Emissions of biogenic volatile organic compounds (BVOCs) from the rhizosphere of Scots pine (*Pinus sylvestris*) seedlings exposed to warming, moderate N addition and bark herbivory by large pine weevil (*Hylobius abietis*)

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Abstract

**Aims** Biogenic volatile organic compound (BVOC) research has been mostly focused on foliar emissions. In this experiment, the main focus was on rhizosphere BVOC emissions of Scots pine seedlings under changing growth conditions.

**Methods** Soil-growing Scots pines were exposed to increased air (0.5°C) and soil (4.0°C) temperature and N addition (30 kg N ha\(^{-1}\) yr\(^{-1}\)) for three growing seasons in a field experiment. In addition to these factors, seedlings were exposed to bark herbivory by large pine weevils in two last seasons. Gas-chromatography and mass-spectrometry was used for analyzing the BVOC samples collected from pine rhizosphere.

**Results** Almost 98 % of BVOCs were non-oxygenated monoterpenes (nMTs), 1 % oxygenated monoterpenes (oMTs), 0.5 % sesquiterpenes (SQTs), and 1 % other BVOCs. In both years, there was an interaction of warming, N addition and bark herbivory on rhizosphere BVOC emissions. In 2015, warming and N addition in single exposures decreased oMT emissions, while herbivory in single exposure increased oMT emissions. In 2016, the three-way interaction showed that the effects of warming, N addition and herbivory on BVOCs were mainly detected in single exposures. In 2016, warming decreased nMT, oMT, SQT and other BVOC emissions; N addition decreased oMT and SQT emissions; and herbivory decreased SQT and other BVOC emissions.

**Conclusions** Warming and N addition in single exposures decreased the rhizosphere BVOC emissions. The effect of bark herbivory on BVOC emissions varied between the years from increase to decrease. It seems that under altered growth conditions Scots pine seedlings may reduce carbon investment to rhizosphere BVOCs.

**Keywords** Monoterpenes · Sesquiterpenes · BVOC · Rhizosphere · Climate change · Scots pine

Introduction

Boreal forests are the largest terrestrial biome in the world (Taggart and Cross 2009). Over 70 % of Finland’s land area is covered with boreal forests with a large proportion of Scots pine stands, making it one of the most significant tree species in the region (Palviainen et al. 2010; Aarne 2013; Viitanen et al. 2018; Mäkisara et al. 2019). Trees produce plant secondary metabolites of which biogenic volatile organic compounds (BVOCs) are released into the atmosphere (Holopainen et al. 2018), and BVOCs emitted by Scots pines comprise largely of monoterpenes (Ghimire et al. 2013, Penuelas et al. 2014, Rasheed et al. 2017, Tiiva et al. 2018, 2019). BVOCs can act as chemical signals for communication within and between plants, herbivores, their predators and parasitoids as well as alleviate the effects of changes in physical environment, e.g. elevated temperature (Holopainen 2004; Holopainen...
and Gershenzon 2010), and BVOCs emitted from foliage and their responses to changes in physical environment have been reported abundantly (Penuelas and Staudt 2010, Holopainen et al. 2018). Roots, rhizobacteria, mycorrhizal fungi and other soil microbes living near the roots are all found to be sources of BVOC emissions in the rhizosphere (Leff and Fierer 2008, Penuelas et al. 2014, Mäki et al. 2019). However, the responses of rhizosphere BVOCs emissions to biotic and abiotic factors are less studied in comparison to the shoot BVOCs emissions. Rhizosphere BVOCs can act as communication signals between rhizobacteria and mycorrhizal fungi and neighboring plants (Hiltpold and Turlings 2008; Ditengou et al. 2015; Šimpraga et al. 2019), control root architecture by promoting lateral root formation (Ditengou et al. 2015) and act as chemical cues for herbivores (Delroy et al. 2016). For instance, monoterpenes α-pinene and limonene are known chemical signals for large pine weevil (Hylobius abietis) (Nordlander et al. 1986; Norlander 1990, 1991; Nordenhem and Norlander 1994), while some monoterpenes (e.g. α-pinene and β-pinene) in rhizosphere have also been reported to have inhibitory effect on soil microbes (Maurer et al. 2008; Adamczyk et al. 2015; Lin et al. 2007) have shown that most of the Scots pine rhizosphere BVOCs remained within a 20 cm radius in the soil around the trunk, and Mäki et al. 2019) have shown that the BVOCs are concentrated in the top organic- and mineral layer of the soil. Monoterpenes and sesquiterpenes readily react with free radicals like OH·, get adsorbed on surfaces or dissolve in soil water, thus, most of the monoterpenes and sesquiterpenes may not diffuse too far from the source into the soil or from soil to the atmosphere (Lin et al. 2007; Hiltpold and Turlings 2008; Šimpraga et al. 2019).

According to the sixth IPCC report (IPCC 2018), the increase in global mean surface temperature has reached 1°C relative to mean temperatures prevailing in 1850–1900, and a further increase of 0.5°C is likely to manifest during the period of 2030–2050, despite climate change mitigation strategies on global scale. Forests are considered to have a significant negative feedback on the climate change by absorbing carbon dioxide from the air, but also by releasing BVOCs that may cool down the climate through aerosol formation in the atmosphere (Paasonen et al. 2013). Increased temperatures generally increase BVOC emissions from conifer shoots by increasing their synthesis, increasing storage space for stored terpenes as well as increasing emissions from storage structures (Holopainen et al. 2018). However, recent publications from potted Scots pine seedlings did not report significant increase in belowground BVOC emissions due to 1.0°C warming in field study (Rasheed et al. 2017) or 2.0°C warming in chamber conditions (Tiiva et al. 2019).

