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Volatile terpenes (VT) content in tree-ring resin, in response to natural infection by *Heterobasidion* spp. in asymptomatic mature Norway spruce (*Picea abies*) trees was investigated. Twenty-three randomly selected mature trees were sampled in a stand in the western Italian alps by extracting cores using an increment borer. Based on fungal isolations from cores and molecular typing using taxon-specific competitive-priming (TSCP)-PCR, 12 out of the 23 trees were identified as infected by *Heterobasidion parviporum*. Tree-ring growth

patterns and VT content in tree rings were determined. Analysis of VT content was performed by means of gas chromatography mass spectrometry on a subset of trees. Results show slightly but not significantly lower tree-ring width in infected compared to non-infected trees in the past two decades. Total concentrations of sesquiterpenes and relative proportions of α-pinene, β-pinene and longifolene were significantly greater in infected trees; while relative proportions of camphene, 3-carene, ρ-cymene, sesquiterpene 15.90 and α-farnesene were significantly lower. This is the first study showing that VTs in tree-ring resin may indicate infection of trees by a fungal forest pathogen, even when trees are mostly asymptomatic.

Introduction

Inducible volatile terpenes (VTs) are abundantly produced and released by different plant organs following abiotic stresses (e.g., Loreto and Schnitzler, 2010; Leonelli et al., 2014) and biotic attacks, including those by insects and pathogens (e.g., Holopainen, 2004; Jansen et al., 2011).

In conifers, VTs are produced and stored in several plant structures, including constitutive resin ducts (CRDs), i.e., species-specific wood anatomical characteristics, and traumatic resin ducts (TRDs). Resin is toxic for most pathogens due to its composition and physical properties (Phillips and Croteau, 1999). In fact, resin contains monoterpenes, diterpenes and sesquiterpenes and some, especially when produced and released abundantly, are known to be insecticidal, antimicrobial and fungicidal (Schuck, 1982; Michelozzi, 1999; Trapp and Croteau, 2001). Conifer resin is produced in bark, phloem and xylem by constitutive and inducible secretory structures, releasing primary and secondary resin, respectively.

In Norway spruce [*Picea abies* (L.) Karst], resin accumulates both in CRDs and in TRDs, which appear within the developing xylem after mechanical wounding, in stem xylem. The formation of TRDs associated with enhanced production of VTs is part of a complex mechanism of plant defence that is activated to induce the successful tree reaction to the attack of pathogens and mechanical damage (Franceschi et al., 2000; Nagy et al., 2000; Fäldt et al., 2003; Krokene et al., 2008; Gärtner and Heinrich, 2009; Danielsson et al., 2011; Brauning et al., 2016). TRDs considerably enhance the oleoresin content of

Norway spruce, considering that they are larger, and thus their volume is much higher, than CRDs. TRDs usually develop in high number in the proximity of the injury caused by mechanical wounding or pathogens, and their number decreases as the distance from the wound increases (Schmidt et al., 2011). TRDs are commonly used for dating events which injure the cambium in geomorphology (e.g., Stoffel, 2008; Butler et al., 2010; Garavaglia and Pelfini, 2011), but their frequency and distribution within tree rings are poorly investigated. In some tree species, most of the resin ducts seem to develop in the latewood (Reid and Watson, 1966), but their distribution is highly variable within the same tree, due to environmental and climatic conditions (Wimmer et al., 1999).

Norway spruce is susceptible to heart rots caused by some fungi included in the *Heterobasidion annosum sensu lato* (*s.l.*) species complex, namely *H. annosum* (Fr.) Bref. and *H. parviporum* Niemelä & Korhonen (Garbelotto and Gonthier, 2013). While the former species is more generalist being able to attack several coniferous tree species, the latter displays a preference for Norway spruce. Regardless of which one of the two species is involved, the disease is mostly asymptomatic in mature trees. In fact, the progressive development of the decay in the heartwood rarely results in the appearance of external symptoms (Garbelotto and Gonthier, 2013). Heart rots caused by *Heterobasidion* spp. are among the most destructive and widespread diseases of Norway spruce in Europe, including the Alpine region (Asiegbu et al., 2005; Gonthier et al., 2012; Giordano et al., 2015). Infection occurs through airborne spores (primary infections) colonising freshly exposed wood surfaces (stumps or wounds in the stem or roots). Subsequently, the fungus can infect uninjured trees by vegetative growth of mycelium through root contacts or grafts (secondary infections) (Garbelotto and Gonthier, 2013).

The production of spores by *Heterobasidion* spp. is more abundant when air temperature are above 5°C (Gonthier et al., 2005). For this reason, climate warming may prolong the time interval favourable for sporulation and infection during the year for *Heterobasidion* spp, The altitude at which pathogens can be found may also be shifted to higher elevations (La Porta et al., 2008).

