Published in final edited form as:

Phytochemistry. 2018 March ; 147: 80–88. doi:10.1016/j.phytochem.2017.12.007.

Diterpenoid fingerprints in pine foliage across an environmental and chemotypic matrix: isoabienol content is a key trait differentiating chemotypes

Astrid Kännaste^{a,*}, Lauri Laanisto^a, Leila Pazouki^{a,b}, Lucian Copolovici^{a,c}, Marina Suhorutšenko^a, Muhammad Azeem^{d,e}, Lauri Toom^f, Anna-Karin Borg-Karlson^{a,e,f}, and Ülo Niinemets^{a,g}

^aInstitute of Agricultural and Environmental Sciences, Estonian University of Life Sciences, Kreutzwaldi 1, Tartu 51014, Estonia

^bDepartment of Biology, University of Louisville, Louisville, KY 40292, USA

^cInstitute of Technical and Natural Sciences Research-Development of “Aurel Vlaicu” University, 2 Elena Dragoi St., 310330, Arad, Romania

^dDepartment of Chemistry, COMSATS Institute of Information Technology, Abbottabad 22060, Pakistan

^eEcological Chemistry Group, Department of Chemistry, KTH, Royal Institute of Technology, 100 44, Stockholm, Sweden

^fInstitute of Technology, University of Tartu, Nooruse 1, Tartu 50411, Estonia

^gEstonian Academy of Sciences, Kohtu 6, 10130 Tallinn, Estonia

Abstract

Diterpenoids constitute an important part of oleoresin in conifer needles, but the environmental and genetic controls on diterpenoid composition are poorly known. We studied the presence of diterpenoids in four pine populations spanning an extensive range of nitrogen (*N*) availability. In most samples, isoabienol was the main diterpenoid. Additionally, low contents of (*Z*)-biformene, abietadiene isomers, manoyl oxide isomers, labda-7,13,14-triene and labda-7,14-dien-13-ol were quantified in pine needles. According to the occurrence and content of diterpenoids it was possible to distinguish ‘non diterpenoid pines’, ‘high isoabienol pines’, ‘manoyl oxide – isoabienol pines’ and ‘other diterpenoid pines’. ‘Non diterpenoid pines’, ‘high isoabienol pines’ and ‘other diterpenoid pines’ were characteristic to the dry forest, yet the majority of pines (>80%) of the bog Laeva represented ‘high isoabienol pines’. ‘Manoyl oxide – isoabienol pines’ were present only in the wet sites. Additionally, orthogonal partial least-squares analysis showed, that in the bogs foliar nitrogen content per dry mass (*N_M*) correlated to diterpenoids. Significant correlations existed between abietadienes, isoabienol and foliar *N_M* in ‘manoyl oxide – isoabienol pines’, and

Corresponding author: Institute of Agricultural and Environmental Sciences, Estonian University of Life Sciences, Kreutzwaldi 1, Tartu 51014, Estonia, astrid.kannaste@emu.ee.

Competing interests

The authors declare that they have no competing interests.

chemotypic variation was also associated by population genetic distance estimated by nuclear microsatellite markers. Previously, the presence of low and high β -3-carene pines has been demonstrated, but the results of the current study indicate that also diterpenoids form an independent axis of chemotypic differentiation. Further studies are needed to understand whether the enhanced abundance of diterpenoids in wetter sites reflects a phenotypic or genotypic response.

Keywords

chemotypic variability; diterpenoids; isoabienol; *Pinus sylvestris* L.; terpenoid signatures; *N* content

1 Introduction

Conifer trees, including the extremely wide-spread Scots pine (*Pinus sylvestris* L.), have characteristically high longevity, which is associated with multiple traits, in particular, with presence of oleoresin in their woody tissues and leaves (Lewinsohn et al. 1991). Oleoresin is contained in the resin ducts and it protects trees against a wide range of herbivores, fungal and bacterial pathogens, providing a very successful broad-spectrum defense system (Croteau et al. 1987; Lewinsohn et al. 1991; Phillips & Croteau 1999). The biosynthetic pathways of oleoresin constituents have been widely investigated (Bohlmann & Keeling 2008; Keeling et al. 2010; Memari et al. 2013; Rosenkranz & Schnitzler 2013). Oleoresin is composed of turpentine fraction consisting of mono- and sesquiterpenoids and rosin fraction consisting of diterpenoids and their derivatives, in particular diterpenoid acids (Phillips & Croteau 1999). Oleoresin can effectively seal the injuries resulting from direct mechanical stresses such as wind damage, but also from browsing by large herbivores, from insect feeding and pathogen damage (Phillips & Croteau 1999; Loreto et al. 2000).

Diterpenoids are toxic to microorganisms and arthropods, and thus play an important role in plant defense (Bailey et al. 1974; Ulubelen et al. 1994; Jasinski et al. 2001; Falara et al. 2010; Koutsaviti et al. 2011; Chaturvedi et al. 2012). For example, labdane-type diterpene (11*E*,13*E*)-labda-11,13-diene-8 α ,15-diol named as WAF-1 contributes to the activation of pathogen- or wound-induced reactions in tobacco leaves (Seo et al. 2003). Additionally, labdane-type diterpenoids (Figure 1) participate in the biosynthesis of diterpenoid acids, also called resin acids (Phillips & Croteau 1999; Lee et al. 2001; Keeling & Bohlmann 2006), composition of which is significantly modified by biotic stress (Kainulainen et al. 1993; Miller et al. 2005). Due to their toxicity, resin acids inhibit mycelial growth of pathogenic fungi carried by bark beetles (Kopper et al. 2005). In Sitka spruce (*Picea sitchensis*), accumulation of dehydroabietic acid is associated with tree resistance against white pine weevil (*Pissodes strobe*) (Robert et al. 2010). Hence, obtaining detailed insight about the factors controlling the content and composition of diterpenoids in oleoresin will help to understand the overall role of diterpenoids in induced defense reactions in gymnosperms.

Pinus species are widely known for their extensive genetic variance (Dering et al. 2017). For example, pines of β -3-carene, α -pinene (Hiltunen et al. 1975; Sjödin et al. 1996, 2000; Bäck et al. 2012; Kännaste et al. 2013;) and isoabienol chemotypes (Gref 1981) are known.