Anthropogenic activities have also led to large scale atmospheric deposition of inorganic nitrogen (N) into the soil (EMEP 2016). Highly populated and industrialized regions receive N deposition of up to 60 kg N ha⁻¹ yr⁻¹ (Dentener et al. 2006), while the northern boreal forests of Fennoscandia region receive atmospheric N deposition in the range 1–12 kg N ha⁻¹ yr⁻¹. The critical N load for boreal forests is considered to be 2.4–10 kg N ha⁻¹ yr⁻¹ (Bobbink et al. 2010; Giordani et al. 2014; Ruohio-Airola et al. 2015). Rhizosphere processes may respond differently to different levels of N addition (Maaroufi et al. 2019). Recently, Rasheed et al. (2017) have reported a general decrease in rhizosphere BVOC emission rates in response to N addition of 120 kg N ha⁻¹ yr⁻¹ from potted Scots pine seedlings growing in a peat:sand mixture in a field experiment. At a lower N addition of 30 kg N ha⁻¹ yr⁻¹, Tiiva et al. (2018, 2019) observed an increase in shoot BVOC emission rates due to moderate N addition of 30 kg N ha⁻¹ yr⁻¹, but no significant effect on below-ground BVOC emissions rates in response to N addition from potted Scots pine growing in sandy forest soil.

Climate warming is also expected to increase insect outbreaks and also increase the feeding period of e.g. large pine weevils, a major forest pest in Finland (Logan et al. 2003; Inward et al. 2012; Schwartzberg et al. 2014; Wainhouse et al. 2014; Norlander et al. 2017). Generalist herbivores, such as large pine weevils, may use constitutively emitted BVOCs from shoots and rhizosphere as cues for foraging and oviposition (Norlander et al. 1986, Šimpraga et al. 2019). Effects of herbivory on BVOC emission rates from Scots pine have been reported extensively, however the bulk of studies focus on aboveground emissions reporting an increase in BVOC emissions in response to herbivory as a main effect or in interaction with abiotic factors (Holopainen and Gershenzon 2010; Heijari et al. 2011; Kivimäenpää et al. 2016; Ghimire et al. 2013, 2017; Tiiva et al. 2018) reported a decrease in monoterpene and SQT emissions from rhizosphere in response to needle feeding by large pine sawfly (Diprion pini) in potted Scots pine seedlings. Rasheed et al. (2017) have reported an increase in α-pinene and a decrease in tricyclene/α-thujene emission rates in response to needle feeding by great web-
spinning pine sawfly (*Acantholyda posticalis*) from rhizosphere of potted Scots pine seedlings in a field experiment. Tiiva et al. (2019), however, did not observe any effects of feeding by large pine weevils on belowground BVOC emission rates of potted Scots pine seedlings in a chamber experiment.

We studied the interactive effects of increasing air and soil temperature (0.5°C and 4°C respectively), moderate N addition to soil of 30 kg N ha$^{-1}$ yr$^{-1}$ and bark herbivory by large pine weevil (*Hylobius abietis*) on the rhizosphere BVOC emissions of young Scots pines, which were grown in forest soil in a three-year-long field experiment. As far as we know, similar field experiments focusing on the interactive effects of warming, moderate N addition and bark herbivory on rhizosphere BVOC emissions measured from Scots pine seedlings growing in natural forest-soil are scarce. Previous field experiments with warming treatment have focused on higher N addition to soil (e.g., 120 kg N ha$^{-1}$ yr$^{-1}$), comparable to forest fertilization in managed forests (Saarisalmi and Määnkönen 2001), and needle herbivory (e.g., Ghimire et al. 2013, Rasheed et al. 2017). Hence, this study differs from the previous field experiments by using moderate N addition levels, comparable to atmospheric deposition in Central Europe (Dentener et al. 2006; Reay et al. 2008), use of forest soil as growth medium, and seedlings grown without pots in the field plots.

Based on the responses of below-ground BVOCs to warming reported by Rasheed et al. (2017) and Tiiva et al. (2019), we hypothesize that warming effects on rhizosphere or below-ground BVOC emissions might be no change or minor changes in emission rates. Different below-ground variables have shown highly variable responses to different levels of N addition (12–120 kg N ha$^{-1}$ yr$^{-1}$; Rasheed et al. 2017, 2020, Maaroufi et al. 2019, Tiiva et al. 2019), it is not clear how moderate N (30 kg N ha$^{-1}$ yr$^{-1}$) addition will affect rhizosphere BVOC emissions of soil-grown Scots pine seedlings. High N addition (120 kg N ha$^{-1}$ yr$^{-1}$) can cause a general decrease in rhizosphere BVOC emissions (Rasheed et al. 2017). Hence, we hypothesize that rhizosphere BVOC responses to moderate N addition may decrease in emission rates. According to Ghimire et al. (2013), Rasheed et al. (2017) and Tiiva et al. (2019), shoot herbivory can modify the BVOC emissions from below-ground such that the emission rates of some BVOCs may increase, while the emission rates of some BVOCs may decrease. In addition, Rasheed et al. (2020) reported two- and three-way interactive effects on shoot and root growth, root-to-shoot ratio, root morpholgy and mycorrhizal colonization from the same seedlings used in the current BVOCs study; therefore, it is possible warming, moderate N addition and bark herbivory may also show some interactive effects on the rhizosphere BVOC emissions.

**Materials and methods**

Experimental site and field exposure system

The experiment was conducted at the research field of Natural Resources Institute Finland (Luke) in Suonenjoki, Central Finland (62°38.5164′N, 27°03.3454′E) during years 2014–2016. There were 16 rectangular plots (1.9 m × 1.4 m) filled with sandy forest soil collected from the nearby mature Scots pine stand in Suonenjoki (online resource 1a). One-year-old Scots pine seedlings (seed origin Sv339; Rasheed et al. (2020)) were planted in the plots (24 experimental seedlings per each plot) in June 2014. On each long side of the experimental plot there were potted seedlings to prevent edge effect (online resource 1b). The seedlings received an initial dose of 60 Kg N ha$^{-1}$ yr$^{-1}$ during the nursery period at Luke.