Defensive strategies and VT production are usually studied under controlled experimental conditions obtained from controlled crosses, and that are artificially inoculated with the pathogen (e.g., Cellini et

al., 2014; Piesik et al., 2015) or in which the pathogen attack is mimicked by treatment with methyljasmonate (e.g., Arnerup et al., 2013). In particular, experiments conducted on Norway spruce revealed that the oleoresin of trees affected by *Heterobasidion* spp. was different to that of non-affected trees in terms of amounts of (+)-α-pinene, (+)-sabinene, (-)-sabinene, δ-3-carene, (-)-limonene and γ-terpinene (Zamponi et al., 2007). However, we are not aware of any studies conducted on the oleoresin content of mature trees infected by *Heterobasidion* spp. in forest stands. Moreover, little is known about VT production in asymptomatic trees. A better understanding of this topic may be crucial for developing strategies allowing the set-up of useful markers enabling the early diagnosis of tree diseases, that could prevent losses in forest productivity, and to assess which factors can influence the climatic signal recorded in tree rings at high altitude (Leonelli et al., 2012).

The main aim of this research was to detect possible differences in VT content in tree-ring resin in response to natural infection by *Heterobasidion* spp. in asymptomatic mature Norway spruce trees. Tree-ring growth was also analysed in infected and non-infected trees in order to investigate if any difference in growth patterns could be attributed to the presence of the pathogen.

Methods

Study site and sampling design

The study site is located in the Western Italian Alps at about 1450 m a.s.l. close to the area called Ermitage (45°47'46.11''N; 6°58'56.39''E), in the municipality of Courmayeur (Aosta Valley Region), where *Heterobasidion* spp. were previously detected in a mature mixed Norway spruce-European larch (*Larix decidua* Mill.) forest stand. About 55% of trees were estimated to be infected (Gonthier et al., 2012). The stand, with a standing volume of 227 m^3 ha^{-1} and a density of 410 trees ha^{-1}, was thinned in 1995. This area and adjacent valleys, i.e., Val Veny and Val Ferret, have been well studied in order to better understand the impact of the climatic and related environmental changes on vegetation (for a review see Bollati et al., 2015).

In an attempt to compare a similar number of infected and putatively non-infected trees, 23 randomly selected trees were sampled at the end of June 2015 by extracting four wood cores at 90° from one another at the base of stems (20 cm above the ground) using a Pressler's increment borer (for details about sampling techniques see, e.g., Pelfini et al., 2007). The minimum and mean distance among sampled trees was 25 m and 80 m, respectively. The diameter at breast height (DBH) of sampled trees ranged between 68 cm and 145 cm (mean 99 cm). Cores were transported to the laboratory in plastic straws and stored at 5°C before subsequent analyses. Two cores were used for isolation and pathogen detection, one for the dendrochronological analyses and one for VT analyses in tree rings (Fig. 1).

Pathogen detection and identification at species level

Cores were sprayed with a benomyl solution (0.010 g benomyl, 500 µL methanol, 1 L distilled water) and incubated for about 10 days at room temperature (25°C ± 2°C) in a moist chamber as described by Gonthier et al. (2003). After incubation cores were inspected under a dissecting microscope (x20 magnification) in order to check for the presence of emerging colonies of the conidial stage of *Heterobasidion* spp.

Fungal isolations were made by transferring infected wood or fungal hyphae onto 6-cm Petri dishes containing a PCNB-based selective medium for *Heterobasidion* spp. (Kuhlman and Hendrix, 1962). All isolates were subsequently subcultured and stored at 5°C on MEA (malt extract agar: 20 g glucose, 20 g malt extract, 2 g peptone, 20 g agar, 1 L distilled water).

DNA from fungal isolates was extracted by a hyphal tipping method (Schweigkofler et al., 2004), modified as follows: fungal mycelium was collected with the tip of a micropipette and suspended in 100 µL of distilled water, frozen on dry ice for 3 minutes, thawed at 75°C, vortexed for 1 minute, and finally centrifuged for 5 minutes at 19,000 g. Freezing and thawing were repeated three times, with the last thaw extended to 15 minutes. Samples were then centrifuged for 5 minutes at 19,000 g and the supernatant was used as template for polymerase chain reactions (PCRs). Identification of *Heterobasidion* isolates at the species level was carried out by a taxon-specific competitive-priming

(TSCP)-PCR (Garbelotto et al., 1996) combined with a PCR-mediated detection of species-specific DNA insertions in the ML5-ML6 DNA region of the mitochondrial large ribosomal RNA (mt LrRNA) gene as described by Gonthier et al. (2001).