Moreover, the resin composition of pines is strongly influenced by environmental factors (Hiltunen et al. 1975; Taft et al. 2015), especially due to the variability in timing and severity of simultaneous abiotic stressors in different ecosystems (Niinemets 2010). Although the genetic variability within and among pine populations has been studied to some extent (Pazouki et al. 2016; Dering et al. 2017), our understanding of the association between chemotypes and environment of pine populations is still limited (Kännaste et al. 2013; Pazouki et al. 2016). *Pinus* species including *P. sylvestris* can occupy highly varying habitats including fertile well-drained mineral soils, excessively drained sandy soils or infertile waterlogged peat soils. Given this high plasticity in coping with habitat conditions, it is surprising that the environmentally-driven variations in key stress-dependent specialized metabolites have been poorly examined across pine populations (Hornoy et al. 2015). Understanding such associations is particularly relevant as long-term stress-induced changes at the molecular level can lead to formation of novel pine chemotypes (phenotypes with different chemical composition) (Jablonka 2013). To our knowledge, chemotypic differences associated with stress-dependent changes in diterpenoids have been only studied in Norway spruce (*Picea abies*) clones exposed to ozone stress (Kainulainen et al. 1995).

Although there are basic controls on specialized metabolism, pathway fluxes to different terpenoid classes are often regulated differently (Dudareva et al. 2004; Tholl 2006; Chen et al. 2011). This is particularly true for differences among cytosol-synthesized sesquiterpenoids and plastid-synthesized mono- and diterpenoids, but even within plastids, mono- and diterpene biosynthesis can be differently controlled by the availability of C₁₀ substrate geranyl diphosphate (GDP) and C₂₀ substrate geranylgeranyl diphosphate (GGDP) (Orlova et al. 2009; Rajabi Memari et al. 2013). In our previous study, which was based on pines from three wet sites and one dry site, we reported a large variation in mono- and sesquiterpenoid signatures over short spatial distances in *P. sylvestris* populations spanning wet organic soils and dry mineral soils (Kännaste et al. 2013). Specifically, α -3-carene was the main monoterpene effectively differentiating the populations (Kännaste et al. 2013). To further differentiate among the *P. sylvestris* chemotypes, we studied here the content and composition of diterpenoids in the same four populations dispersed over a large gradient of soil conditions (Kännaste et al. 2013). We hypothesized that in addition to α -3-carene, diterpenoids form an additional axis of chemical differentiation for *P. sylvestris* populations. Based on the nutrient-dependent variation in mono- and sesquiterpenoid content across the sites (Kännaste et al. 2013), we suggested that diterpenoid contents in pine needles scale negatively with site N availability. In addition, given that the genetic distance among the studied populations has been estimated on the basis of microsatellite markers (Pazouki et al. 2016), we further hypothesized that chemotype-level differentiation is associated with genetic distance among the studied pine populations.

2 Results and Discussion

2.1 Variations of diterpenoid contents and compositions

Diterpenoids were present in more than 90% of 218 analyzed samples (Table 1). High isoabienol content was characteristic to the majority of pine samples. Moreover, isoabienol contents were similar in the bog samples, but foliage isoabienol content was greater in dry

forest samples than in bogs Laeva and Parika (Figure 2). Isoabienol has been previously found in the needles of *Juniperus communis* (Adams et al. 2010) and in the wood of *Cunninghamia konishii* (Cheng et al. 2012); but also in *P. sylvestris* samples from Sweden and Finland (Gref 1981; Manninen et al. 2002). In the Finnish and Swedish samples, it was only found in a few cases (Gref 1981; Manninen et al. 2002). Thus, widespread and high rate of this diterpenoid in Estonian samples was surprising.

Besides isoabienol, *P. sylvestris* foliage contained abietadiene, abieta-8(14),13(15)-diene, (*Z*)-biformene, labda-7,14-dien-13-ol, labda-7,13,14-triene, manool, manoyl oxide and 13-*epi*-manoyl oxide (Figure 1). The total content of these diterpenoids remained below 1 mg g⁻¹ (Figure 2). Generally, diterpenoids are not routinely analyzed together with mono- and sesquiterpenoids in conifer studies. This is partly because of different extraction procedures required for diterpenoids, and thus, diterpenes not normally present in the essential oil fraction obtained by steam distillation (Ormeño et al. 2011), and also because different GC-MS analysis conditions required for diterpenes. We argue that future studies should also include detailed analysis of all fractions of conifer oleoresin.

Diterpenoids with similar molecular structures (Figure 1) may be biosynthesized via the common pathway. In addition to the total contents of diterpenoids, it was possible to evaluate the total leaf dry mass investment in diterpenoids biosynthesis (Tables 2, Figures 3 and 4). Correlation analyses (Table 2) demonstrated strong covariances between isoabienol and (*Z*)-biformene contents per dry mass ($r = 0.81$, $P < 0.001$), isoabienol and labda-7,14-dien-13-ol ($r = 0.71$, $P < 0.001$), (*Z*)-biformene and labda-7,14-dien-13-ol ($r = 0.61$, $P < 0.001$), abieta-8(14),13(15)-diene and labda-7,13,14-triene ($r = 0.62$, $P < 0.01$), labda-7,13,14-triene and manoyl oxide ($r = 0.62$, $P < 0.01$), and manool and 13-*epi*-manoyl oxide ($r = 0.81$, $P < 0.05$). The change from isoabienol synthesis to labda-7,14-dien-13-ol or vice versa seems to be related to hydration/dehydration of the respective diterpenoid (Figure 1). In fact, biochemical hydration/dehydration would also explain high correlation values between isoabienol and (*Z*)-biformene (Table 2).

The coexistence of isoabienol and manoyl oxide revealed two different positive correlations among these compounds (Figure 3). The first correlation (Figure 3A) was characteristic only to 'manoyl oxide – isoabienol samples' from the wet sites ($r = 0.62$, $P < 0.001$), while the second correlation appeared within 'high isoabienol samples' from both the wet sites and the dry forest ($r = 0.89$, $P < 0.001$) (Figure 3B, pine chemotypes discussed below). Hence, manoyl oxide and isoabienol may be biosynthesized via different pathways. Cyclization/decyclization would explain high correlation values between isoabienol and manoyl oxides. However, in the perfume industry, ambreinolide, which has similar structure as manoyl oxide, is synthesized via oxidation of isoabienol (Kukovinets et al. 2001). Moreover, in a recent genetic engineering study, the diterpenoid profile was shown to depend on the substrate. For example, the combination of *Z*-abienol synthase NtABS from *Nicotiana tabacum* (Sallaud et al. 2012) and copalyl diphosphate synthase from *Salvia fruticosa* yielded (*Z*)-biformene, while the combination of NtABS and 8-hydroxy copalyl diphosphate synthase from *Cistus creticus* yielded (*Z*)-abienol (Ignea et al. 2015). A recent review demonstrates that the capacity for multi-substrate use is widespread among species (Pazouki & Niinemets 2016).