Half of the plots were warmed 24-hours a day using IR heaters (1250mm long x 94mm wide, Comfortintra CIR105-220, 230–400 V, Frico AB, Sweden; online resource 1c) while the rest were kept at ambient temperature in the field. The warming exposure lasted for 94 days (2.7–3.10.2014), 151 days (4.5–1.10.2015) and 123 days (3.5–2.9.2016) during 2014–2016. Soil and air temperatures, soil moisture content and relative humidity were all measured in each plot with sensors (Campbell scientific Ltd, UK) and the amount of rain was measured in weather station (Campbell scientific Ltd, UK) at the center of the field site. Data logger (Campbell scientific Ltd, UK) was used to monitor and log the data from the sensors in the field. The overall average increase in air and soil temperature was 0.5 °C and 4.0 °C, respectively. The more detailed air humidity, soil moisture and monthly rain sums are published in Rasheed et al. (2020). N addition (30 kg N ha$^{-1}$ yr$^{-1}$, Yara Peatcare TM, slow release 1, N:P:K, 9:4:5) to the soil was performed in four warmed as well as four ambient temperature plots at the start of each exposure season in 2014–2016.

Bark herbivory treatment (H, see Appendix for further explanation of treatment abbreviations) was performed...
with large pine weevils (*Hylobius abietis*) in 2015 and 2016. In 2015, herbivory treatment lasted for 24 hours (1-7.2015) and in 2016 for 7 days (22-29.6.2016). In 2015, a clear plastic cup of 250 ml was cut into half and placed around the base of the stem. The top part of the cage touching the stem was sealed with cotton wool hole allowing air exchange between the outside and inside air, while the cage part touching the soil was sealed with wet quartz sand to ensure that the weevils could not escape from the cage. In 2015, two weevils were placed around each of 16 seedlings (H+ treatment) and there were empty cages around another 16 seedlings (H- treatment); one seedling per each plot per treatment. In 2016, four weevils were used per seedling, and there were two H+ treatment seedlings and one H- treatment seedling from each plot. In 2016, clear plastic bags (60 cm × 60 cm, 75 L, and 10 × 10 cm window covered with fine mesh cloth for air exchange) were used as cages. Bag was enclosing the crown of the seedling inside it and bag was tightened around the base of the stem with a wire. The damaged surface due to herbivory was photographed and the area (mm²) was measured using ImageJ program (version 1.47) from the digital images, and the intensity of feeding damage was calculated as the percentage of bark surface area of the main shoot (Rasheed et al. 2020). In 2015 and 2016, the (mean ± standard error) of damaged surface area was (42.7 ± 20.0) mm² and (498.5 ± 54.4) mm² respectively; and the (proportion ± standard error) of damaged surface area was (0.41 ± 0.17)% and (2.83 ± 0.33)% respectively.

The treatments in the field were C = control (ambient temperature without N addition), T = temperature increase alone, N = N addition alone, and TN = temperature increase and N addition in combination in non-herbivory (H-) and herbivory (H+) treatments; (n = 4 per each treatment). See Appendix for further explanation of treatments abbreviations.

Rhizosphere BVOC collections

Rhizosphere BVOC sampling was performed on 23.9.2015 for 48 seedlings and 1-2.9.2016 for 64 seedlings in total (i.e., 3 seedlings per each plot in 2015 and 4 seedlings per each plot in 2016) and the sampling system is illustrated in Fig. 1. Seedlings were harvested with their root systems and rhizosphere soil attached to roots (soil block around the roots had a volume 20 cm × 20 cm × 25 cm). Excess soil from the roots was gently shaken off from the roots of the harvested seedling and then the root system, still attached to the shoot, was carefully enclosed into a pre-cleaned (120°C, 60 min) polyethylene terephthalate (PET) cooking bag (Rainbow, ITS foil, film & paper products, The Netherlands). The bag was enclosed at the base of the stem using a garden wire, and the bag size was 35 × 43 cm (volume 4.0 L). The bag was ventilated through Teflon tubing with a flow of filtered and MnO₂ scrubbed air (flow rate 400 ml min⁻¹) for 10 minutes prior sampling. A 10-minute sample collection was started immediately after the ventilation. The sample was pulled through a purified stainless-steel tube (Perkin Elmer, Boston, MA, USA) filled with adsorbents (Tenax TA and Carbopack B, 100 mg of each, mesh 60/80, Supelco, Bellefonte, PA, USA). The tube was attached to one corner of the bag with a garden wire. Custom-made sampling boxes containing the filters and pumps (12V Retsch-Thomas, Puchheim, Germany) were used in the samplings. The flow rate through the tube was set to 200 ml min⁻¹, while inflow (400 ml min⁻¹) of purified air was maintained into the bag during the sampling. A higher inflow was used to create overpressure and to prevent outside VOCs from leaking into the system (Kivimäenpää et al. 2013). The flows were adjusted using an M-5 bubble calibrator (A.P. Buck, Orlando, FL, USA).

Air temperature and relative humidity inside the bag was monitored during the whole sampling time (DS1923-F5# Hygrochron, Maxim Integrated, San Jose, CA, USA). A blank sample was collected using an empty bag in each measurement set. The sample tubes were sealed with brass caps immediately after the sampling, refrigerated (+ 5°C) and analysed within two days.