Dendrochronological analysis

The cores were prepared for tree-ring dating and ring-width measurements following standard methods (Stokes and Smiley, 1968), usually applied in dendrochronological studies conducted in mountain environments and in the nearest geographical areas (e.g., Pelfini et al., 2007; Garavaglia et al., 2010). Tree-ring widths were measured to the nearest 0.01 mm using the LINTAB system with the TSAPWin software (Frank Rinn, Heidelberg, Germany), and the obtained series were visually and statistically cross-dated using the COFECHA software (Grissino-Mayer, 2001) in order to find and correct any dating error in the dataset. Two main ring-width mean chronologies were built: one, named “pathogen”, using the trees found to be infected by *Heterobasidion* spp., and one, named “no pathogen”, using trees putatively non-infected by the pathogen.

To analyse tree-ring growth trends in the two groups of trees, the raw ring-width series were standardized using the software Arstan (Holmes, 1992) and a residual chronology for each category was prepared applying a negative exponential curve.

VT analysis in tree rings

Five trees infected and five trees putatively non-infected by *Heterobasidion* spp. were selected for the analyses of VTs. Selection was mainly based on the overall conditions of the cores: priority was given to the cores with no broken tree rings, at least in the terminal part of the core, and characterised by easily identifiable tree rings. The last five tree rings of each core (corresponding to the years from 2010 to 2014) were split from each other using a scalpel, for a total of 50 samples (Fig. 1).

VT relative content was determined by means of gas chromatography mass spectrometry. For this procedure, about 25 mg of cortical and xylem tissues were placed into a sterilised vial, and 200 µL of

pentane with tridecane as internal standard was added to each vial, after which the vials were put in a Soltec ultrasound machine Sonica 2200 S3 at the temperature of 30°C for 60 minutes. The vials were left in a Gerhardt Thermoshake THO 5 for 24 hours, and the extracts were then filtered with 0.45 µm PTFE syringe filters and injected (3 µL) in the GC-MS system. An Agilent 7820 GC-chromatograph equipped with a 5977A MSD mass spectrometer with EI ionisation operating at 70 eV was used for analysis. A chromatographic column J&W Innovax 50 m, 0.20 mm, ID 0.4 µm DF was used. The GC injection temperature was 250°C, splitless mode, and the oven was programmed at 40°C for 1 minute, followed by a ramp of 5°C/minute to 200°C, and of 10°C/minute to 260°C. This high temperature was held for 5 minutes. Mass spectra were acquired within the 29-350 M/Z interval with an Agilent 5977 MSD spectrometer at three scans s^{-1}. VT identification was done on the basis of both peak matching with library spectral database (NIST 08) and kovats indeces as retrived in literature for the identified compounds.

Total absolute amounts (total concentrations) of monoterpenes (total MTs) and sesquiterpenes (total SQTs) were expressed as milligrams of terpenes per grams of fresh tree tissue and they were analysed by non-parametric Mann-Whitney U Test, in order to test differences between the two groups "pathogen" and "no pathogen".

The relative amount (proportions or percentages) of each monoterpene was expressed as a percentage of total monoterpenes (monoterpene profiles), while the relative amount of each sesquiterpene was expressed as a percentage of the sum of mono- and sesquiterpenes (terpene profiles). The average and standard error (SE) of the percentage were calculated for each compound and compared between "pathogen" and "no pathogen" trees.

In order to analyse variations in total concentrations of terpenes of Norway spruce tree rings between different sampling years we performed the statistical Friedman Test. Friedman test results (Supplementary material: tables S1 and S2) showed no significant variations in total MTs, SQTs, MTs + SQTs and the relative content of terpenes between different sampling years,; based on these results, mean value of total MTs, SQTs , MTs + SQTs and relative content of terpenes were calculated within

treatment from 2010 to 2014. Mean values were not normally distributed (Kolmogorov-Smirnov one-sample test) and were analysed using the Mann-Whitney U Test for comparison among disease treatments of the plants. A 0.05 threshold was used as cut-off value for all analyses. Statistical analyses were carried out using SPSS (statistical package for social science, SPSS software, v.22.0, SPSS Inc., Chicago, USA).

Results

Pathogen detection and identification at species level

Out of the 23 sampled trees, 12 were infected by *Heterobasidion* spp. (52%) while the remaining 11 samples were putatively non-infected by the pathogen. None of the cores analysed displayed visible symptoms of wood decay. Based on the molecular diagnostic assay, all infected trees were colonized by *H. parviporum*.