2.2 Separation of pines according to diterpene profiles

Isoabienol and manoyl oxide were the dominant diterpenoids, which affected the grouping of pines into clusters. Hence, based on the isoabienol and manoyl oxide contents per needle dry mass, the 'diterpenoid containing pines' were, in turn, classified into 'manoyl oxide – isoabienol pines', 'high isoabienol pines' and 'other diterpenoid pines' in PCA plots (Figure 4A and 4B). Normalization of diterpenoid contents per total diterpenes showed that the cluster of 'other diterpenoid pines' consisted of 'abieta-8(14),13(15)-diene – manoyl oxide pines' (Figure 5A) and 'abietadiene pines' (Figure 5B). 'Manoyl oxide – isoabienol pines' contained on average 93% of isoabienol and 6% of manoyl oxide (Figure 5C), but among 'high isoabienol pines' it was again possible to distinguish two different chemotypes (Figure 5D and 5E). Both chemotypes were dominated by isoabienol, and the content of manoyl oxide remained below 1%. Nevertheless, the diterpenoid profiles of those chemotypes differed considerably (Monte Carlo test, $P < 0.05$).

2.3 Diterpenoids profiles in relation to site

In our previous study (Kännaste et al. 2013) the distribution of pine chemotypes according to the presence or absence of 3-carene was not related to the provenance of samples, although across the bogs monoterpenoid profiles from Laeva samples differed considerably from those from Kakerdaja and Parika (Kännaste et al. 2013). In the present study, majority of bog samples contained high amounts of isoabienol (Table 1, Figure 5) and were classified either as 'manoyl oxide – isoabienol pines' or 'high isoabienol pines' (Table 1). Conifer resin composition is mainly controlled genetically, but it is also influenced by environmental factors (Hiltunen et al. 1975; Holliday et al. 2010; Taft et al. 2015). Pazouki et al. (2016) concluded that the high genetic variation in pines was related to pollen dispersal in bogs. However, based on nuclear microsatellite markers (nSSR markers), pines samples from dryer and wetter areas of various bogs clustered differently (Pazouki et al. (2016). Moreover, Mantel test based on nSSR markers (Pazouki et al. 2016) and diterpenoid contents per dry mass (Mantel test ($r = 0.49$, $P < 0.05$, Figure 6) showed that the distribution of pines' samples across the sites (Table 1) could be related to the high acclimatization ability of pines to local environment via epigenetic factors (Bussotti et al. 2015). According to Baradat and Yazdani (1988) and Taft et al. (2015) the impact of environmental factors on terpenoids is selective. Hence, taking into account the age of trees, site differences (see Kännaste et al. 2013), and the results of earlier genetic studies (Baradat & Yazdani 1988, Jablonka 2013, Hornoy et al. 2015, Taft et al 2015, Pazouki et al. 2016), we conclude that the genetic factors have been involved in pine chemotype diversification (Table 1, Figure 5) by either suppressing or upregulating the biosynthesis pathways of diterpenoids (Table 2, Figure 3-5).

In wet sites nitrogen (N) availability is one of the main stress factors (Niinemets & Lukjanova 2003; Portsmouth et al. 2005; Niinemets 2010; Kännaste et al. 2013). It is well known, that limited N availability slows down the normal growth of plants (Ingestad & Kähr 1985; Kainulainen et al. 2000), but N fertilization increases the amount of resin cells and the content of resin acids in the mature needles of *P. sylvestris* (Kainulainen et al. 1996). When the relationships between foliar diterpenoids (mg g^{-1}) and N_M (%) were evaluated (Orthogonal partial least-squares (OPLS) algorithm, Figure 7), the R^2Y - and Q^2Y -values (model fitting coefficients indicating its predictive performance) for each bog were above

0.9 (Figure 7). Additionally, the impact of N_M on diterpenoid content was tested with Pearson correlation. In 'manoyl oxide – isoabienol pines' from Kakerdaja and Parika, N_M correlated negatively with the sum of abietadiene and abieta-8(14),13(15)-diene ($r = -0.79$, $P < 0.01$, $n = 11$); and positively with isoabienol ($r = 0.49$, $P < 0.05$, $n = 13$). In previous studies, N_M correlated also with foliar mono- and sesquiterpenoids (Kainulainen et al. 2000; Kännaste et al. 2013). In addition, Laeva samples had the lowest N_M and the lowest content of other diterpenoids (Figure 2), while the dry forest samples contained the highest amount of isoabienol (Figure 2) and N_M (Kännaste et al. 2013).

Finally, abietadienes, manoyl oxide and isoabienol may play essential role in pine defense reaction (Figure 2 and 5) and their co-existence may be even synergistic (Figure 2). For example, isoabienol is an antimicrobial (Koutsaviti et al. 2011) and antifungal agent (Cheng et al. 2012). In plant leaves infected by fungi, dehydroabietinal, which may originate from abietadiene (Keeling & Bohlmann 2006), activates a systemic acquired resistance mechanism (Chaturvedi et al. 2012; Dempsey and Klessig 2012). In conifers, the quantity of abietic acid, which is biosynthesized from abietadiene (LaFever et al. 1994; Phillips and Croteau 1999), determines the tree ability to overcome the mass-attack of mountain bark beetles (*Dendroctonus ponderosae*) (Boone et al. 2013).

The induction of manoyl oxide may be partly related to the fact that diterpenoid is the precursor of forskolin (Garcia-Granados et al. 2007; Pateraki et al. 2014). The latter metabolite increases the content of intracellular cyclic adenosine monophosphate (cAMP), which in turn is known to be associated with a group of root pathogen induced resistance genes involved in the defense mechanism of Norway spruce (Jøhnk et al. 2005). In higher plants cAMP controls K^+ -flow in the mesophyll protoplasts (Gehring 2010). In flooded areas the ability of plants to keep K^+ -flow under control is essential in order to survive (Wang et al. 2013).

2.4 Conclusion

Isoabienol dominated in the needles of *P. sylvestris*, although the blends of diterpenoids varied strongly across pine chemotypes. Similarly to mono- and sesquiterpenoids (Kännaste et al. 2013), variations in diterpenoid profiles were related to foliar N_M content. Additionally, distribution of pine chemotypes across the sites reflected significant environmental plasticity. Taken together, based on earlier studies (Baradat & Yazdani 1988; Jablonka 2013, Hornoy et al. 2015, Taft et al. 2015, Pazouki et al. 2016), we suggest that the growth environment of pines plays essential role in the adaptation of pines to local environment by developing unique diterpenoid profiles.

3 Experimental

3.1 GC-MS analysis

Diterpenoids were quantified with a β -DEX 120 (0.25 mm i.d. $\times$ 30 m, film thickness 0.25 μ m, Supelco, Bellefonte, PA, USA) chiral column with the following GC oven program: 60 °C for 5 min, 5 °C min⁻¹ to 80 °C for 20 min, 2 °C min⁻¹ to 200 °C for 1 min, and 10 °C min⁻¹ to 220 °C for 10 min. The MS operated in electron-impact mode (EI) at 70 eV, and in

the mass scan range of m/z 33–400 in total ion current (TIC) mode. Transfer line temperature was set at 240 °C and ion-source temperature at 150 °C (Kännaste et al. 2013).