BVOC identification and calculation of emission rates

The BVOC samples were analyzed by gas chromatograph-mass spectrometer (GC-MS, Hewlett-Packard GC 6890, MSD 5973, USA). BVOCs in the adsorbent tubes were desorbed with a thermal desorber (Perkin-Elmer ATD400, Automatic Thermal Desorption system, Wellesley, MA, USA) connected to the GC with heat transfer line at +200°C. The adsorbent tubes were first heated at +280°C temperature for 10 min, cryofocused at -30°C on a cold trap and then injected in a split mode into an HP-5MS capillary column (model 19091S-105, 50 m × 0.20 mm i.d × 0.33 μm film thickness, Agilent, USA) with helium used as the carrier gas. The oven temperature program was set at +40°C for 1 min, and scheduled to increase by 5°C min⁻¹ to +210°C and subsequently by 20°C min⁻¹ to +250°C and hold 3
min, at 1 mL min\(^{-1}\) column flow. Mass spectra were obtained by scanning technique from 33 to 400 m/z.

BVOCs were identified according to the mass spectra in the Wiley data library, and quantified by comparing the peak areas to those of standard s using an in-lab prepared standard solution containing 24 different compounds (Fisher chemicals, HPLC grade) dissolved in methanol: \(\alpha\)-pinene, camphene, sabinene, \(\beta\)-pinene, \(\beta\)-myrcene, 3-carene, D-limonene, 1,8-cineole, \(\gamma\)-terpinene, terpinolene, linalool, E-DMNT, camphor, borneol, terpen-4-ol, \(\alpha\)-terpineol, bornyl acetate, longifolene, trans-\(\beta\)-farnesene, \(\alpha\)-humulene, \(\alpha\)-copaene, trans-caryophyllene, aromadendrene and \(\beta\)-elemene. For compounds that were not present in the standard mixture, peak area of the compound with similar molecular mass, structure and retention time were used. There were nine compounds that was not possible to identify based on Wiley data library (mass spectra for these compounds are shown in online resource2). The software Enhanced ChemStation (Agilent Technologies, Santa Clara, CA, USA) was used to analyse the chromatograms. The quantities of BVOCs determined from blank samples were subtracted from the rhizosphere BVOC emission results. The presented dataset includes the BVOCs that were identified with \(\geq 90\%\) certainty.

The emission was calculated and normalized over root dry mass (g) with the Eq. (1):

\[
E = \frac{(C_{\text{out}} - C_{\text{in}})F}{\text{rootdrymass}} \times 60\text{min}
\]  

(1)

Where \(E\) = emission rate, \(C_{\text{out}}\) = BVOC concentration (ng/L), \(C_{\text{in}}\) = BVOC concentration in clean air (assumed to be 0) and \(F = 0.4\ L/min\) (inflow of clean air). All resulting emissions are presented as \(\mu g\ g^{-1}\ h^{-1}\). The detected compounds were grouped into non-oxygenated monoterpenes (nMT), oxygenated monoterpenes (oMT), sesquiterpenes (SQT) and other BVOCs.

The emission rates were standardized for 15°C (288.15 K) using the algorithm in Eq. (2) (Guenther et al. 1993) to make a fair comparison between BVOCs collected in varied temperatures in the outdoor set-up:

\[
Es = \frac{E}{\exp((T - Ts))}
\]  

(2)

Where \(Es\) = standardized emission rate, \(E\) = emission rate from Eq. (1), \(\beta\) (the constant of the algorithm) = 0.1 for MTs, oMTs and other BVOCs and 0.17 for SQTs, \(T\) is the average temperature in kelvin (K) prevailing at the time of sample collection and \(Ts = 288.15\) K (Guenther et al. 2012).

### Statistical analysis

The emission rates of individual compounds as well as the sum of emission rates of each BVOC group (nMTs, oMTs, SQTs and other BVOCs) were aggregated over the plot identity, after which there were four true replicates per each treatment (\(n = 4\)). The main and interaction effects of warming, N addition and herbivory (fixed factors) on the emission rates of the nMTs, oMTs, SQTs and other BVOCs from 2015 to 2016 were tested with Linear Mixed Models (LMM) ANOVA with plot identity as a random factor. Residuals were tested for normality, and in case of non-normal distribution of residuals, transformations were performed on the data before the LMM ANOVA tests. \(P\)-values \(\leq 0.05\) were considered statistically significant and \(P\)-values \(\leq 0.1\) were considered marginally significant. All interactions shown by LMM ANOVA tests with \(P\)-value \(\leq 0.1\) were further tested with simple main effects (SME), i.e., post hoc test for two- or three-way interactions with Bonferroni corrections. In LMM ANOVA designs, each fixed factor had two levels, i.e. temperature factor had T- = no warming and T+ = warming levels, N factor had N- = no N addition and N+ = N addition levels, and herbivory factor had H- = no herbivory and H+ = herbivory levels. From here on these terms are used in the interpretations of the SME test results (Appendix shows treatments abbreviations and SME test terms).

In order to study the separation of the BVOC profiles due to experimental treatments, we analyzed the rhizosphere BVOC profiles (73 compounds in 2015 and 81 compounds in 2016) using multivariate Principal component analysis (PCA) and Partial Least Square Discriminant Analysis (PLS-DA). PCA and PLS-DA both showed many outliers within the BVOC emission profiles, indicating that the variation among the samples was high. Omission of the outliers in PCA and PLS-DA resulted overfitting of the models. Therefore, only those compounds belonging to the major BVOC groups showing three-way interactions in LMM ANOVA were tested in more detail with either independent samples t-test (normally distributed data) or independent samples Mann-Whitney U-test (non-normally distributed data).
data). Hence, if LMM ANOVA and SME test showed a significant effect of warming without N addition (N-) in non-herbivory seedlings (H-) for a BVOC group, then the effect of warming in N- and H- treated seedlings was tested on individual compounds belonging to that BVOC group using t-test or Mann-Whitney U-test. IBM SPSS Statistics for Windows (version 23.0, IBM Corp., Armonk, N.Y., USA) was used for LMM ANOVA, SME test, independent t-test, and Mann-Whitney U tests. SIMCA® 15 was used to perform PCA analysis and PLS-DA was performed using MetaboAnalyst 5.0 (Xia et al. 2009).