Dendrochronological analysis

The tree-ring width mean chronologies covered the period 1902-2015 for “pathogen” trees and 1901-2015 for “no pathogen” trees. Median age was similar for the two series, i.e., 65 years for “pathogen” trees and 64 years for “no pathogen” trees. The two mean chronologies showed similar growth trends, especially after 1970 when more than five trees contributed to the chronology (Fig. 2, continuous line). “Pathogen” trees were characterised by slightly, but not significantly, lower tree-ring width in the last 15 years compared to “no pathogen” trees. The two residual chronologies show similar growth patterns along the entire considered time interval, with the more recent relative peaks of positive growth in 1998 (“pathogen” trees) and 2000 (“no pathogen” trees) (Fig. 3).

VT analysis in tree rings

Changes in total concentrations

Mann-Whitney U test results showed that mean values of SQTs were significantly different ($\chi^2 = 5.8$; $P < 0.05$) between "pathogen" and "no pathogen" trees, while mean values of MTs ($\chi^2 = 0.9$; $P = 0.35$) and MTs plus SQTs ($\chi^2 = 0.8$; $P = 0.34$) did not show significant differences between the two groups (Fig. 4).

Changes in the relative content of terpenes (terpene profiles)

The Mann-Whitney U test showed significant differences in the relative content of 8 terpenes between tree rings of "pathogen" and "no pathogen" trees. As regards the monoterpenes, α-pinene ($\chi^2 = 4.8$; $P < 0.05$) and β-pinene ($\chi^2 = 5.8$; $P < 0.05$) were significantly higher in "pathogen" trees compared to "no pathogen" trees, while camphene ($\chi^2 = 6.8$; $P < 0.01$), 3-carene ($\chi^2 = 6.8$; $P < 0.01$), and ρ-cymene ($\chi^2 = 6.81$ $P < 0.05$) were significantly higher in "no pathogen" compared to "pathogen" trees. The monoterpenes sabinene ($\chi^2 = 1.8$; $P = 0.18$), myrcene ($\chi^2 = 1.7$; $P = 0.17$), limonene ($\chi^2 = 0.3$; $P = 0.60$), β-phellandrene ($\chi^2 = 0.1$ $P = 0.75$), cineole ($\chi^2 = 2.6$; $P = 0.11$) and γ-terpinene ($\chi^2 = 0.9$; $P < 0.35$) did not show statistically significant differences between the two groups.

Among the analysed sesquiterpenes, sesquiterpene 15.90 ($\chi^2 = 3.9$; $P < 0.05$) and α-farnesene ($\chi^2 = 3.9$; $P = 0.05$) showed higher proportions in "no pathogen" compared to "pathogen" trees, while higher relative contents of longifolene were observed in infected compared to non-infected samples ($\chi^2 = 5.7$; $P < 0.05$). α-Humulene ($\chi^2 = 0.3$; $P = 0.6$) and β-caryophyllene ($\chi^2 = 1.8$; $P = 0.18$) did not show significant differences between the analysed categories (Fig. 5).

Discussion

This study represents the first attempt to detect possible differences in mono- and sesquiterpene content in annual tree rings of adult asymptomatic Norway spruce trees in response to natural infection by a fungal pathogen, i.e., *Heterobasidion* spp.

All *Heterobasidion* infected trees were colonized by *H. parviporum* and none by *H. annosum*, thus confirming that the overwhelming majority of Norway spruce decays in the area are caused by the former species, as previously documented (Gonthier et al., 2003). Although the dates of infection of

trees remain unknown, which may complicate the interpretation of the results of this work, all lines of evidence suggest infection occurred relatively recently, possibly in the last 15 years. First, none of the cores analysed displayed visible symptoms of decay, pointing to a recent upward colonization of the fungus from the point of infection in the roots. Second, the infection courts for primary infections by means of airborne spores, i.e. stumps, have been most likely created during thinning performed in 1995. Third, and incidentally, the mean ring-width chronology of trees infected by *H. parviporum* showed lower values starting from the late 1990s compared with non-infected trees, and this may suggest infection of trees occurred at that time. In fact, growth reduction in conifers is common during infection by fungi, e.g. *Heterobasidion parviporum* (Gori et al., 2013). This pattern was also observed by Cherubini et al. (2002) on *Pinus mugo* Turra trees killed by *H. annosum* and *Armillaria* sp. Although these authors found a more remarkable difference in ring-width between infected and non-infected trees than we did in this study, it should be noted that pine trees compared to Norway spruce trees are more susceptible to root rot and mortality rather than heart rot (Garbelotto and Gonthier, 2013), and this may explain the higher levels of growth reduction in pines than in Norway spruce trees (Mallett and Volney, 1999).

The progressive reduction in tree-ring width can affect the climatic signal recorded in tree rings, thus negatively influencing dendroclimatic reconstructions (Trotter et al., 2002). Our results, even if limited to only a small number of trees, support previous investigations conducted on conifers, revealing that Norway spruce infected by *Heterobasidion* spp. shows lower tree-ring width compared to non-infected trees (Cherubini et al., 2002).

