

RESEARCH ARTICLE

Aboveground herbivory elevates belowground rhizosphere volatile organic compound concentrations in ambient and polluted air, independent of volatile sampling methodology

Tihomir Simin¹  | Luke Bell² | James M. W. Ryalls² | Robbie D. Girling^{2,3}  |
Oliver E. I. Welling¹ | Neil J. Mullinger⁴ | Muhammad Usman Rasheed¹ |
James D. Blande¹

¹Department of Environmental and Biological Sciences, University of Eastern Finland, Kuopio, Finland

²School of Agriculture, Policy and Development, University of Reading, Whiteknights, Early Gate, Reading, UK

³Centre for Sustainable Agricultural Systems, Institute for Life Sciences and the Environment, University of Southern Queensland, Toowoomba, QLD, Australia

⁴UK Centre for Ecology and Hydrology, Midlothian, UK

Correspondence

Tihomir Simin, Department of Environmental and Biological Sciences, University of Eastern Finland, Yliopistonranta 8, 70211 Kuopio, Finland.

Email: tihomir.simin@uef.fi

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Societal Impact Statement

The effect of air pollution on plants is still underexplored, especially belowground. We used a field facility that released diesel exhaust and ozone at concentrations equivalent to those near a busy road in Reading, UK, to measure belowground gas exchange of Scots pine together with feeding by large pine weevils, a major pest of conifers. We found that blends and concentrations of belowground volatile organic compounds (VOCs) change significantly upon exposure to air pollution and aboveground insect herbivory. This impact of the rhizosphere in systemic plant-stress responses is important, considering that VOCs can affect local pollution levels, weather and climate.

Summary

- One of the major challenges regarding air pollution is that we do not have a clear understanding how it affects the functioning of natural systems, including the mechanisms underpinning inter- and intraspecific ecological interactions. Expanding our knowledge of these interactions in this area can serve as a basis upon which future nature protection policies could be built. Volatile organic compounds (VOCs) are synthesised by plants and have roles in inter- and intraspecific interactions and in protection against herbivores. They also have important roles in biosphere–atmosphere interactions. Rhizosphere VOCs play key roles in plant defence and soil ecology but are poorly understood due to difficulties in measurement. This study examined how aboveground herbivory by the large pine weevil (*Hylobius abietis*) influences belowground VOC concentrations in Scots pine (*Pinus sylvestris*) under ambient and polluted conditions.
- Experiments were conducted within a Free-Air Diesel and Ozone Enrichment (FADOE) platform, using two field-based VOC collection methods (a noninvasive soil collar and a VOC-mole™ inserted into the soil).

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- Herbivory significantly increased total belowground VOC concentrations after 38 h (by 165%), indicating a systemic plant response. In rings receiving the combined air pollution treatment, concentrations of benzenoid VOCs and oxygenated monoterpenes were also elevated (by 136% and 217%, respectively) relative to rings with ambient air.
- Both sampling methods produced comparable VOC profiles, supporting their interchangeable use according to experimental needs. These results suggest a role for rhizosphere VOCs in plant defence and provide robust field methods for studying plant-stress interactions.

KEYWORDS

diesel, free-air diesel and ozone enrichment, *Hylobius abietis*, nitrogen oxides, ozone, *Pinus sylvestris*, rhizosphere, volatile organic compounds

1 | INTRODUCTION

Covering one third of the world's forested area (Brandt et al., 2013) boreal forests provide important ecosystem services for people, including timber production, regulation of water, soil and air quality, recreation and availability of nonwood forest products, such as mushrooms and berries accessible through broad everyman's rights (Gauthier et al., 2015; Pohjanmies et al., 2017). Moreover, dominated by coniferous tree species such as Scots pine (*P. sylvestris*; Gauthier et al., 2015), boreal forests emit volatile organic compounds (VOCs) that influence atmospheric chemistry and biosphere-atmosphere feedbacks (Satake et al., 2024). While aboveground VOC emissions from foliage are well documented (Laotawornkittikul et al., 2009; Liu et al., 2023; Rieksta et al., 2023; Satake et al., 2024; Seco et al., 2022; Simin et al., 2021, 2022), belowground concentrations in the rhizosphere, comprising roots, fungi, microorganisms and soil, remain understudied due to practical measurement challenges. The rhizosphere is a dynamic zone where VOCs play roles in plant defence and interactions with soil microbes and herbivores, yet their response to stressors such as herbivory and air pollution remains poorly understood (Lin et al., 2007; Rasheed et al., 2017, 2021).