Results

BVOC emission profiles of the Scots pine rhizosphere

In total, 26 nMTs, 17 oMTs, 22 SQTs and 8 other BVOCs (non-isoprene-based compounds) were detected from samples in 2015, while 27 nMTs, 23 oMTs, 21 SQTs and 10 other BVOCs were emitted from the rhizosphere of the seedlings in 2016. In both years, nMTs comprised approximately 98 % of the total BVOC emissions, while oMTs and other BVOCs both comprised approximately 1 % and SQTs comprised less than 0.5 % of the total BVOC emissions. Of nMTs, α-
pinene and δ-3-carene were the most dominant compounds, together comprising over half of the total nMT emissions (online resources 3 and 4). The oMT emissions were dominated by myrtenal, verbenone, thymyl methyl ether and eucarvone (comprised over half of total oMTs) in both sampling years (online resources 3 and 4). Of the total SQT emissions, α-longipinene and junipene comprised over half of the emissions in 2015 and 2016 (online resources 3 and 4). The other BVOCs’ emissions mainly consisted of toluene in both the years (online resources 3 and 4). GLVs were not detected in the rhizosphere emissions.

BVOC emission rate responses to treatments in 2015

There was a three-way interaction (T × N × H interaction; see Appendix) of warming, N addition and herbivory (Fig. 2; Table 1) on the total oMT emission rates in 2015. SME tests showed that warming without N addition in herbivory seedlings (T+ vs. T-: N- and H+) and N addition without warming in herbivory seedlings (N+ vs. N-: T- and H+) decreased the total oMT emission rates (Fig. 2; Table 1). SME test also showed that herbivory without warming or N addition (H+ vs. H-: T- and N-) increased oMT emission rates (Fig. 2; Table 1). There were no significant treatment effects on nMTs, SQTs or other BVOCs in 2015.

Of the individual oMT compounds, emission rate of 1,8-cineol (oMT) decreased due to warming at prevailing soil N levels in herbivory seedlings (T+ vs. T-: N- and H+; Fig. 3a; Table 2) and fell below the detection limit due to N addition without warming in herbivory seedlings (N+ vs. N-: T- and H+; Table 2; Fig. 3b). Herbivory alone, at ambient temperature and prevailing N conditions in soil, increased 1,8-cineole emissions from rhizosphere (H+ vs. H-: T- and N-; Fig. 3c; Table 2). Emission rate of D-camphor significantly increased due to warming without N addition in herbivory seedlings (T+ vs. T-: N- and H+; Fig. 3a; Table 2). Emission rate of bornyl acetate was below detection limit at ambient temperature without N addition in herbivory seedlings, but its emission rate significantly increased due to warming without N addition in herbivory seedlings (T+ vs. T-: N- and H+; Fig. 3a; Table 2).

BVOC emission rate responses to treatments in 2016

There was a three-way interactive effect (T × N × H) on total nMT (Fig. 4a), oMT (Fig. 4b), SQT (Fig. 4c) and other BVOC (Fig. 4d) emission rates in 2016. Warming effect was consistent across four different BVOC groups, as warming without N addition in non-herbivory seedlings decreased the total nMT, oMT, SQT and other BVOC emission rates from the rhizosphere (T+ vs. T-: N- and H-; Fig. 4a-d; Table 1). N addition decreased nMT, oMT and SQT emission rates without warming in non-herbivory seedlings (N+ vs. N-: T- and H-; Fig. 4a-c; Table 2), while herbivory decreased SQT and other BVOC emission rates without warming in prevailing N conditions (H+ vs. H-: T- and N-; Fig. 4c-d; Table 2).

Of the individual BVOC compounds, N addition decreased tricyclene/α-thujene (nMT) and α-longipinene (SQT) emission rates under ambient temperature in non-herbivory seedlings (N+ vs. N-: T- and H-; Fig. 5a; Table 2). Carvacol (oMT) emissions from rhizosphere without N addition and warming in non-herbivory seedlings was below detection limit, but it significantly increased due to N addition in the above conditions (N+ vs. N-: T- and H-; Fig. 5a; Table 2). In seedlings exposed to herbivory without warming in prevailing N conditions, cis-caryophyllene (SQT) emissions from rhizosphere were below detection limit (H+ vs. H-: T- and N-; Fig. 5b; Table 2).

Discussion

Monoterpene and sesquiterpene profile of rhizosphere resembles to that of Scots pine shoots

The BVOC emissions from the rhizosphere in both the years were dominated by monoterpenes, such as α-pinene and δ-3-carene, this result being consistent with the previous below-ground BVOC studies from Scots pines (Ludley et al. 2009; Ghimire et al. 2013; Rasheed et al. 2017). The overall profile from rhizosphere was also very similar when compared to the shoot BVOC emission profile of Scots pines, where α-pinene and δ-3-carene dominated the shoot BVOC emissions and monoterpenes in general comprise over 90% of the emission profiles (Räisänen et al. 2009; Bäck et al. 2012; Kivimäenpää et al. 2016, 2018; Tiiva et al. 2018). Monoterpene composition is very similar in different plant parts of Scots pine (Kivimäenpää et al. 2018 and references therein). Thus, the similarity of nMT emission blend here with shoot emissions is mostly due to high proportion of roots in the rhizosphere.
samples where excess soil was removed. The high abundance of SQTs such as \( \alpha \)-longipinene and junipene is also in agreement with the earlier studies by Ghimire et al. (2013) who reported them to be the most abundant SQTs emitted from the root-soil blocks of potted Scots pine seedlings. SQT emission rates were generally low, which is consistent with Napierala-Filipiak et al. (2002), and in our study representing an average of only 0.5% of total emissions in 2015 and 2016. The low emission rates of SQTs can also be attributed to their low volatility due to their relatively high molecular mass, and SQTs are often classified as semi-volatile organic compounds (Mofikoya et al. 2019). Toluene is reported to be emitted in high quantities from young Scots pine shoots (Misztel et al. 2015), and here toluene was also one of the most abundantly emitted non-isoprene-based BVOC from the Scots pine rhizosphere. Tiiva et al. (2019) have reported tridecane (other BVOC) emissions from the

