment (**Table 9**).

DISCUSSION

This study investigated whether the drought tolerance of Norway spruce can be improved through repeated lime applications. Our results showed that there was no general increase in drought tolerance after liming. Differences in the radial growth response during and after drought years depended on drought intensity and were in most cases to a greater proportion explained by site than by liming. Furthermore, the timing of the identified drought events of different intensity in combination with the number and dosage of lime applications must be considered when interpreting our results.

TABLE 9 | Model results (glm) testing the influence of time period after liming (Δt) on BAI recovery (Rc) and resilience (Rt) of *P. abies* after severe drought (SPI −1.5 to −1.99).

	Predictor (severe droughts only)						
	Treatment	**Site**	**Δt**	**Δt × Treatment**	**AIC**	**Delta**	**AIC weight**
Rc Models	**X**	**X**	0.0012		292	0	0.99
(*N* = 139)	**X**	**X**	0.0020	x	301	9	0.01
Rs Models	**X**	**X**	**0.0287**		221	0	0.97
(*N* = 139)	**X**	**X**	**0.0227**	x	228	7	0.03

X indicates significant factorial predictors, x denotes non-significant factorial predictors, bold figures indicate the slope of a significant continuous predictor.

Growth Resistance (Rt)

There was no general treatment effect of liming on resistance of radial growth. In moderate droughts, the response of Rt to liming in Norway spruce was almost negligible; only 3% higher than in control trees. This weak response of Rt to liming corresponded with small growth changes in moderate drought years across all sites investigated.

In contrast, site-specific growth depressions in years classified as severe drought have been much more prominent (up to −40%). Nevertheless, we found also for severe droughts neither a general nor a site-specific improvement of Rt in trees of limed treatments. To our knowledge, there are no other studies on the effect of liming on the drought responses of "Llorets growth indices" (Rt, Rc, and Rs) that could be used for comparison of our findings. However, it was demonstrated previously that the drought resistance of radial growth in Norway spruce could also not be improved by thinning (Kohler et al., 2010; Sohn et al., 2012), which is a silvicultural option to improve stability and vitality of trees through enlarging their growing space. These studies showed that radial growth reduction in larger trees with presumably larger individual root systems (from heavy thinning) was as large as that of trees with less growing space. It demonstrated that the size of the root system does not confer any advantages, if the entire soil profile falls dry during drought years. We may assume that the same would apply to root systems that may be more intense as a result of liming. The advantage that liming could provide during drought years is therefore an increase in deep roots that would ensure that tree may access soil or ground water for longer periods. However, that was obviously not the case.

Severe droughts were dated between 1985 and 2003, only at Bad Waldsee we identified a severe drought in 2015 (**Table 5**). This means that our models for severe droughts mainly included data from the first 18 years of the experiment considering the short to mid-term effect of the first lime application in 1985 only (3 t dolomite ha^{-1}) whereas the fine-root distributions shown in **Figure 1** were assessed in 2015, and therefore influenced through the second, distinctly higher application rate of lime in 2003 (6 t dolomite ha^{-1}).

Importantly, our results on the resistance of radial growth in severe drought years do not confirm findings of previous studies where an increase of weather-sensitivity of radial growth in limed trees was detected (van der Perre et al., 2012). This had been explained with the shallow root system of Norway spruce and the limited depth effect of a single lime application. Other studies had found even more shallow root systems of Norway spruce after liming, indicating an increased risk of drought stress through the treatment (Kreutzer, 1995; Helmisaari and Hallbäcken, 1999). However, based on results from our study, we can exclude an increased risk for drought stress after once-only liming in the mid- to long-term at all study sites. Unfortunately, we have no data on possible changes in fine-root distributions within the 18-year period after the first liming. Yet, it has already been shown that even after single lime applications, a deeper and more homogeneous root penetration as well as higher fine-root densities of Norway spruce can be achieved in the long-term (Schäffer, 2006). However, the lime dosage in that study was distinctly higher (10 t ha^{-1}) than the commonly applied quantity of 3 t ha^{-1}, which was also the dosage of the first liming in our study.