The large pine weevil (*Hylobius abietis*) is a major pest in coniferous forests and creates particular issues with clear cutting of forested areas. This is because after the trees are cut, the stumps are targets for *Hylemya abietis* oviposition and can hence lead to pest outbreaks in new plantations. The large pine weevils can girdle and kill young seedlings (Véle, 2022). Adults consume bark, with one weevil capable of consuming 75 mm² of bark per day, potentially killing dozens of seedlings over its 4-year lifespan (Lalík et al., 2021). Its widespread distribution, from southern Spain to northern Finland, underscores its importance as a pest, with traditional chemical controls being increasingly restricted (López-Villamor et al., 2019). Herbivory by the large pine weevil is known to increase aboveground VOC emissions (Heijari et al., 2011; Simin et al., 2026; Yu et al., 2022), serving as a defence mechanism and communication signal (Simin et al., 2026; Yu et al., 2022, 2024), but whether this response extends belowground is

unclear. Due to complexities involved with sampling and analysis methods of soil VOCs, we are only beginning to understand belowground plant-plant interactions (Ali et al., 2026; Peñuelas et al., 2014). Furthermore, Kristensen et al. (2020) in their meta-analysis found that herbivory reduces belowground carbon allocation by plants and increases atmospheric losses of elements. They have also recommended closing the knowledge gaps from boreal forests on the effects of aboveground insect herbivory on belowground processes.

Even though it is often far from centres of human activity, boreal forest health will be increasingly affected by air pollution, including tropospheric ozone (O₃) and nitrogen oxides (NO_x). This is because pollutants can be carried across vast distances from their origin and can affect remote pristine environments, even crossing large bodies of water like the Pacific Ocean (Han et al., 2019; Nguyen et al., 2022). While the impact of air pollution on emission of aboveground VOCs is established (Farré-Armengol et al., 2016; Grulke & Heath, 2020; Nguyen et al., 2022; Pinto et al., 2010), its effect on rhizosphere concentrations represents a knowledge gap.

The soil can be a significant source of VOCs and NO_x and is therefore important for formation and removal of tropospheric O₃ (Hui et al., 2023; Peñuelas et al., 2014; Yang et al., 2024), but studies on the effect of air pollution on belowground VOC concentrations are scarce. A study from southern Spain found that when VOCs and semi-volatile compounds are emitted from industrial sources and urban traffic exhaust, they deposit into the soil and are subsequently released back into the atmosphere (Domínguez-Morueco et al., 2018). Yu and Blande (2021) showed that O₃ exposure increased the loss of carbon resources through belowground VOC emission by 794% (compared to control) in summer, indicating a significant shift in carbon allocation patterns.

Belowground Scots pine VOC collection has previously been performed in laboratory-based studies, by enclosing the rhizosphere in a polyethylene terephthalate (PET) bag after careful removal from the soil (Lin et al., 2007; Yu & Blande, 2021). However, this method is not a viable option if seedlings are not to be harvested. Installing a plastic collar by the seedlings for the purpose of collecting rhizosphere VOCs

via an attached PET bag has been performed on rhizospheres of silver birch (Maja et al., 2014) and Scots pine (Tiiva, Julkunen-Tiitto, et al., 2018). Soil collars have also been used to study the flux of belowground VOCs in boreal forests (Mäki et al., 2019). Therefore, this represents a more established option for sampling underground VOCs in the field. Other methods of VOC collection include burying adsorbent cartridges in the soil, similar to the work of Lin et al. (2007), but the technique could be improved by using specialised steel probes with a series of holes through which soil gases can pass, thus facilitating the sampling of belowground VOCs. However, these probes have not been used before in field-based rhizosphere ecological studies coupled to air pollution.

Plant–plant interactions mediated by VOCs are an important component of ecological communication. Aboveground herbivore-induced VOCs can prime neighbouring plants for enhanced defence, influence resource allocation and affect community-level processes (Ninkovic et al., 2019). Far less is known about whether such signals also operate belowground, or whether belowground VOC concentrations themselves respond to damage on neighbouring individuals (Ali et al., 2026; Peñuelas et al., 2014). Field-based experiments that simultaneously examine above- and belowground VOC responses, especially under realistic air-pollution conditions, remain scarce (Ali et al., 2026; Peñuelas et al., 2014; Rasheed et al., 2023). Addressing this gap is particularly relevant in boreal forests, where damage from the large pine weevil is common and atmospheric pollution is increasing.

Our study addresses these issues by testing (i) whether aboveground herbivory by the large pine weevil increases belowground VOC concentrations in Scots pine; (ii) whether exposure to damaged neighbouring (emitter) plants affects belowground VOC concentrations in receiver plants; and (iii) whether air pollution alters the concentration of VOCs and CO₂ in the soil. We also compared two field-based VOC collection methods (a noninvasive soil collar and a sub-surface VOC-mole™) to assess their efficacy, interchangeability and sensitivity to air pollution.

2 | MATERIALS AND METHODS

2.1 | Experimental site and setup

The study was conducted at the University of Reading Free-Air Diesel and Ozone Enrichment (FADOE) facility in Sonning, UK (51°28'54.1"N 0°53'43.4"W; Figure 1a). The facility, covering an area of 13,124 m², includes a central control unit and 12 rings (8 m diameter) that deliver four treatments: elevated ozone (O₃), elevated NO_x (diesel exhaust), combined O₃ and diesel exhaust and ambient air control. This means that each air pollution treatment was represented by three separate rings ($n = 3$). The air is pumped to each ring using regenerative blowers (R4110-2, Gast, Benton Harbor, MI, USA); the ozone treatment is provided by three ozone generators (CD2000P, ClearWater Tech, San Luis Obispo, CA, USA); and the diesel treatment is provided by a diesel generator (SSDK12W Kubota 12.6 kVA, SGS Engineering Ltd, UK). Each ring is surrounded by pipes that deliver the

air, controlled by a system of relays. For a detailed description of the FADOE facility, refer to Mofikoya et al. (2024).