3.2 Identification and quantification of diterpenoids

Due to the high content in pine needles (Figure S1) isoabienol was extracted and identified by NMR analysis (see supporting information). Other diterpenoids (Table 4) were identified by analyzing the needle extracts and a C_7 - C_{30} hydrocarbon standard (Sigma Aldrich, St. Louis, MO, USA) with GC-MS using a ZB5-MS column (0.25 mm i.d. $\times$ 30 m, 0.25 μ m film Zebron, Phenomenex, Torrance, CA, USA). The GC oven program was: 40 °C for 1 min, 5 °C min⁻¹ to 220 °C for 5 min. The use of the hydrocarbon standard enabled the calculation of retention indices (RIs), which were compared to published data (Adams 2001, NIST library and MassFinder 3 database) in addition to the mass spectrums of diterpenoids (Figure S2).

Isoabienol was used as a diterpenoid standard for calibrating the GC-MS. Calibration samples were prepared by diluting known quantities of isoabienol in hexane, then analyzing 1 μ l of each sample to create a calibration factor that was used for quantifying the peak areas of diterpenoids in pine samples. Contents of diterpenoids were calculated according to the linear regression ($y = 18897009 x$) by dividing the peak areas of diterpenoids with the calibration factor and dry weight of pine needles. Detection limit of diterpenoids was 10 pmol/ μ l and the linear range of the MS detector was up to 100 μ g.

3.3 Study sites

The study was conducted in four naturally-regenerated *P. sylvestris* dominated sites in Estonia. Pine samples from the bogs of Parika, Kakerdaja, and Laeva were compared to the ones from the dry forest of Männiku (Table 3). Location of populations, as well as tree characteristics and main floral species at tree-, shrub- and grass layer are shown in Table 3. For a detailed overview about the sites together with soil pH-values is presented in our previous study (Kännaste et al. 2013).

3.4 Foliage collection for chemical analyses

The sampling in all sites was conducted during June 2-25, 2010. Sampled trees were chosen randomly. Samples were collected not further than 50 m from the trajectory (Figure S3). In the bog sites, sampling started from the drier and less acidic edges, and progressed towards the wetter and more acidic bog interior in equidistal intervals. In the bogs of Kakerdaja and Parika sampling stopped near bog lakes (Figure S3). In the Laeva bog, the transition mire gradually transformed into raised bog between samples 45 and 60 (Figure S3). In the dry forest, the samples were taken randomly.

Terminal branches were taken from open-growing trees on the southern side of the crown. One-year-old foliage from multiple shoots was collected. Finally, one pine sample analyzed by GCMS corresponded to one pine tree. 32 Samples from dry forest of Männiku to 70 samples from bog of Kakerdaja were collected from each site. The plant material was placed in sealed plastic bags and kept in an ice-box until transported to the laboratory (within two hours after collecting). The samples for terpene analyses were kept at – 80 °C until analyzed.

3.5 Sample homogenization for diterpenoid analyses

For each chemical analyses, a sample of 140 ± 20 mg of fresh plant material from randomly chosen needles was weighted into a 2 ml tube with 2.8 mm stainless steel beads (Bertin Technologies, Aix-en-Provence, France).

The tubes filled with liquid nitrogen and plant material were immediately homogenized with Precellys 24 homogenizer (Bertin Technologies, Aix-en-Provence France) at 6500 rotation per minute for three 60 s periods at 20 °C. After homogenization, 1 ml of GC-grade hexane (Sigma Aldrich, St. Louis, MO, USA) was added and mixed, then left at 6 °C for extraction (Kännaste et al. 2013). The extraction protocol was optimized by extracting the diterpenoids for 1, 3, 5, 24 and 48 hours (Figure S2). The analysis showed, that the content and composition of diterpenoids did not alter during the sub-sample removal and rapid determination of needle mass for analyses (Figure S4). Hence, diterpenoids were extracted by following an extraction protocol of 3 hours.

After the extraction procedure supernatant was immediately injected into Shimadzu QP2010 Plus GC-MS using an auto-injector AOC-20i (Shimadzu Corporation, Kyoto, Japan). Samples were analyzed with a split ratio of 5:1. Repeated analyses of different replicates taken randomly from the same samples demonstrated that the variation among diterpenoid concentrations caused by within-sample variability and slight inaccuracies during the extraction (mass determination, imperfect extraction, potential loss of sample during the extraction) was less than 10%.

3.6 Molecular analysis

Details of nSSR analysis are described in a previous study (Pazouki et al. 2016).

3.7 Foliar N analysis

The needle N content per dry mass was estimated with Vario MAX CNS analyzer (Elementar Analysensysteme GmbH, Hanau, Germany).

3.8 Data handling and statistical analysis

The composition of diterpenoids in pine samples was evaluated by principal component analysis (PCA) (Wold et al. 1987), where the diterpenoid dataset was mean-centered and log-transformed. The differences in diterpenoid profiles were tested by Monte-Carlo permutation test and redundancy data analysis (RDA). The relationship between genetic and chemical axes of PCA analysis was tested by Gaussian response curves fitted by generalized linear model. Multivariate analyses were conducted by Canoco 5.0 software (ter Braak and Smilauer, Biometris Plant Research International, The Netherlands). All statistical tests were considered significant at $P < 0.05$.

Mantel test with 1000 random permutations was carried out for studying the correlation between the matrix of pairwise genetic differentiation and a matrix of diterpenoid distance using GenAlEx v6 (Peakall and Smouse 2006).

In Pearson correlation analyses diterpenoid contents per dry weight were used to gain insights on the relationships among diterpenoids (Fäldt et al. 2003; Semiz et al. 2007; Kännaste et al. 2013). These analyses were conducted with R (R Foundation for Statistical Computing, Vienna, Austria). Additionally, orthogonal partial least-squares (OPLS) analysis (Trygg and Wold 2002) was used to analyse the relationship between diterpenoids and N_M . OPLS allows to model separately the variations of the predictors correlated and orthogonal to the response (Thévenot et al. 2015). OPLS analysis was carried out using *ropls* R package, function 'opls' (Thévenot et al. 2015).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

This study was funded by the European Social Fund (post-doctoral grant MJD 14), the Estonian Ministry of Science and Education (IUT 8-3, PUT 1409), European Union through the European Regional Fund (The Centre of Excellence in Environmental Adaptation TK107 and The Centre of Excellence, EcolChange, TK131) and Mobilitas Program Top Researcher Grant No. MTT2 "Chemical Ecology" (to LT and AKBK) and the European Research Council (advanced grant 322603, SIP-VOL+). Finally, we would like to acknowledge prof. Thomas D. Sharkey for linguistic corrections.