Within our 30-year observation period, we identified only two extreme droughts, namely in 2003 at site Bad Waldsee and in 2015 at site Horb. For the extreme drought at Bad Waldsee, we found also no treatment effect on the resistance of BAI suggesting that the assumptions discussed above already for severe droughts can be applied here, too. The second liming that occurred at this site in 2003, is unlikely to have had any direct impact on the growth reaction in that drought year as again slowly soluble dolomite rock powder was applied, even if the dosage of 6 t ha^{-1} was the double quantity of the first application in 1985.

In contrast, there was a strongly positive effect of liming on Rt (+20%) for the extreme drought 2015 at Horb. Several explanations are possible for this phenomenon. The root survey from 2015 showed increased fine-root densities at Horb after the two lime applications in 1985 and 2003, both in the upper mineral soil (0 to 5 cm depth) and at medium depth (20–25 cm) which may explain the higher resistance of limed trees at Horb in 2015, if the increased rooting density led to a later onset of drought stress (**Figure 1**).

Furthermore, the better Rt in limed trees could also have been influenced by the third lime application at Horb, where a mixture of dolomite rock powder and readily soluble wood ash was carried out in May 2015. Wood ash admixtures are considered to improve rapidly the supply of plant available potassium (K) (Wilpert et al., 2013). In previous studies, a distinct and prompt increase of foliar K concentrations in Norway spruce was detected in response to wood ash application within a short-term period (Schäffer, 2002; Wilpert, 2002). Foliar K content is assumed to play an important role in the drought stress tolerance of trees, since cellular K^+ fluxes control the opening and closing of stomata (Kadereit and Strasburger, 2013). Unfortunately, foliar element analyses of limed and control trees in or after the drought 2015 are not available yet. However, potassium availability is limited in many forest soils in Baden-Württemberg owing to a selective potassium depletion on soil aggregates surfaces which has been observed especially on loamy sites (Hildebrand, 1990; Wilpert et al., 1993; Wilpert and Hildebrand, 1994). At Horb, the soil texture class was monitored as clay-loam (**Table 1**), so that limitations in the foliar potassium nutrition cannot be excluded there. Nevertheless, whether improved stomatal control may be responsible for such a strong increase in radial growth resistance is questionable.

The 35-year age difference between trees limed and the control stand could potentially also have influenced the different Rt response to extreme drought at Horb. However, this can be largely excluded as in none of the Rt models, regardless of drought intensity, the variable "stand age at drought" has been found to be a significant predictor (**Table 6**). Likewise, the predictor "site index" – which was a significant predictor of Rt when used in a mono-variable model – had no explanatory power at all in the Rt model considering the interaction between "site" and "treatment." This indicated that the improved Rt was not a general site index effect but a site-specific treatment effect which has been developed after 30 years at Horb. Otherwise, the site index should have been a significant predictor of Rt already in previous droughts, which was not the case (**Table 6**).

Recovery and Resilience of Growth (Rc and Rs)

The response of Rc and Rs to liming was different depending on the drought intensity. After moderate drought, we found no treatment effect on both response variables. All sites showed resilience indices of at least 1.0, which means that growth returned completely to its respective pre-drought level at all sites. This was not unexpected as it corresponded to the only little growth changes of Rt in moderate drought years so that the absence of a treatment effect also for Rc and Rs was not surprising.

After severe droughts, both Rc and Rs were found to be significantly higher in trees from limed treatments compared to control trees. This means that liming had an impact on Llorets-growth indices similar as found in previous studies for thinning: no effect on growth resistance, but significant better growth recovery of treated Norway spruces in post drought years. However, the effect size of heavy thinning was much stronger with a Rc improvement that was mostly higher than 25% (Kohler et al., 2010; Sohn et al., 2012). This higher recovery was only possible because trees in these thinning experiments that were carried out at other sites, both from thinned and unthinned plots, were more strongly affected during the drought (lower Rt values of less than 0.5) than in the liming experiments. In our study, the treatment effect of liming was only of moderate size with an Rc and Rs improvement of +8% and +6%, respectively. Otherwise, it must be seen that this positive response to liming was the result of once-only applications of dolomite rock powder in the common dosage of 3 t ha^{-1}. Thus, our results provided evidence that already after a single lime application a measurable improvement of drought tolerance in Norway spruce can be achieved in the short- to medium-term. Since we have no data on fine-root distribution, sap flow or foliar element contents for this period, it is not possible to point to the underlying mechanisms.