Total concentrations of both monoterpenes and sesquiterpenes were lower in trees infected by *H. parviporum* compared to putatively non-infected ones and, for sesquiterpenes, the difference between "pathogen" and "no pathogen" trees appeared to be significant. Both mono- and sesquiterpenes have an important role in counteracting pathogen infection in Norway spruce trees. However, the Friedman Test did not show any significant difference in the terpene content between different years (Supplementary material), suggesting that this method does not allow the identification of any difference in terpene content following pathogen infection at the yearly resolution.

The relative content (percentage) of the monoterpenes α-pinene and β-pinene and of the sesquiterpene longifolene are significantly higher in infected compared to non-infected trees. In particular, the monoterpenes α-pinene and β-pinene are known for their role in conifer defence strategies in stems and roots (Huber et al., 2005). These results are in agreement with research performed by Zamponi et al. (2007) on branches of Norway spruce trees experimentally inoculated with *H. parviporum*. In that study, α-pinene and β-pinene were significantly different between infected and non-infected trees, which is also in agreement with our study. However, there were some differences between our study and the results obtained by Zamponi et al. (2007), i.e., we did not detect a significant increase in the relative content of 3-carene and myrcene following *Heterobasidion* attack. These differences could be due to the tissues colonized by the pathogens in the two studies, i.e. heartwood vs sapwood, respectively. In fact, while branches, hence sapwood, was inoculated with *Heterobasidion* spp. by Zamponi et al. (2007), it is likely that our adult Norway spruces were colonized by *H. parviporum* in the heartwood as it occurs as a general rule (Garbelotto and Gonthier, 2013).

The relative content of the monoterpenes camphene, 3-carene and ρ-cymene and of the sesquiterpenes sesquiterpene 15.90 and α-farnesene was significantly lower in infected compared to non-infected trees. This can be a consequence of the defence mechanism activated by the tree following infection: the plant reduces the production of the biologically less active compounds and increases the synthesis of the more toxic terpenes (Michelozzi, 1999). When the infection begins, Norway spruce trees start increasing the level of several terpenes in order to contrast the pathogen attack but if the defence mechanism is not successful (Luchi et al., 2005), then the tree reduces the production of the terpenes that are less effective for restricting the pathogen, because their production has a relevant cost for the tree itself (e.g., Ghimire et al., 2016).

Conclusions

In summary, this study reveals that both dendrochronological and VT analyses may indicate fungal infection in adult trees. In particular, the tree-ring mean chronology showed lower values in infected

compared to non-infected trees in the more recent years and the relative content of some terpenes, i.e., α-pinene, β-pinene and longifolene showed significantly higher values in infected compared to non-infected trees. This is the first study suggesting that VT composition in tree rings may be an indicator of fungal disease and this is particularly important in the case of Norway spruce, where external symptoms of infection, for example by *H. parviporum,* are usually poor. A future study considering different geographical regions and trees from diverse genetic lineages, as well as a larger sample size, should be carried out to identify which markers can be used for the identification of diseased trees.

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Conflict of interest statement

None declared.

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Figure captions

Figure 1. Experimental design of the analyses.

Figure 2. Ring-width mean chronologies for “pathogen” and “no pathogen” trees. Discontinuous lines characterize the curve built with less than five trees.

Figure 3. The two residual chronologies “pathogen” and “no pathogen”. Discontinuous lines characterize the curve built with less than five trees.

Figure 4. Mean (+ SE) values of total monoterpenes (MTs), sesquiterpenes (SQTs) and mono + sesquiterpenes (MTs + SQTs) concentrations detected in tree rings of “pathogen” and “no pathogen” trees. Values of columns with different letters differ significantly ($P < 0.01$).

Figure 5. Average percentage of terpenes in “pathogen” and “no pathogen” tree rings. Statistical difference was determined by Mann-Whitney test. Error bars indicate SE. Values of columns with different letters differ significantly (the values of statistical significance are reported in the text).