![Graph](image_url)

**Fig. 2** The emission rates (µg g\(^{-1}\) h\(^{-1}\)) of total oxygenated monoterpenes (oMTs) in 2015. *P*-value for the interaction (LMM ANOVA) is shown. The bars represent means and error bars represent standard error of mean \((n = 4\) per treatment). See Appendix for treatment abbreviations.

| Tested variable | Interaction | SME comparison | SME test *-value | Change |
|-----------------|-------------|----------------|------------------|--------|
| 2015 oMTs       | T × N × H   | T+vs. T\(^-\): N- & H\(^+\)† | 0.004            | ↓ 51%  |
|                 |             | N+vs. N-: T- & H+ | <0.001           | ↓ 70%  |
|                 |             | H+vs. H-: T- & N- | 0.001            | ↑ 181% |
| 2016 nMTs       | T × N × H   | T+vs. T\(^-\): N- & H- | 0.022            | ↓ 77%  |
|                 |             | N+vs. N-: T- & H- | 0.049            | ↓ 65%  |
| 2016 oMTs       | T × N × H   | T+vs. T\(^-\): N- & H- | 0.035            | ↓ 70%  |
|                 |             | N+vs. N-: T- & H- | 0.029            | ↓ 73%  |
| 2016 SQTs       | T × N × H   | T+vs. T\(^-\): N- & H- | 0.013            | ↓ 67%  |
|                 |             | N+vs. N-: T- & H- | 0.003            | ↓ 84%  |
|                 |             | H+vs. H-: T- & N- | 0.049            | ↓ 52%  |
| 2016 Other BVOCs| T × N × H   | T+vs. T\(^-\): N- & H- | 0.005            | ↓ 84%  |
|                 |             | H+vs. H-: T- & N- | 0.022            | ↓ 67%  |

See Appendix for statistical terms’ abbreviations
† Tested factor within the tested interaction in SME test
* Grouping factors in SME test.
rhizosphere area of potted Scots pines based on soil collar measurements, and here also trace amounts of tridecane were detected in Scots pine rhizosphere.

Warming and N addition consistently decreased BVOC emissions from the rhizosphere.

In 2015, emission rates of oMTs were decreased due to warming alone in herbivory seedlings. In 2015, the emission rates of oMTs were also decreased due to N addition alone in herbivory-treated seedlings. In 2016, warming and N addition decreased the emission rates of all the BVOC groups in single exposures in non-herbivory seedlings. BVOC emissions are known to be sensitive to environmental factors such as prevailing temperature and N availability in soil (Holopainen et al. 2018). Previously, increase in emissions of shoot level BVOCs from 1 to 2°C warming and N addition of (30–120 kg N ha\(^{-1}\) yr\(^{-1}\)) from young Scots pine has been reported (Kivimäenpää et al. 2016; Ghimire et al. 2017; Tiiva et al. 2018). However, warming of 1-2°C in either single exposure or in combination with N addition to soil and herbivory by great web-spinning pine sawfly (Rasheed et al. 2017) or large pine weevils (Tiiva et al. 2019), did not show any significant effects on below-ground BVOC emission rates from potted Scots pine.

![Fig. 3](image)

**Fig. 3**  
(a) Warming effects on emission rates (µg g\(^{-1}\) h\(^{-1}\)) of 1,8-cineol, D-camphor and bornyl acetate without N addition in herbivory seedlings (T+ vs. T- in N- and H+); (b) N addition effects on 1,8-cineol without warming in herbivory seedlings (N+ vs. N+: T- and H+), and (c) herbivory effects on 1,8-cineol without warming and N addition (H- vs. H+: T- and N-) in 2015. The bars represent means and error bars represent standard error of mean (n = 4 per each factor level) and differ significantly (data are tested with t-test or Mann-Whitney U-test, P < 0.05, t-test for Mann-Whitney U-test). Note axis break in a. See Appendix for treatment and statistical terms’ abbreviations.

| Tested VOC          | Group | Interaction                  | P-value | Change   |
|---------------------|-------|------------------------------|---------|----------|
| 2015                |       |                              |         |          |
| 1,8-Cineol          | oMT   | T+ vs. T-: N- & H+           | 0.029   | ↓ 96%    |
|                     |       | N+ vs. N+: T- & H+           | 0.029   | ↓ 100%   |
|                     |       | H+ vs. H+: T- & N-           | 0.020   | ↑ 4150%  |
| D-camphor           | oMT   | T+ vs. T-: N- & H+           | 0.029   | ↑ 783%   |
| Bornyl acetate      | oMT   | T+ vs. T-: N- & H+           | 0.029   | ↑ 100%   |
| 2016                |       |                              |         |          |
| Tricyclene/α-thujene| nMT   | N+ vs. N+: T- & H-           | 0.029   | ↓ 92%    |
| Carvacol            | oMT   | N+ vs. N+: T- & H-           | 0.029   | ↑ 100%   |
| α-Longipinene       | SQT   | N+ vs. N+: T- & H-           | 0.041   | ↑ 83%    |
| Cis-Caryophyllene   | SQT   | H+ vs. H+: T- & N-           | 0.029   | ↓ 100%   |