References

- Adams, RP. Identification of essential oil components by gas chromatography / quadrupole mass spectroscopy. Allured Publishing Corporation; Carol Stream, USA: 2001.
- Adams RP, Beauchamp PS, Dev V, Bathala RM. The leaf essential oils of *Juniperus communis* L. varieties in North America and the NMR and MS data for isoabienol. Journal of Essential Oil Research. 2010; 22:23–28.
- Bailey JA, Vincent GG, Burden RS. Diterpenes from *Nicotiana glutinosa* and their effect on fungal growth. Journal of General Microbiology. 1974; 85:57–64. [PubMed: 4436652]
- Baradat P, Yazdani R. Genetic expression for monoterpenes in clones of *Pinus sylvestris* grown on different sites. Scandinavian Journal of Forest Research. 1988; 3:25–36.
- Bohlmann J, Keeling CI. Terpenoid biomaterials. Plant Journal. 2008; 54:656–669. [PubMed: 18476870]
- Boone CK, Keefover-Ring K, Mapes AC, Adams AS, Bohlmann J, Raffa KF. Bacteria associated with a tree-killing insect reduce concentrations of plant defense compounds. Journal of Chemical Ecology. 2013; 39:1003–1006. [PubMed: 23807433]
- Bussotti F, Pollastrini M, Holland V, Brüggemann W. Functional traits and adaptive capacity of European forests to climate change. Environmental and Experimental Botany. 2015; 111:91–113.
- Bäck J, Aalto J, Henriksson M, Hakola H, He Q, Boy M. Chemodiversity of a Scots pine stand and implications for terpene air concentrations. Biogeosciences. 2012; 9:689–702.
- Chaturvedi R, Venables B, Petros RA, Nalam V, Li M, Wang X, Takemoto LJ, Shah J. An abietane diterpenoid is a potent activator of systemic acquired resistance. Plant Journal. 2012; 71:161–172. [PubMed: 22385469]
- Chen F, Tholl D, Bohlmann J, Pichersky E. The family of terpene synthases in plants: a mid-size family of genes for specialized metabolism that is highly diversified throughout the kingdom. Plant Journal. 2011; 66:212–229. [PubMed: 21443633]
- Cheng S-S, Chung M-J, Lin C-Y, Wang Y-N, Chang S-T. Phytochemicals from *Cunninghamia konishii* Hayata act as antifungal agents. Journal of Agricultural and Food Chemistry. 2012; 60:124–128. [PubMed: 22129092]

- Croteau R, Gurdewitz S, Johnson MA, Fisk HJ. Biochemistry of oleoresinosis: monoterpene and diterpene biosynthesis in Lodgepole Pine saplings infected with *Ceratocystis clavigera* or treated with carbohydrate elicitors. *Plant physiology*. 1987; 85:1123–1128. [PubMed: 16665815]
- Dempsey DA, Klessig DF. SOS - too many signals for systemic acquired resistance? *Trends in Plant Science*. 2012; 17:538–545. [PubMed: 22749315]
- Dering M, Kosiński P, Wyka TP, Pers-Kamczyc E, Boratyński A, Boratyńska K, Reich PB, Romo A, Zadworny M, Jętkowiak R, Oleksyn J. Tertiary remnants and Holocene colonizers: Genetic structure and phylogeography of Scots pine reveal higher genetic diversity in young boreal than in relict Mediterranean populations and a dual colonization of Fennoscandia. *Diversity and Distributions*. 2017; 23:540–555.
- Dudareva N, Pichersky E, Gershenzon J. Biochemistry of plant volatiles. *Plant Physiology*. 2004; 135:1893–1902. [PubMed: 15326281]
- Falara V, Pichersky E, Kanellis AK. A Copal-8-ol diphosphate synthase from the angiosperm *Cistus creticus* subsp. *creticus* is a putative key enzyme for the formation of pharmacologically active, oxygen-containing labdane-type diterpenes. *Plant Physiology*. 2010; 154:301–310. [PubMed: 20595348]
- Fäldt J, Martin D, Miller B, Rawat S, Bohlmann J. Traumatic resin defense in Norway spruce (*Picea abies*): Methyl jasmonate-induced terpene synthase gene expression, and cDNA cloning and functional characterization of (+)-3-carene synthase. *Plant Molecular Biology*. 2003; 51:119–133. [PubMed: 12602896]
- Garcia-Granados A, Martinez A, Parra A, Rivas F. Manoyl-oxide biotransformations with filamentous fungi. *Current Organic Chemistry*. 2007; 11:679–692.
- Gehring C. Adenyl cyclases and cAMP in plant signaling - past and present. *Cell Communication and Signaling*. 2010; 8:1–5. [PubMed: 20181064]
- Gref R. Variation in isoabienol content in *Pinus sylvestris* needles. *Canadian Journal of Botany*. 1981; 59:831–835.
- Hiltunen R, Tigerstedt PMA, Juvonen S, Pohjola J. Inheritance of 3-carene quantity in *Pinus sylvestris* L. *Farmaseuttinen Aikakauslehti*. 1975; 84:69–72.
- Holliday JA, Ritland K, Aitken SN. Widespread, ecologically relevant genetic markers developed from association mapping of climate-related traits in Sitka spruce (*Picea sitchensis*). *New Phytologist*. 188:501–514.
- Hornoy B, Pavy N, Gérardi S, Beaulieu J, Bousquet J. Genetic adaptation to climate in white spruce involves small to moderate allele frequency shifts in functionally diverse genes. *Genome Biology and Evolution*. 2015; 7:3269–3285. [PubMed: 26560341]
- Ignea C, Ioannou E, Georgantea P, Loupassaki S, Trikkas FA, Kanellis AK, Makris AM, Roussis V, Kampranis CS. Reconstructing the chemical diversity of labdane-type diterpene biosynthesis in yeast. *Metabolic Engineering*. 2015; 28:91–103. [PubMed: 25498547]
- Ingestad T, Kähr M. Nutrition and growth of coniferous seedlings at varied relative nitrogen addition rate. *Physiologia Plantarum*. 1985; 65:109–116.
- Jablonka E. Epigenetic inheritance and plasticity: The responsive germline. *Progress in Biophysics & Molecular Biology*. 2013; 111:99–107. [PubMed: 22975443]
- Jasinski M, Stukkens Y, Degand H, Purnelle B, Marchand-Brynaert J, Boutry M. A plant plasma membrane ATP binding cassette-type transporter is involved in antifungal terpenoid secretion. *Plant Cell*. 2001; 13:1095–1107. [PubMed: 11340184]
- Jøhnk N, Hietala AM, Fossdal CG, Collinge DB, Newman MA. Defense-related genes expressed in Norway spruce roots after infection with the root rot pathogen *Ceratobasidium bicorne* (anamorph : *Rhizoctonia* sp.). *Tree Physiology*. 2005; 25:1533–1543. [PubMed: 16137939]
- Kainulainen P, Holopainen JK, Oksanen J. Effects of gaseous air pollutants on secondary chemistry of Scots pine and Norway spruce seedlings. *Water Air and Soil Pollution*. 1995; 85:1393–1398.
- Kainulainen P, Holopainen J, Palomaki V, Holopainen T. Effects of nitrogen fertilization on secondary chemistry and ectomycorrhizal state of Scots pine seedlings and on growth of grey pine aphid. *Journal of Chemical Ecology*. 1996; 22:617–636. [PubMed: 24227573]