Each ring contained four 155-L containers (53 cm in height, 67 cm in diameter; Nuova Pasquini & Bini, Altopascio, Italy) in which 2-year-old Scots pine seedlings from the Meggernie provenance in Scotland (56°35'25.8"N 4°18'14.4"W; grown from seed by and purchased from Alba Trees Ltd, Gladsmuir, UK) were planted in a total of 48 containers across 12 rings (Figure 1b). The soil mixture in the containers consisted of two parts coarse sand and one part natural ericaceous soil with a low pH (4.5–5.5; Premium Topsoil Supplies, Broadbridge Heath, UK). Four seedlings were planted in each container on 4 May 2023, one in the centre and three around the edges, approximately 120° apart (Figure 1c). The central seedling served as the emitter, while one of the three surrounding receiver trees had a small plastic collar (length: 4.5 cm, diameter: 10 cm; OsmoDrain, Wavin, Doncaster, UK) inserted 2 cm into the soil next to it to standardise the measurements of soil respiration and soil VOC concentrations. The average distance between emitter and receiver seedlings was 26.7 cm.

The trees were watered as needed to maintain soil moisture between 10% and 17%. After potting, the trees were left to acclimate and develop new growth for 26 days. Measurements were conducted in sets of four rings at a time: The first set was measured from 18 June to 21 June 2023 and the second from 28 June to 1 July 2023. Due to time and resource constraints, four rings were omitted; we therefore sampled eight containers per air pollution treatment ($n = 8$).

The herbivory treatment for the H1 (interactions) experiment involved placing three adults of the large pine weevil, *Hylobius abietis*, onto the bark of selected trees and enclosing the insects and the bark in a white mesh bag (Eurokangas, Orimattila, Finland). The weevils were starved for 24 h before placement. They were collected from tree trunks in Scotland the previous year, kept in captivity at low temperature (16°C at night and 9°C during the day) and fed pine and spruce branches regularly. Exposure to damaged emitters involved placing the weevils onto the emitter tree, present in the middle of each container, for 5 days (weevils were omitted from undamaged control emitters) and then measuring the receiver trees' responses. Herbivory involved placing the weevils onto the receiver trees for 38 h and measuring bark damage, in addition to repeating the measurements from the first phase. There were two containers with herbivory treatment on emitter plants and with control emitters in each ring, so in total, we had four replicates for each air pollution and interactions treatment ($n = 4$).

For more information on the experimental setup and the effects of interactions on aboveground VOC emissions, please see Simin et al. (2026).

2.2 | Soil respiration measurements

Soil (rhizosphere) respiration was measured using an EGM-4 Environmental Gas Monitor for CO₂ (PP Systems, Amesbury, MA, USA) equipped with an SRC-1 soil respiration chamber and soil temperature probe. The soil respiration chamber was positioned on the plastic soil