See Appendix for statistical terms’ abbreviations.
seedlings. N addition of 120 kg N ha$^{-1}$ yr$^{-1}$ has been reported to consistently decrease rhizosphere nMT emissions (Rasheed et al. 2017); and moderate N addition of 30 kg N ha$^{-1}$ yr$^{-1}$ in interaction with warming of 2°C above ambient temperature has been reported to marginally decrease other BVOC emissions (Tiiva et al. 2019) from soil collar measurements in pot-grown young Scots pines. Our study together with above previous reports show that the BVOCs from shoot and rhizosphere of Scots pine may behave antagonistically in response to warming and N addition, i.e. shoot level emissions increase while those of rhizosphere decrease. Warming and N addition to soil, alone and in interaction with each other, have been reported to increase root growth from the same experiment (Rasheed et al. 2020). Thus, the consistent decreasing effect of these abiotic factors on rhizosphere BVOC emissions implies that seedlings actively invested carbon resources in below-ground growth but not so much in rhizosphere BVOC synthesis. Given that large pine weevils use nMTs from Scots pine rhizosphere as cues for oviposition (Norlander et al. 1986), decreasing effect of warming and N addition on the emission rates of nMTs may affect to some extent to large pine weevils’ ability to locate Scots pine roots for oviposition too.

In addition to Scots pine roots, SQTs and oMTs have also been shown to be emitted from the ectomycorrhizal fungi growing in the rhizosphere (Bäck et al. 2010, Penuelas et al. 2014, Ditengou et al. 2015). Studies performed with Scots pines have suggested that high

![Fig. 4](image-url) Treatment effects on the emission rates (µg g$^{-1}$ h$^{-1}$) of total (a) non-oxygenated monoterpenes (nMTs), (b) oxygenated monoterpenes (oMTs), (c) sesquiterpenes (SQTs) and (d) other VOCs (oVOCs) in 2016. $P$-values for the interactions (LMM ANOVA) are shown. The bars represent means and error bars represent standard error of mean ($n = 4$ per treatment). See Appendix for treatment abbreviations.
N addition both tend to decrease the host plants’ dependence on mycorrhizal fungi as the mycorrhizal colonization has been observed to decrease in response to N addition to soil (Rasheed et al. 2017, 2020). Here decreased emissions of SQTs and oMTs from the rhizosphere in response to N addition may thus follow the N response observed in the mycorrhizal colonization. Furthermore, in this field experiment, warming, N addition to soil and bark herbivory all in single exposures decreased the SQT emissions from rhizosphere, but increased specific root length, thus increasing soil exploration efficiency of the roots (Ostonen et al. 2007; Rasheed et al. 2020). This indicates that seedlings were investing more carbon to fine root growth than production of BVOCs.

Individual BVOC responses to warming and N addition

Warming without N addition in non-herbivory seedlings decreased 1,8-cineol emissions and increased D-camphor and bornyl acetate emissions simultaneously in 2015. 1,8-Cineol, D-(+)-camphor and bornyl acetate all have antimicrobial and insecticidal properties and may have a protective role in roots Klepzig and Schlyter 1999; Park et al. 2003; Chen et al. 2013, Penuelas et al. 2014). Bornyl acetate is also a precursor for synthesis of D-camphor, which explains the similar trends in the emission of these two compounds. 1,8-cineol and D-(+)-camphor can act synergistically as insecticides such that 1,8-cineol is a more potent insecticide in presence of D-camphor (Chen et al. 2013). Hence, although warming reduced the emission rate of 1,8-cineol, emission rates of D-(+)-camphor and bornyl acetate were increased, most likely, compensating for the decrease in insecticidal properties of the overall BVOC blend. 1,8-cineol has been shown to inhibit root growth (Koitabashi et al. 1997), and it is possible that under warming and N addition treatment in the presence of herbivory, Scots pine actively reduced 1,8-cineol emissions, as they invested carbon resources in root growth (Rasheed et al. 2020).

Emission rates of tricyclene/α-thujene (nMT) and α-longipinene (SQT) decreased, while carvacol (oMT) increased due to N addition without warming and herbivory in 2016. Concentrations and emission rates of tricyclene/α-thujene have been shown to increase from shoots of Scots pine seedlings (Kainulainen et al. 1996; Kivimäenpää et al. 2016), and decrease in emissions from rhizosphere of Scots pine seedlings (Rasheed et al. 2017) due to N addition. The decrease in α-longipinene emissions due to N addition is a novel finding and the role of α-longipinene in Scots pine is less understood. However,
emission rates of α-longipinene have been shown to increase due to feeding by large pine weevil (Semiz et al. 2016) or exogenous methyl jasmonate (signal compound of insect induced defense pathway) treatment (Heijari et al. 2008) in pine shoots.

Carvacol may have fungicidal properties (Neri et al. 2007; Brilliet al. 2019). Increase in carvacol emissions in N addition coincided with a decrease in mycorrhizal colonization reported by Rasheed et al. (2020) due to N addition. This finding implies that root level emission of carvacol may play a role in controlling the mycorrhizal colonization of young Scots pine.

Bark herbivory affects oMT, SQT and other BVOC emissions from Scots pine rhizosphere

The increasing effect of herbivory on oMTs in 2015, and the decreasing effect of herbivory on SQTs and other BVOCs in 2016 from rhizosphere emissions of young Scots pines were detected only without warming and N addition. Large pine weevils use nMTs from Scots pine for locating hosts for oviposition. Particularly, α-pinen (nMT) acts as an attractant for large pine weevils (Norlander et al. 1986, Norlander 1990), but there were no effects of herbivory on nMT emissions in either year. This implies that Scots pine seedlings do not respond to moderate feeding by large pine weevils by changing nMT emission rates from the rhizosphere. Mycorrhizal fungi can be also a source of SQT emissions (Ditengou et al. 2015). The decrease in total SQT emissions reported here coincides with the decreased mycorrhizal colonization reported from the same experimental seedlings by Rasheed et al. (2020), indicating that herbivory could reduce mycorrhizal colonization which also in turn may reduce the emissions of mycorrhizal SQTs. Tiiva et al. (2019) have reported a decreasing effect of weevil feeding on 1,3-dimethylbenzene, decanal and tridecane from soil collars due to herbivory by large pine weevils. Here the decreasing effect of weevil feeding on total other BVOC emissions was significant in single exposure regime, but statistical analysis of individual compounds did not identify any compounds that could explain the above change.