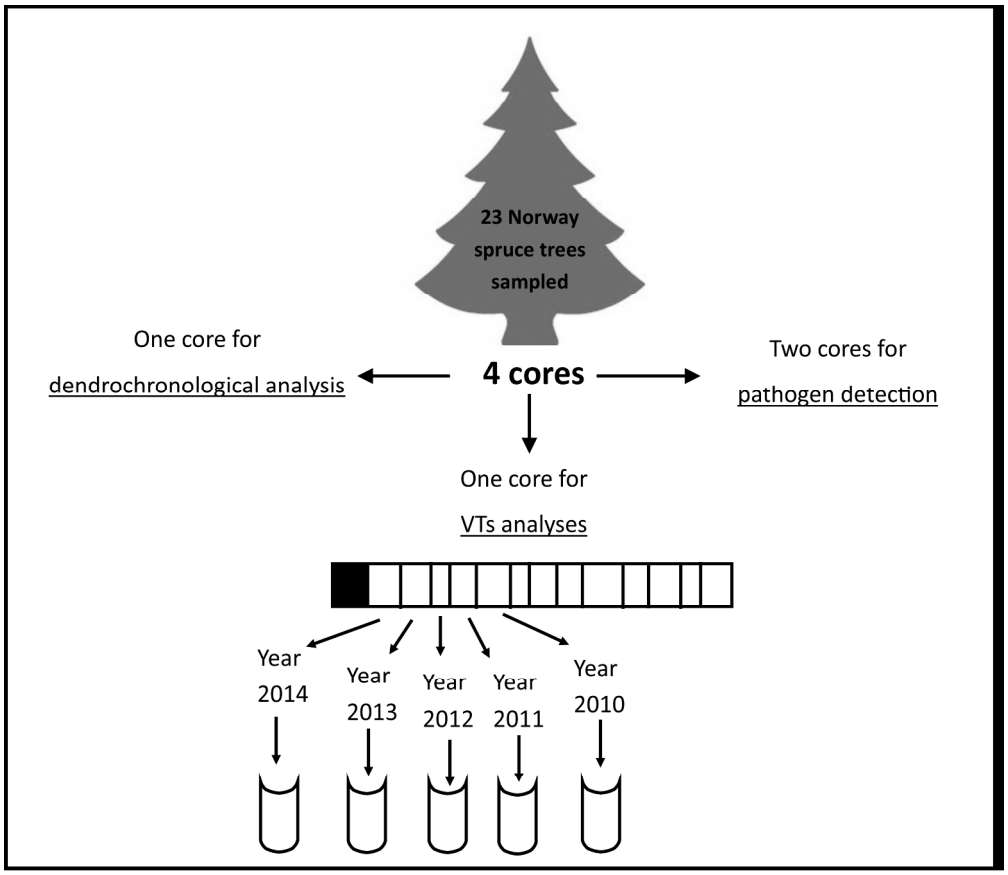

Figure 1. Experimental design of the analyses.

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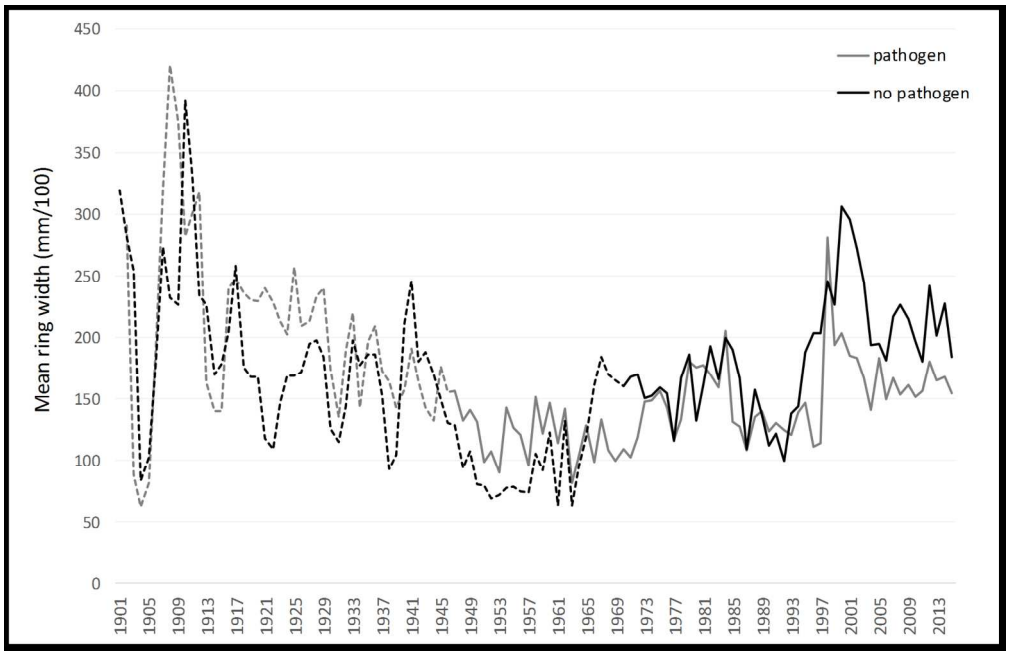

Figure 2. Ring-width mean chronologies for "pathogen" and "no pathogen" trees. Discontinuous lines characterize the curve built with less than five trees.