- Kainulainen P, Satka H, Mustaniemi A, Holopainen JK, Oksanen J. Conifer aphids in an air-polluted environment. II. Host plant quality. *Environmental Pollution*. 1993; 80:193–200. [PubMed: 15091864]
- Kainulainen P, Utriainen J, Holopainen JK, Oksanen J, Holopainen T. Influence of elevated ozone and limited nitrogen availability on conifer seedlings in an open-air fumigation system: effects on growth, nutrient content, mycorrhiza, needle ultrastructure, starch and secondary compounds. *Global Change Biology*. 2000; 6:345–355.
- Keeling CI, Bohlmann J. Diterpene resin acids in conifers. *Phytochemistry*. 2006; 67:2415–2423. [PubMed: 16996548]
- Keeling CI, Dullat HK, Yuen M, Ralph SG, Jancsik S, Bohlmann J. Identification and functional characterization of monofunctional ent-copalyl diphosphate and ent-kaurene synthases in white spruce reveal different patterns for diterpene synthase evolution for primary and secondary metabolism in gymnosperms. *Plant Physiology*. 2010; 152:1197–1208. [PubMed: 20044448]
- Kopper BJ, Illman BL, Kersten PJ, Klepzig KD, Raffa KF. Effects of diterpene acids on components of a conifer bark beetle-fungal interaction: Tolerance by *Ips pini* and sensitivity by its associate *Ophiostoma ips*. *Environmental Entomology*. 2005; 34:486–493.
- Koutsaviti A, Milenkovi M, Tzakou O. Antimicrobial activity of the essential oil of Greek endemic *Stachys spruneri* and its main component, isoabienol. *Natural Product Communications*. 2011; 6:277–280. [PubMed: 21425694]
- Kukovinets OS, Zainullin RA, Odinkov VN, Kisilitsyn MI, Roshchin VI, Galin FZ, Tolstikov GA. Ozonolysis of alkenes and study of reactions of polyfunctional compounds: LXIV. Synthesis of ambreinolide and 8 α ,13-Epoxy-14,15,16-trisnorlabd-12-ene proceeding from isoabienol ozonolysis. *Russian Journal of Organic Chemistry*. 2001; 37(2):235–237.
- Kännaste A, Copolovici L, Pazouki L, Suhhorutsenko M, Niinemets Ü. Highly variable chemical signatures over short spatial distances among Scots pine (*Pinus sylvestris*) populations. *Tree Physiology*. 2013; 33:374–387. [PubMed: 23513034]
- LaFever RE, Vogel BS, Croteau R. Diterpenoid resin acid biosynthesis in conifers: enzymatic cyclization of geranylgeranyl pyrophosphate to abietadiene, the precursor of abietic acid. *Archives of biochemistry and biophysics*. 1994; 313:139–49. [PubMed: 8053674]
- Lee H-J, Ravn MM, Coates RM. Synthesis and characterization of abietadiene, levopimaradiene, palustradiene, and neoabietadiene: hydrocarbon precursors of the abietane diterpene resin acids. *Tetrahedron*. 2001; 57:6155–6167.
- Lewinsohn E, Gijzen M, Croteau R. Defense mechanisms of conifers: differences in constitutive and wound-induced monoterpene biosynthesis among species. *Plant Physiology*. 1991; 96:44–9. [PubMed: 16668184]
- Lewinsohn E, Gijzen M, Savage TJ, Croteau R. Defense mechanisms of conifers: relationship of monoterpene cyclase activity to anatomical specialization and oleoresin monoterpene content. *Plant Physiology*. 1991; 96:38–43. [PubMed: 16668182]
- Loreto F, Nascetti P, Graverini A, Mannozi M. Emission and content of monoterpenes in intact and wounded needles of the Mediterranean pine, *Pinus pinea*. *Functional Ecology*. 2000; 14:589–595.
- Manninen AM, Tarhanen S, Vuorinen M, Kainulainen P. Comparing the variation of needle and wood terpenoids in Scots pine provenances. *Journal of Chemical Ecology*. 2002; 28:211–228. [PubMed: 11868675]
- Memari, HR., Pazouki, L., Niinemets, Ü. The biochemistry and molecular biology of volatile messengers in trees. *Biology, controls and models of tree volatile organic compound emissions*. Niinemets, Ü., Monson, RK., editors. Springer, Printforce; the Netherlands: 2013. p. 547
- Miller B, Madilao LL, Ralph S, Bohlmann J. Insect-induced conifer defense. White pine weevil and methyl jasmonate induce traumatic resinosis, *de novo* formed volatile emissions, and accumulation of terpenoid synthase and putative octadecanoid pathway transcripts in Sitka spruce. *Plant Physiology*. 2005; 137:369–382. [PubMed: 15618433]
- Niinemets Ü. Responses of forest trees to single and multiple environmental stresses from seedlings to mature plants: past stress history, stress interactions, tolerance and acclimation. *Forest Ecology and Management*. 2010; 260:1623–1639.