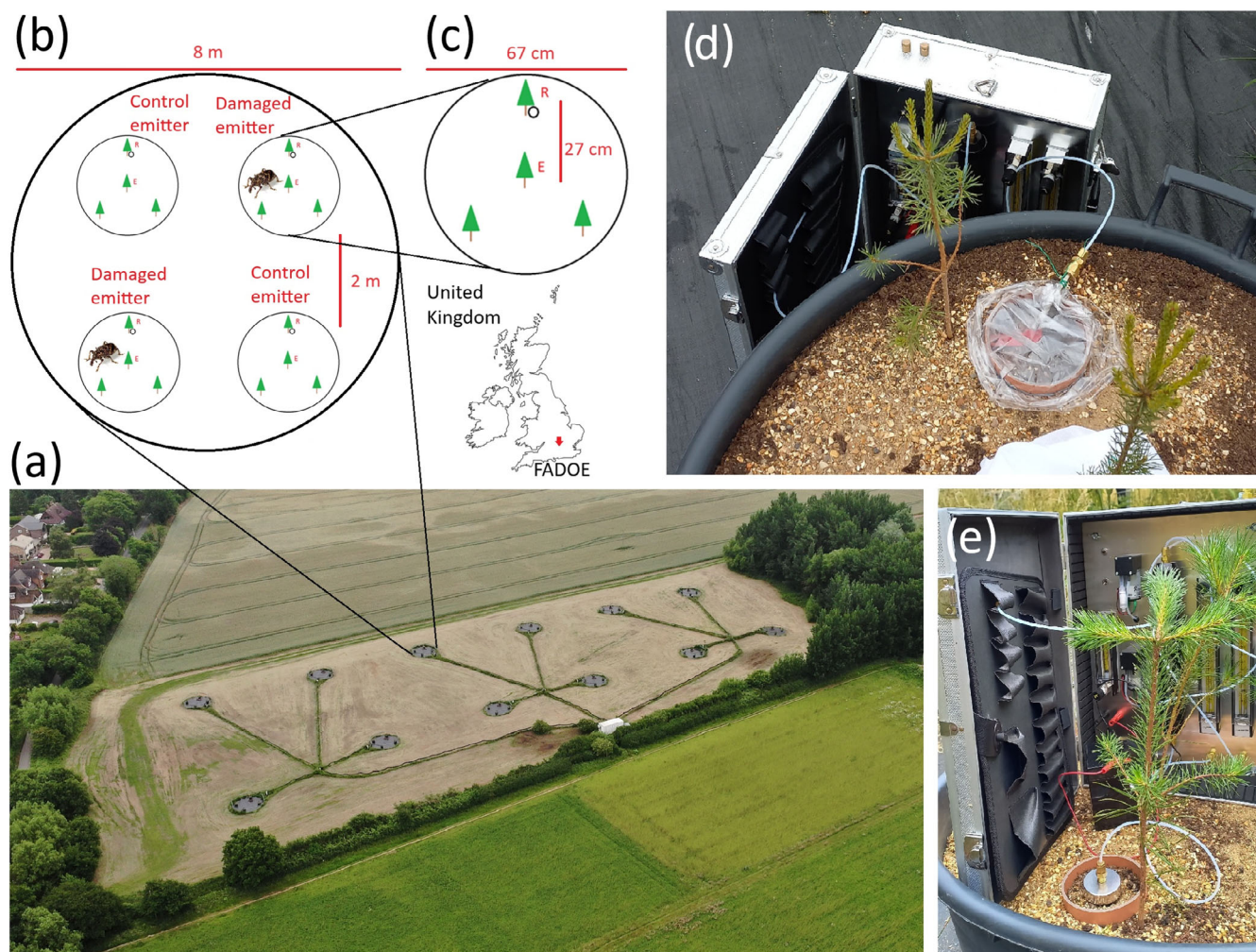


FIGURE 1 Experimental setup and two volatile organic compound (VOC) collection methods. (a) Free Air Diesel and Ozone Enrichment (FADOE) research facility in Sonning, UK, 51.48, -0.89 ; 95 m.a.s.l. (b) Positioning of containers inside each FADOE ring: Weevils were placed on emitter seedlings in two out of four containers in each ring, while the other two were used as control. (c) Scots pine layout inside each FADOE ring: An emitter seedling was planted in the middle of a container and a receiver seedling along with a soil collar next to it were positioned at the edge of the container. (d) VOC collection from the soil collar: A polyethylene bag was placed around the collar, and the VOC were collected on adsorbent cartridges—This method was used during the interactions experiment because of its nondestructive nature. (e) VOC collection using the VOC-mole™: It was inserted into the middle of the soil collar and VOCs were collected on the cartridge positioned inside—This method was used following the experiment because it disturbs the rhizosphere of the Scots pine. Map lines delineate study areas and do not necessarily depict accepted national boundaries. Photo credits: (a) University of Reading; (d), (e)—Tihomir Simin.

collars next to the receiver tree. A circular piece of polyethylene foam was placed between the chamber edge and the collar to prevent outside air seeping in. The volume of the air within the chamber and soil collar was set to 1800 mL. A linear fit was chosen to obtain the final CO_2 efflux values by the instrument. For ambient and $\text{O}_3 + \text{D}$ rings, $n = 6$ and for O and D rings $n = 4$.

2.3 | Soil collar VOC collection and analysis

For the soil collar VOC measurements, a PET bag (25 × 38 cm; Cooke & Miller, Gem Imports Ltd, Hoyland, UK) was secured around the soil collar and a temperature logger (Thermochron, OnSolution

Pty Ltd, Castle Hill, Australia) was inserted (Figure 1d). The air was pulled out of the bag through the adsorbent cartridge (200 mg Tenax TA, Markes International Ltd, Llantrisant, UK) at the rate of 200 mL/min for 30 min without flushing the bag beforehand. After sampling, the cartridges were sealed and stored at 5°C until analysis. A new PET bag was used for each measurement. Six in situ blanks were performed during the interactions experiment on a soil collar inserted into a container without Scots pine seedlings (placed in one of the ambient air rings) to account for VOCs derived from the soil mixture itself, sampling materials and the analysis instrument (Figure S1). VOC concentrations in the blanks were subtracted from the soil collar samples temporally, meaning that a blank was collected for a group of sample measurements and was used for subtraction from the samples

of those days. A new blank was used to subtract the VOC concentrations from samples on following dates: 13 June–20 June, 21 June, 22–23 June, 28 June, 29 June and 30 June–1 July 2023. Soil collar measurements were nondestructive and taken once during the between-plant interactions experiment (analysed on the GC–MS instrument in Kuopio, Finland) and another time following the experiment, on 3 July 2023 to complement and compare with the VOC-mole™ measurements (analysed on the Gas Chromatography Time-Of-Flight Mass Spectrometry (GCxGC-TOF-MS) instrument in Reading, England).