224x144mm (300 x 300 DPI)

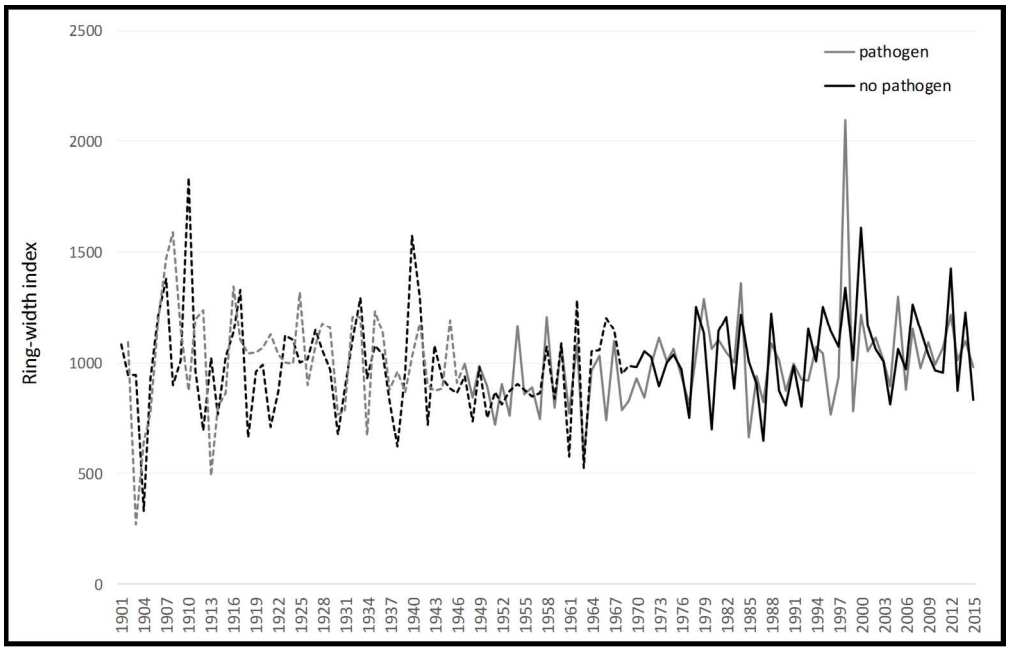

Figure 3. The two residual chronologies "pathogen" and "no pathogen". Discontinuous lines characterize the curve built with less than five trees.

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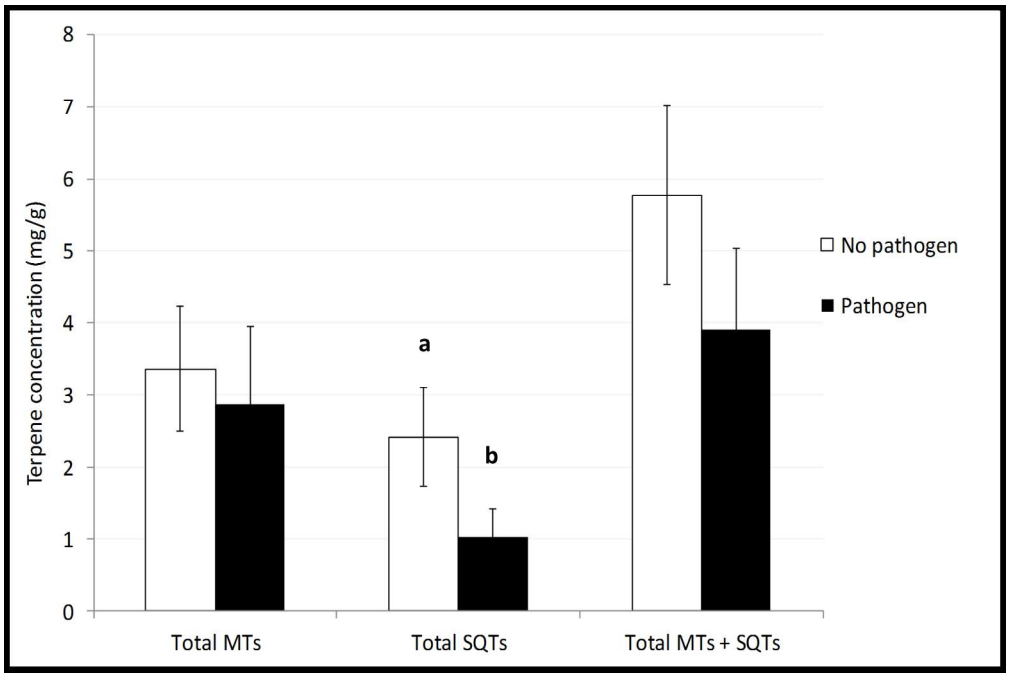