- Niinemets Ü, Lukjanova A. Needle longevity, shoot growth and branching frequency in relation to site fertility and within-canopy light conditions in *Pinus sylvestris*. *Annals for Forest Science*. 2003; 60:195–208.
- Orlova I, Nagegowda DA, Kish CM, Gutensohn M, Maeda H, Varbanova M, Fridman E, Yamaguchi S, Hanada A, Kamiya Y, Krichevsky A, et al. The small subunit of snapdragon geranyl diphosphate synthase modifies the chain length specificity of tobacco geranylgeranyl diphosphate synthase in planta. *Plant Cell*. 2009; 21:4002–4017. [PubMed: 20028839]
- Ormeño E, Goldstein A, Niinemets Ü. Extracting and trapping biogenic volatile organic compounds stored in plant species. *Trends in Analytical Chemistry*. 2011; 30:978–989.
- Pateraki I, Andersen-Ranberg J, Hamberger B, Heskes AM, Martens HJ, Zerbe P, Bach SS, Moller BL, Bohlmann J. Manoyl oxide (13R), the biosynthetic precursor of forskolin, is synthesized in specialized root cork cells in *Coleus forskohlii*. *Plant Physiology*. 2014; 164:1222–1236. [PubMed: 24481136]
- Pazouki L, Shanjani PS, Fields PD, Martins K, Suhhorutšenko M, Viinalass H, Niinemets Ü. Large within-population genetic diversity of the widespread conifer *Pinus sylvestris* at its soil fertility limit characterized by nuclear and chloroplast microsatellite markers. *European Journal of Forest Research*. 2016; 135:161–177.
- Pazouki L, Niinemets Ü. Multi-substrate terpenoid synthases: their occurrence and physiological significance. *Frontiers in Plant Science*. 2016; 7:1–16. [PubMed: 26858731]
- Peakall R, Smouse PE. GENALEX V. 6: genetic analysis in excel. Population genetic software for teaching and research. *Molecular Ecology Notes*. 2006; 6:288–295.
- Phillips MA, Croteau RB. Resin-based defenses in conifers. *Trends in Plant Science*. 1999; 4:184–190. [PubMed: 10322558]
- Portsmouth A, Niinemets Ü, Truus L, Pensa M. Biomass allocation and growth rates in *Pinus sylvestris* are interactively modified by nitrogen and phosphorus availabilities and by tree size and age. *Canadian Journal of Forest Research*. 2005; 35:2346–2359.
- Rajabi Memari, H., Pazouki, L., Niinemets, Ü. The biochemistry and molecular biology of volatile messengers in trees. Biology, controls and models of tree volatile organic compound emissions. Niinemets, Ü., Monson, RK., editors. Springer; Berlin: 2013. p. 47-93.
- Robert JA, Madilao LL, White R, Yanchuk A, King J, Bohlmann J. Terpenoid metabolite profiling in Sitka spruce identifies association of dehydroabietic acid, (+)-3-carene, and terpinolene with resistance against white pine weevil. *Botany-Botanique*. 2010; 88:810–820.
- Rosenkranz, M., Schnitzler, J-P. Genetic engineering of BVOC emissions from trees. Biology, controls and models of tree volatile organic compound emissions. Niinemets, Ü., Monson, RK., editors. Springer; Berlin: 2013. p. 95-118.
- Sallaud C, Giacalone C, Toepfer R, Goepfert S, Bakaher N, Roesti S, Tissier A. Characterization of two genes for the biosynthesis of the labdane diterpene *Z*-abienol in tobacco (*Nicotiana tabacum*) glandular trichomes. *Plant Journal*. 2012; 72:1–17. [PubMed: 22672125]
- Semiz G, Heijari J, Isik K, Holopainen JK. Variation in needle terpenoids among *Pinus sylvestris* L. (Pinaceae) provenances from Turkey. *Biochemical Systematics and Ecology*. 2007; 35:652–661.
- Seo S, Seto H, Koshino H, Yoshida S, Ohashi Y. A diterpene as an endogenous signal for the activation of defense responses to infection with tobacco mosaic virus and wounding in tobacco. *Plant Cell*. 2003; 15:863–873. [PubMed: 12671083]
- Sjödin K, Persson M, Borg-Karlson A-K, Norin T. Enantiomeric compositions of monoterpene hydrocarbons in different tissues of four individuals of *Pinus sylvestris*. *Phytochemistry*. 1996; 41:439–445.
- Sjödin K, Persson M, Fäldt J, Ekberg I, Borg-Karlson A-K. Occurrence and correlations of monoterpene hydrocarbon enantiomers in *Pinus sylvestris* and *Picea abies*. *Journal of Chemical Ecology*. 2000; 26:1701–1720.
- Taft S, Najjar A, Godbout J, Bousquet J, Erbilgin N. Variations in foliar monoterpenes across the range of jack pine reveal three widespread chemotypes: implications to host expansion of invasive mountain pine beetle. *Frontiers in Plant Science*. 2015; 6
- Thévenot EA, Roux A, Xu Y, Ezan E, Junot C. Analysis of the human adult urinary metabolome variations with age, body mass index, and gender by implementing a comprehensive workflow for

- univariate and OPLS statistical analyses. *Journal of Proteome Research*. 2015; 14(8):3322–3335. [PubMed: 26088811]
- Tholl D. Terpene synthases and the regulation, diversity and biological roles of terpene metabolism. *Current Opinion in Plant Biology*. 2006; 9:297–304. [PubMed: 16600670]
- Trygg J, Wold S. Orthogonal projections to latent structures (O-PLS). *Journal of Chemometrics*. 2002; 16(3):119–128.
- Ulubelen A, Topcu G, Eris C, Sönmez U, Kartal M, Kurucu S, Bozok-Johansson C. Terpenoids from *Salvia sclarea*. *Phytochemistry*. 1994; 36:971–974. [PubMed: 7765213]
- Wang M, Zheng Q, Shen Q, Guo S. The critical role of potassium in plant stress response. *International Journal of Molecular Sciences*. 2013; 14:7370–7390. [PubMed: 23549270]
- Wold S, Esbensen K, Geladi P. Principal component analysis. *Chemometrics and Intelligent Laboratory Systems*. 1987; 2:37–52.

Figure 1.
Molecular structures of diterpenoids quantified in *Pinus sylvestris* L. needles.

Figure 2. Total contents of isoabienol and other diterpenoids (mg g⁻¹, mean ± SE) in *P. sylvestris* needles of Parika (P), Kakerdaja (K), Laeva (L) and Männiku (M) populations (Table 3 and Figure S1 for the sites). Lowercase letters indicate significant differences across the sites ($P < 0.05$ according to one-way ANOVA followed by a Tukey's test).

Figure 3.

Correlation between isoabienol and manoyl oxide expressed per unit dry mass in *P. sylvestris* samples of 'manoyl oxide - isoabienol pines' and 'high isoabienol pines'. Correlation was significant at level of $P < 0.001$ (***).

Figure 4.

Loading-plot (A) and score-plot (B) of PCA analysis based on the diterpenoids per dry mass (mg g^{-1}) in the needles of *P. sylvestris* called 'high isoabienol pines' (yellow circle), 'manoyl oxide – isoabienol pines' (pink circle) and 'other diterpenoid pines' (green circle) (see Table 1). In the score plot, each symbol represents one sample of one tree of Parika (triangle symbols), Laeva (diamond symbols), Kakerdaja (circle symbols) and Männiku (red star symbols) sites. In the loading plot the quantified amounts of diterpenoids (see Table 4) increase with the distance from the origin of the coordinate system. In total nearly 100% of

data variance was explained by the first and the second principal components (PCA 1 97 %, PCA 2 2.9 %).