Adsorbent cartridges were transported to Kuopio, Finland, and analysed using gas chromatography–mass spectrometry (GC-2010 Plus + GCMS-QP2020; Shimadzu Corporation, Kyoto, Japan) after thermal desorption (TD-30R; Shimadzu corporation, Kyoto, Japan). The carrier gas was helium. Cartridge desorption was performed for 10 min at a temperature 300°C and flowrate of 50 mL/min, following a pre-purge for 1 min at 20 mL/min. The joint, valve and transfer lines were kept at 250°C. The VOCs from the cartridges were desorbed onto the cold trap kept at a temperature of –20°C, which were then further desorbed into the GC column at 300°C for 1 min using the splitless injection mode. The temperature in the GC oven was kept at 40°C for 1 min, rising to 125°C by 5°C min^{–1}, and then to 260°C at 10°C min^{–1}. A ZB-5MSplus capillary column made of diphenylsiloxane and dimethylsiloxane (60.0 m length, 0.25 mm diameter, 0.25-µm film thickness; Phenomenex Inc., Torrance, USA) was used for compound separation. The pressure was kept at 191.5 kPa, total flow was 2.9 mL/min, column flow was 1.88 mL/min, linear velocity was 35 cm s^{–1}, and purge flow was kept at 1 mL/min. Separated compounds were then ionised by the MS ion source kept at 200°C, while the interface temperature was 280°C and detected on the quadrupole at the threshold of 50 kV. To prevent detector overload with methanol and other low-mass compounds, solvent cutoff time was set to 3.4 min, so chromatograms started at this time and continued until 35 min, which was the total programme time for GC–MS analysis of our soil collar VOC samples.

Chromatograms were analysed using PARADISE (Quintanilla-Casas et al., 2023; v.6.0.0.18; <https://ucphchemometrics.com/paradise/>). The identification of VOCs was based on 34 authentic internal standards Table S1 and NIST 20 library mass spectra (National Institute of Standards and Technology, Gaithersburg, MD, USA) for compounds without standards. Compounds for which the NIST 20 Match Factor was <800 were identified to the level of a broad compound group. Sample concentrations were quantified proportionally based on the internal standards. The VOCs were classified into non-oxygenated monoterpenes (MTs), oxygenated monoterpenes (oMTs), sesquiterpenes (SQTs), green leaf volatiles (GLVs), benzenoids (BZs) and other non-isoprenoids (ONIs). Compounds for which no standard was available were quantified using the most appropriate standard, based on compound structure and mass, as in Simin et al. (2022).

VOC concentrations in blanks temporally corresponding most closely with the samples were subtracted from the Scots pine VOC sample concentrations, and high-concentration compounds in blanks were considered as measurement system contaminants and omitted.

2.4 | VOC-mole™ VOC collection and analysis

On 3 July 2023, a VOC-mole™ (Markes International Ltd, Llantrisant, UK) was inserted into the centre of the soil collar to collect sub-surface VOCs (Figure 1e). An adsorbent-filled cartridge (200 mg Tenax TA, Markes International Ltd, Llantrisant, UK) was attached and the collection time was set to 30 minutes at an outflow rate of 200 mL/min. The VOC-mole™ is a destructive method for VOC collection, so measurements were conducted following the interactions experiment.

Analysis of VOC compounds was conducted using two-dimensional GCxGC-TOF-MS. The system is composed of an automated extraction and enrichment unit (Centri; Markes International Ltd., Bridgend, UK), coupled to an 8890 GC system (Agilent, Stockport, UK) with INSIGHT-Flow modulator (SepSolve Analytical Ltd., Peterborough, UK), a Flame Ionisation Detector (FID; Agilent), and BenchTOF2 mass spectrometer (SepSolve Analytical Ltd.). Sample separation was achieved using a WAX-HT 20 m × 18 mm ID × 0.18-µm film (polyethylene glycol; MEGA S.r.l., Legnano, Italy) as the primary column phase, and a BPX50 2.5 m × 0.25 mm ID × 0.1-µm film (50% phenyl polysilphenylene-siloxane; Trajan Scientific & Medical, Ringwood, VI, Australia) as the secondary column phase. Helium was used as the carrier gas.

Adsorbent cartridges containing the VOC samples extracted using the VOC-mole™ were placed in the Centri loading racks for analysis. Cartridges were dry purged using nitrogen gas for 5 min with trap in-line at a flowrate of 50 mL/min to remove water. Cartridges were desorbed for 3 min at 260°C. VOC compounds from the cartridges were transferred to the focussing trap (Material Emissions, U-T12ME-2S; Markes International Ltd.) with trap and split flows set to 50 mL/min and a trap temperature of 20°C. Following probe desorption, the trap was purged for 5 min at 50 mL/min with nitrogen gas. The trap was then desorbed and fired by heating to 280°C (100°C/sec) for 5 min. A 5:1 split was applied with a split flow of 5 mL/min. The transfer line flow path was heated at 180°C, and sample was injected into the GC system.