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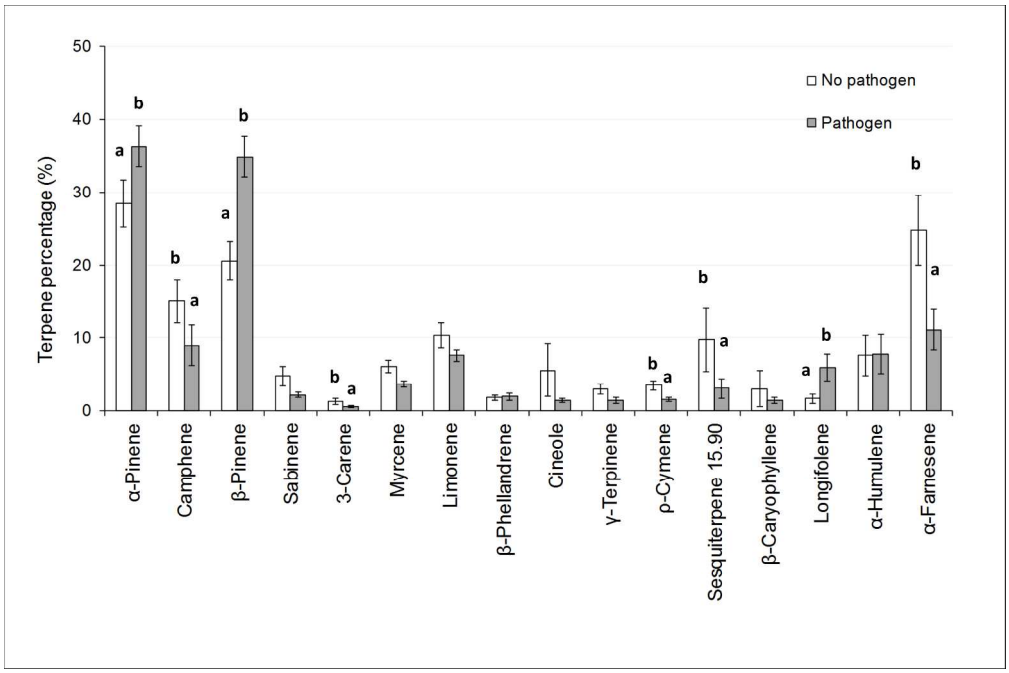

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256x170mm (300 x 300 DPI)

Table S1. Statistical results of the Friedman Test examining the variations in total concentration of terpenes of Norway spruce tree rings between different sampling years (d.f.=4: years from 2010 to 2014) within the same disease treatment; MTs: monoterpenes; SQTs: sesquiterpenes; NS: not significant; N° samples: 5 for each group.

Terpene	Treatment	$\chi^2(p)$
MTs	pathogen	5.0 NS
	no pathogen	4.0 NS
SQTs	pathogen	0.2 NS
	no pathogen	8.5 NS
MTs + SQTs	pathogen	2.2 NS
	no pathogen	3.8 NS

Table S2. Statistical results of the Friedman Test examining the variations in the relative content of monoterpenes of Norway spruce tree rings between different sampling years (d.f.=4: years from 2010 to 2014) within the same disease treatment; NS: not significant; N° samples: 5 for each group.

Terpene	Treatment	$\chi^2(p)$
α-pinene	pathogen	4.2 NS
	no pathogen	3.4 NS
camphene	pathogen	3.7 NS
	no pathogen	2.6 NS
β-pinene	pathogen	3.7 NS
	no pathogen	7.6 NS
sabinene	pathogen	3.4 NS
	no pathogen	5.8 NS
δ-3-carene	pathogen	4.5 NS
	no pathogen	1.0 NS
myrcene	pathogen	3.4 NS
	no pathogen	4.6 NS
limonene	pathogen	4.3 NS
	no pathogen	5.7 NS
β-phellandrene	pathogen	3.5 NS
	no pathogen	4.6 NS
cineole	pathogen	4.0 NS
	no pathogen	8.2 NS
□-terpinene	pathogen	6.7 NS
	no pathogen	4.2 NS
p-cymene	pathogen	3.5 NS
	no pathogen	7.4 NS
sesquiterpene 15.90	pathogen	6.2 NS
	no pathogen	5.4 NS
□-caryophyllene	pathogen	3.7 NS
	no pathogen	1.8 NS
longifolene	pathogen	5.1 NS
	no pathogen	8.8 NS
α-humulene	pathogen	2.6 NS
	no pathogen	3.8 NS
α-farnesene	pathogen	1.6 NS
	no pathogen	7.4 NS