Figure 5.

Diterpenoid profiles (% , mean $\pm$ SE) in the needles of *P. sylvestris* of 'abieta-8(14), 13(15)-diene – manoyl oxide type' (A), 'abietadiene type' (B), 'manoyl oxide – isoabienol type' (C) and 'high isoabienol type' (D and E). On PCA plot 'abieta-8(14),13(15)-diene – manoyl oxide pines' and 'abietadiene pines' formed a single cluster called 'other diterpenoid pines' (see Table 1 and Figure 4A and 4B). The diterpenoid profiles of 'high isoabienol pines' (D and E) differed considerably (Monte Carlo permutation test, $p < 0.05$).

Figure 6. Relationship between genetic and chemical distances according to nSSR markers and diterpenoids (mg g^{-1}). Relationship was significant at $P < 0.05$ (*).

Figure 7.
OPLS model based on foliar diterpenoids (mg g^{-1}) and nitrogen contents (%) of *P. sylvestris* samples of bog Kakerdaja.

Table 1

Distribution of non- and diterpenoid containing *Pinus sylvestris* L. samples across the sites

Population	Number of samples	Non-diterpenoid pines ¹ (%)	Diterpenoid containing pines (%)		
			'Manoyl oxide - isoabienol pines' ² (%)	'High isoabienol pines' ² (%)	'Other diterpenoid pines' ² (%)
Laeva (L)	69	4	6	81	9
Parika (P)	47	2	26	53	19
Kakaerdajaja (K)	70	7	17	61	15
Männiku (M)	32	341	0	44	22

The first three sites are temperate raised bogs, while the Männiku site is a dry pine forest on sandy soil.

¹ Samples were grouped according to the presence of diterpenoids to either 'non-diterpenoid pines' or 'diterpenoid-containing pines'.

² According to the content of isoabienol and manoyl oxide pines grouped as follows: needle extracts of 'manoyl oxide - isoabienol pines' contained $8.9 \pm 1.3 \text{ mg g}^{-1}$ of isoabienol; needle extracts of 'high isoabienol pines' contained $27.3 \pm 2.1 \text{ mg g}^{-1}$ of isoabienol; finally 'other diterpenoid pines' contained isoabienol at very low level of $0.068 \pm 0.026 \text{ mg g}^{-1}$ (average $\pm$ SE).

Table 2

Correlation coefficients between diterpenoids (mg g⁻¹) in *P. sylvestris* samples.

Diterpenoids	Abietadiene	Abieta-8(14),13(15)-diene	(Z)-Biformene	Isoabienol	Labda-7,14-dien-13-ol	Labda-7,13,14-triene	Manool	Manoyl oxide	13- <i>epi</i> -Manoyl oxide
Abietadiene	1	0.43**	0.49**	0.47***	0.40**	0.006	0.70	0.18	0.51***
Abieta-8(14),13(15)-diene		1	0.50**	0.20	0.26	0.62**	-	0.045	0.17
(Z)-Biformene			1	0.81***	0.61***	-0.14	-0.12	0.33**	0.40**
Isoabienol				1	0.71***	0.002	0.36	-	0.40***
Labda-7,14-dien-13-ol					1	0.18	0.36	0.12	0.33*
Labda-7,13,14-triene						1	0.54	0.62***	0.20
Manool							1	-0.30	0.81*
Manoyl oxide								1	0.13
13- <i>epi</i> -Manoyl oxide									1

Level of significance at *P*-value of 0.05 (*), 0.01 (**) or 0.001 (***).

Table 3

Location, tree characteristics and flora at sites of studied populations of *P. sylvestris*.

Population	Altitude (m)	Growth conditions	GPS coordinates	Characteristics of trees		Flora
				height m	stem diameter cm	
Laeva (L)	44	transition mire / raised bog	N 58 26.102 E 26 16.124	1.4 - 6	3 - 20.5	tree layer – <i>P. sylvestris</i> , <i>Betula pubescens</i> ; shrub / dwarf-shrub layer characteristic to transitional bog – <i>Salix lapponum</i> , <i>Oxyccoccus palustris</i> , shrub / dwarf-shrub layer characteristic to raised bog- <i>Chamaedaphne calyculata</i> and <i>Ledum palustre</i> ; grass / moss layer- <i>Phragmites communis</i> , <i>Eriophorum vaginatum</i> , <i>Carex</i> spp and various <i>Sphagnum</i> spp
Parika (P)	46	raised bog	N 58 30.091 E 25 46.823	0.8 - 6.5	3 - 17	tree layer – <i>P. sylvestris</i> , <i>B. pubescens</i> , <i>Betula nana</i> ; shrub / dwarf-shrub layer – <i>O. palustris</i> , <i>Empetrum nigrum</i> and <i>Andromeda polifolia</i> ; grass / moss layer - <i>Eriophorum</i> spp., <i>Carex</i> spp. and <i>Sphagnum</i> spp.
Kakaerdaja (K)	80	raised bog	N 59 11.321 E 25 30.613	0.4 – 4.5	3.5 - 20	tree layer - <i>P. sylvestris</i> ; shrub / dwarf shrub layer - <i>E. nigrum</i> , <i>A. polifolia</i> and <i>L. palustre</i> ; grass /moss layer - <i>Scheuchzeria palustris</i> , <i>Rubus chamaemorus</i> and various <i>Sphagnum</i> spp.
Männiku (M)	57	dry forest / heath	N 59 21.555 E 24 42.605	8 - 15	9 - 40	tree layer - <i>P. sylvestris</i> and <i>Picea abies</i> ; shrub layer - <i>Sorbus aucuparia</i> and <i>Frangula alnus</i> ; grass / moss layer - <i>Calluna vulgaris</i> , <i>Thymus sepyllum</i> , <i>Pleurozium schreberi</i>

Table 4

Diterpenoids in *P. sylvestris* samples.

No	Diterpenoid	Retention index	
		RI	RI ^c
1	Abietadiene	2088 ^a	2102
2	Abieta-8(14),13(15)-diene	2154 ^a 2152 ^b	2160
3	(<i>Z</i>)-Biformene	1988 ^b	1942
4	Isoabienol	2124 ^b	2121
5	Labda-7,14-dien-13-ol	2096 ^b	2097
6	Labda-7,13,14-triene	1978 ^b	1983
7	Manool	2057 ^a 2070 ^b	2064
8	Manoyl oxide	1998 ^a 2007 ^b	2009
9	13- <i>epi</i> -Manoyl oxide	2017 ^a 2023 ^b	2030

^aRetention indices according to Adams 2001.^bRetention indices according to software MassFinder 3 library.^cRetention indices calculated by injecting the hydrocarbon standard of C7 to C30 (Sigma- Aldrich, St Louis, MO, USA) to GC-MS.