GC runtime was 74 min with an initial 45°C temperature hold for 2 min. An oven temperature ramp programme was set to 3.5°C min^{–1} to 280°C with a final 5-min hold. The inlet was set to splitless mode and maintained at 250°C. Total flow was 6.5 mL/min at 29 psi with a septum purge flow of 3 mL/min. The GC column flows and parameters were as follows: Column 1 (primary)—0.5 mL/min (constant flow; 29 psi), Column 2 (secondary)—20 mL/min (constant flow; 21.4 psi), Column 3 (bleed line to controller)—6.1 mL/min (constant flow; 16.6 psi), Column 4 (bleed line to atmosphere)—0.5 mL/min (constant flow; 1.5 psi), Column 5 (transfer line to TOF)—4 mL/min (constant flow; 7.6 psi) and Column 6 (transfer line to FID)—46.8 mL/min (constant pressure; 7.6 psi). The INSIGHT-Flow modulator was balanced for a flush time of 125 ms and a modulation period of 3 s. The FID was heated to 300°C and supplied with 35 mL/min of hydrogen with a make-up flow of nitrogen (up to 47 mL/min) and an airflow of 300 mL/min.

TOF acquisition was conducted with a filament voltage of 1.8 V, transfer line temperature of 250°C and an ion source temperature of

250°C. Ionisation voltage was -70 eV, and scan range was set from 40 to 450 m/z (data rate acquisition speed 50 Hz). Data were collected as High Definition (HD) spectra from the MS channel, as well as a parallel FID signal. Spectra were corrected for temperature and used CO_2 as a lock mass.

Prior to experimental sequences, the TOF was checked for air/water counts ($<150,000$), and an instrument tune was performed using an internal tuning mix. A C_7 – C_{30} alkane (Merck-Sigma, Gillingham, UK) was loaded onto a conditioned cartridge (1 μ L; 1:20 dilution in hexane) run under identical conditions with the exception that a filament delay (1 V) was applied for 240 s to prevent detector overloading by hexane. A blank trap fire was initiated before sample introduction to ensure the focussing trap was free of any contamination.

TOF data were analysed using ChromSpace (v.2.1.7.; SepSolve Analytical Ltd.). Files were batch processed using a Dynamic Background Correction (DBC) algorithm to remove baseline signal noise from chromatograms (peak width setting was set to 0.2 s (Wesoly et al., 2024). Integration was performed using a tile sum method, where two-dimensional chromatograms were divided into overlapping ‘tiles’ used for downstream chemometrics (Eggermont et al., 2023). Tile window widths were set to 12 s (^1tR) and 0.75 s (^2tR) with a 25% overlap.

Chemometrics of the integrated tile sum data were analysed using ChromCompare+ (CC+; v.2.1.7., SepSolve Analytical Ltd.). Raw ion data were processed and filtered to identify ions explaining the largest amount of discrimination (Spadafora et al., 2023) between sampling methods (VOC-mole™ vs. soil collar) and experimental treatments (control/ambient, diesel, ozone and diesel + ozone). Ion features were initially filtered using a minimum intensity cut-off of 2500 counts. The remaining ion features were normalised using Probabilistic Quotient Normalization (PQN) based on the mean ion abundance across all samples (Gorrochategui et al., 2016).

The normalised data were then filtered using the feature discovery algorithm for the top 20 discriminating ions between extraction methods and experimental treatments. These features' retention times (^1tR and ^2tR) were then cross-referenced with DBC chromatograms in ChromSpace to identify the associated chromatogram peaks. Compounds were tentatively identified using the NIST 23 library (National Institute of Standards and Technology, Gaithersburg, MD, USA). A match factor and reverse match factor of 800 was used as a threshold for tentative identification, and the m/z of the ions identified by CC+ were required to be present in both the observed mass spectra and the library mass spectra for a reliable association to be determined. CC+ was then used to generate visualisations for principal component analysis, volcano plots and box-plots (with 95% confidence intervals).

2.5 | Statistical analysis

Linear mixed models (LMMs) were used to analyse all receiver-tree-response data using IBM SPSS Statistics software (v.29.0.2.0 [20]; The International Business Machines Corporation, Armonk, NY,

USA). We performed LMMs on total VOCs, individual VOC groups, soil respiration rate and bark herbivory as response variables and pollution treatment (ambient, ozone, diesel and diesel + ozone), exposure to emitter plants (damaged or control) and direct herbivory (before and after herbivory on receiver trees) as fixed factors. Random factors included the pot number nested within the FADOE ring number and seasonality (18 June–21 June; 28 June–1 July). The significance of main effects and their interactions was assessed at various levels: $p < 0.001$ (***), $p < 0.01$ (**), $p < 0.05$ (*) and $p < 0.1$ (†). Data were log-transformed ($\log [x + 1]$) if the assumption of normality of residuals was violated.