Influence of Time Span Between Liming and Drought (Δt) on Rc and Rs

We could not find any influence of Δt on the recovery or resilience of radial growth after severe droughts. For this, different explanations are possible. As already mentioned, severe droughts occurred predominantly in the 18-year period after the first lime application. Perhaps this time period was not long enough to detect any time effect of liming in our models, especially when considering that the observed effect of liming on Rc and Rs was only of moderate size.

Furthermore, increasing stand ages within the 18-year period could have blurred possible time effects of liming on Rc and Rs. However, we could not find any significant effect of the predictor "stand age at drought event" in our models. In the literature, contrasting results on the role of stand age on climate sensitivity of tree growth have been reported. No general effect of tree age on climate sensitivity of radial growth has been found in Zang et al. (2014) while in other studies both increased sensitivity (e.g., Martinez-Vilalta et al., 2012) or also decreased sensitivity with advancing tree age were described (Szeicz and MacDonald, 1995). Compared to these studies, the period of changing stand age was relatively short so that the absent "stand age effect" in our models was reasonable.

A liming-specific Δt effect could also be obscured by the general recovery of soil chemical and forest nutritional conditions which has been reported for many forest sites in Germany in the second nation-wide forest soil survey (Wellbrock et al., 2016). This improvement of environmental conditions for forest growth was mainly caused by the reduction of the airborne deposition of sulfur which led also in Baden-Württemberg - where the sites of our study were located – to a measurable increase of pH-values in many forest soils (Hug et al., 2005). A general trend of increasing growth and vitality of Norway spruce since the mid of the 1980s has also been reported in Uhl et al. (2013).

CONCLUSION

This is the first study that tested the influence of repeated lime applications on the growth response of Norway spruce trees to droughts of different intensity across a variety of sites in south-western Germany. We showed that liming did not generally reduce the impact of drought during the event. This would only be possible, if liming increased water holding capacity of the soil, improved the maximum rooting depth to increase the soil volume from which trees can extract water, or fostered physiological drought responses such as stomatal control to reduce transpiration. The latter two may have occurred to some extent at one site that was additionally treated with wood ash 30 years after the first lime application, leading to high concentrations of soluble potassium in soil.

Despite the lack in stress reduction during drought events, liming improved the drought tolerance of trees. In contrast to the assumptions in previous studies, our results indicated that even a single lime application can lead to moderate improvements of growth recovery and resilience after severe droughts. Even if this liming effect was only moderate, it may help to reduce the stress period in trees, which in turn may reduce the vulnerability to secondary, drought-related pests and pathogens. This is particularly relevant in case of Norway spruce.

However, the relatively small influence of liming on drought tolerance of Norway spruce will not facilitate the cultivation of this species at sites with high drought risk. Apart from the above mentioned increase in vulnerability to pest and pathogens, also distinctly higher risk of drought induced stem-crack damages must be expected, especially in younger and fast growing stands of Norway spruce on fertile soils (Kohler et al., 2017). Thus, Norway spruce dominated stands growing at sites with high drought risks should be converted into mixed stands with more stable and climate-change tolerant trees species (Spiecker, 2003; Vitali et al., 2018).

AUTHOR CONTRIBUTIONS

MK made substantial contributions to the conception and design of the study, analysis and interpretation of data, and is the lead author of the manuscript. JK was mainly responsible for data acquisition and has participated in data analysis and writing. JH made substantial contributions to data acquisition and interpretation of data. PH, LJ, and HP contributed to data acquisition, data interpretation and writing. JB had the idea for this study. KW and JB contributed to data interpretation and writing.

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