Emission rate of 1,8-cineol from rhizosphere increased in response to short-term (24-hours) herbivory. The overall proportion of 1,8-cineol from Scots pine rhizosphere appears to be higher compared to shoot level emission profile in a previous study (Bäck et al. 2012). 1,8-Cineol is assumed to be selected by evolution due to its antimicrobial and insecticidal properties (Penuelas et al. 2014), and thus, herbivory-induced emissions of 1,8-cineol from rhizosphere might act as natural protection against herbivory. The proportion of 1,8-cineol was around 10 % of total oMT emissions in 2015, while in 2016 the proportion of 1,8-cineol was around 2.5 % of the total oMT emissions, which may explain the lack of herbivory effect on oMT emissions from rhizosphere in 2016, despite the herbivory treatment lasting 7-days. This also implies that seedlings may have different responses towards different duration and/or intensity of herbivory, i.e. 24-hour feeding by large pine weevil triggers increased emissions of 1,8-cineol while 7-day feeding by the same herbivore induced decreased emissions of cis-caryophellene from Scots pine rhizosphere.

Cis-caryophyllene (SQT) emissions decreased below detection limit due to herbivory at ambient temperature without N addition in 2016. Emissions of trans-β-caryophyllene, which has insecticidal properties (Yuqing et al. 2010), has been reported to increase due to weevil damage in pine shoots (Heijari et al. 2011; Kovalchuk et al. 2015; Semiz et al. 2016) have shown that trans-β-caryophellene was detected only from the shoot of Scots pine seedlings damaged by large pine weevils as defense pathways were activated. This suggests that under bark feeding by large pine weevils, Scots pine shifts from cis-caryophellene emission in roots to trans-β-caryophellene emission in shoots.

Conclusions

We report here that BVOC emissions from rhizospheres of young Scots pines are likely to respond to warming, N addition and bark herbivory all, but mainly when these three factors are not occurring together. Whenever there was a warming or N addition effect on the total emissions of the four studied BVOC groups (nMTs, oMTs, SQTs and other BVOCs), they consistently decreased emissions from the rhizosphere. Herbivory increased the total oMT emissions in 2015 and decreased the total SQT and other BVOCs emissions in 2016. Current results suggest that warming may induce increased emission of antimicrobial and insecticidal compounds such as D-camphor and bornyl acetate. Bark herbivory may induce an increase in emission of 1,8-cineol and decrease the emission of cis-caryophyllene from the rhizosphere.
Appendix

Table 3  Treatment abbreviations for eight treatments performed in the field

| Abbreviation | Herbivory (H) | Treatment description |
|--------------|--------------|-----------------------|
| C            | H-          | Control: ambient temperature + ambient level of soil N (no herbivory) |
| T            | H-          | Warming treatment (air: 0.5°C above ambient, soil: 4.0°C above ambient (no herbivory)) |
| N            | H-          | N addition 30 kg N ha\(^{-1}\) yr\(^{-1}\) to soil (no herbivory) |
| TN           | H-          | Warming and N addition combined (no herbivory) |
| C            | H+          | Control treatment and herbivory combined (large pine weevil (Hylobius abietis)) |
| T            | H+          | Warming treatment and herbivory combined |
| N            | H+          | N addition treatment and herbivory combined |
| TN           | H+          | Warming and N addition treatments and herbivory combined |

Only statistically significant or marginally statistically significant main and interaction effects found in LMM ANOVA tests are shown below. For main effects, Bonferroni test was performed for comparison, and for three-way (T × N × H) interaction, comparisons were performed with Simple Main Effects (SME) test with Bonferroni correction (statistical terms used in the text, tables and figures).

| Main/Interaction effect | Comparisons | Interpretation |
|-------------------------|-------------|----------------|
| T                       | T + vs. T-  | T main effect comparison irrespective of other factors |
| N                       | N + vs. N-  | N main effect comparison irrespective of other factors |
| H                       | H + vs. H-  | H main effect comparison irrespective of other factors |
| T × N × H               | T + vs. T- in N + and H+ | Effect of T in N and H+ treated seedlings |
|                         | T + vs. T- in N- and H+ | Effect of T in H- treated seedlings grown at ambient level N |
|                         | T + vs. T- in N+ and H- | Effect of T in H+ treated seedlings grown at ambient level N |
|                         | T + vs. T- in N- and H- | Effect of T in H+ treated seedlings grown at ambient level N |
|                         | N + vs. N- in T+ and H+ | Effect of N in H+ treated seedlings grown at ambient temperature |
|                         | N + vs. N- in T+ and H- | Effect of N in H+ treated seedlings grown at ambient temperature |
|                         | N + vs. N- in T- and H+ | Effect of N in T+ treated seedlings grown at ambient temperature |
|                         | N + vs. N- in T- and H- | Effect of N in T+ treated seedlings grown at ambient temperature |
|                         | H + vs. T- in H+ and N+ | Effect of H in T+ and N+ treated seedlings |
|                         | H + vs. T- in H+ and N- | Effect of H in T+ and N- treated seedlings |
|                         | H + vs. T- in T+ and N+ | Effect of H in T+ and N+ treated seedlings |
|                         | H + vs. T- in T+ and N- | Effect of H in T+ and N- treated seedlings |

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