We ran the machine learning algorithm Random Forests (RF) to assess how pollution treatment, emitter exposure and direct herbivory altered the relative contributions of individual VOCs to the total belowground VOC concentration profile (hereafter called ‘VOC blend’) and to identify compounds important for the blend differences (Biau & Scornet, 2016; Breiman, 2001). We used the ‘randomForest’ function from the *randomForest* package in R statistical framework—version 2024.12.0 (RStudio Team, PBC, Boston, MA, USA). RF shows if samples fall into distinct clusters and how clusters are related to a certain treatment.

We ran separate supervised RF with herbivory (H vs. Control [C]; $n = 36$), emitter exposure (damaged vs. undamaged; $n = 37$) and pollution treatment after herbivory (ambient vs. ozone ($n = 8$), diesel ($n = 8$) or diesel + ozone ($n = 6$)) as the response variable and individual VOC concentrations as the predictor variables. 100,000 bootstrap samples (N_{tree}) with 10 variables (VOCs; $mtry$) randomly selected at each node. For optimal results, it is usually recommended to set the $mtry$ value equal to the square root of the number of variables ($n = 95$; Biau & Scornet, 2016). RF is randomly filtering a subset of samples and variables and returns an out-of-bag (OOB) error rate and a confusion matrix for each classification and selection method. There is no need for a separate test set for unbiased results or for cross-validation of results because RF leaves out variables at each tree (Biau & Scornet, 2016). Importance of each VOC for the classification, expressed as the mean decrease in accuracy (MDA) was also obtained using the ‘cmdscale’ function in the *randomForest* R package. Specific VOCs with high importance in separating the VOC blends have higher MDA values. We also tested all compounds used in RF utilising the paired t-test (for comparing Herbivory and Control) and Dunnett's test (for comparing ozone, diesel and diesel + ozone pollution treatments to ambient) using *dplyr* and *multcomp* packages in R.

3 | RESULTS

3.1 | Interactions experiment—Effect of herbivore feeding and air pollution on belowground gases

Air pollutants alone exhibited only a marginal effect on soil CO_2 emission ($F_{3,58} = 2.21$, $p = 0.096$, LMM), but the interaction between pollution and herbivory was significant ($F_{3,58} = 10.12$, $p < 0.001$, LMM), as we observed a 27% increase in soil respiration between plants in

ambient rings before herbivory and plants in rings with combined ozone and diesel exhaust ($O_3 + D$) after herbivory (Figure S2).

We identified 95 individual VOCs in the Scot's pine belowground VOC blend with an average 74% being ONIs, 17% monoterpenes (MT), 4% benzenoids (BZ) and 3.5% GLV, with a trace amount of oxygenated MTs (oMT) and sesquiterpenes (SQT; Table S2).

Direct herbivory increased the total VOC concentrations by 165%, as well as the concentrations of MTs by 246% and ONIs by 188% ($F_{1,45} = 18.70$, $p_{TOTAL} < 0.001$, $F_{1,45} = 26.06$, $p_{MT} < 0.001$, and $F_{1,45} = 7.97$, $p_{ONI} = 0.007$, respectively, LMM; Figure 2). The effect of air pollution alone affected the concentrations of BZs ($p = 0.001$, $F = 8.32$, LMM; 136% increase in $O_3 + D$ rings, compared to ambient rings), oMTs ($p < 0.001$, $F = 6.55$, LMM; 217% increase in $O_3 + D$ rings, compared to ambient rings) and the ONIs ($F_{3,45} = 4.85$, $p = 0.005$, LMM). The combined effect of herbivory and pollution was significant for most VOC groups, except for BZs and GLVs ($F_{3,45} = 5.29$, $p_{TOTAL} = 0.003$; $F_{3,45} = 5.29$, $p_{MT} = 0.015$; $F_{3,42} = 3.89$, $p_{oMT} < 0.001$; $F_{3,41} = 12.17$, $p_{SQT} < 0.001$; $F_{3,45} = 6.20$, $p_{ONI} = 0.033$, LMM). Exposure to damaged emitters did not have a significant effect on belowground VOC concentrations.

Supervised RF revealed a clear separation of belowground VOC blends between the two herbivory treatments (Figure 3a) and the pollution treatments following herbivory (Figure 3b), but no clear separation of blends of plants exposed to damaged versus control emitters (Figure S3). Compounds with the highest MDA separating the VOC blends before and after herbivory mostly belonged to the MT group: α -pinene (334% increase after herbivory; $t_{473,-35} = -2.38$, $p = 0.025$, t -test), terpinolene (286% increase; $t_{35,-8} = -3.18$, $p = 0.004$, t -test), α -phellandrene (287% increase; $t_{19,-5} = -3.41$, $p = 0.002$, t -test), 3-carene (320% increase; $t_{392,-71} = -2.97$, $p = 0.006$, t -test) and β -pinene (501% increase; $t_{84,2} = -1.95$, $p = 0.062$, t -test), with some significant oxygenated VOCs, such as geranyl acetone (ONI; 2738% increase, $t_{270,-78} = -3.74$, $p < 0.001$, t -test) and 2-octyldecanol (ONI; 707% increase, $t_{100,-16} = -2.91$, $p < 0.001$, t -test; Figure 3c).

The second RF analysis revealed that VOC blends from the ozone treatment were similar to the one from ambient rings and showed that the diesel exhaust addition was what made the clear difference between the blends, while the VOC blend of plants from $O_3 + D$ rings were positioned between the O_3 and D blends (Figure 3b). Overall, the compounds with the highest MDA in the second RF were related

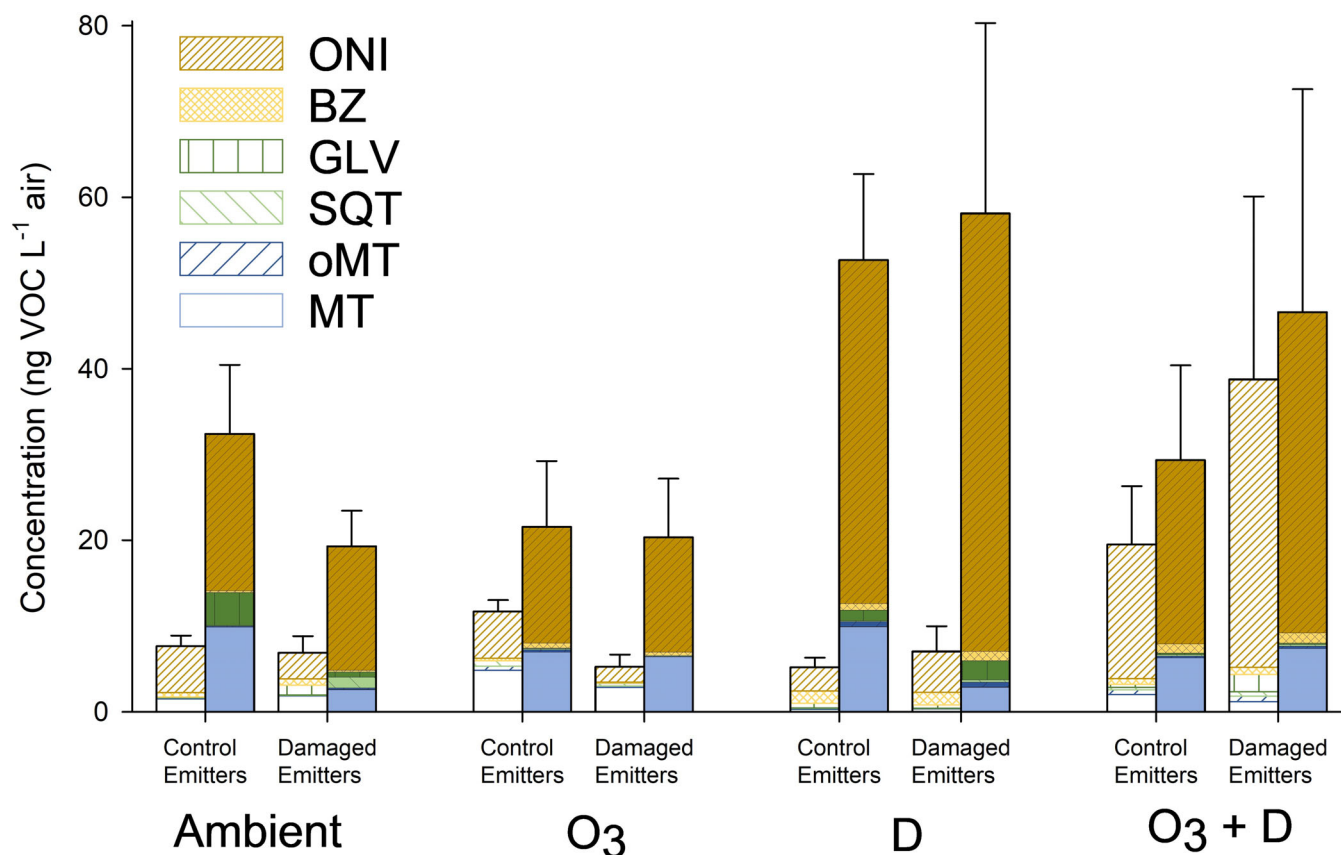


FIGURE 2 Individual and combined effects of in situ air pollution (ozone, O_3 and diesel exhaust, D) exposure on the belowground volatile organic compound (VOC) concentration of Scots pine exposed to damaged and control emitters. Damaged emitters were exposed to herbivory by 2 large pine weevils for 5 days, while control emitters were left undamaged. Filled bars represent herbivory phase of receiver seedlings (unfilled = Phase 1 (pre-herbivory), filled = Phase 2 (post-herbivory)). BZ, benzenoids; GLV, green-leaf volatiles; MT, non-oxygenated monoterpenes; oMT, oxygenated monoterpenes; ONI, other non-isoprenoids; SQT, sesquiterpenes. $n = 4$. Error bars show standard error of the mean of total VOC emissions.

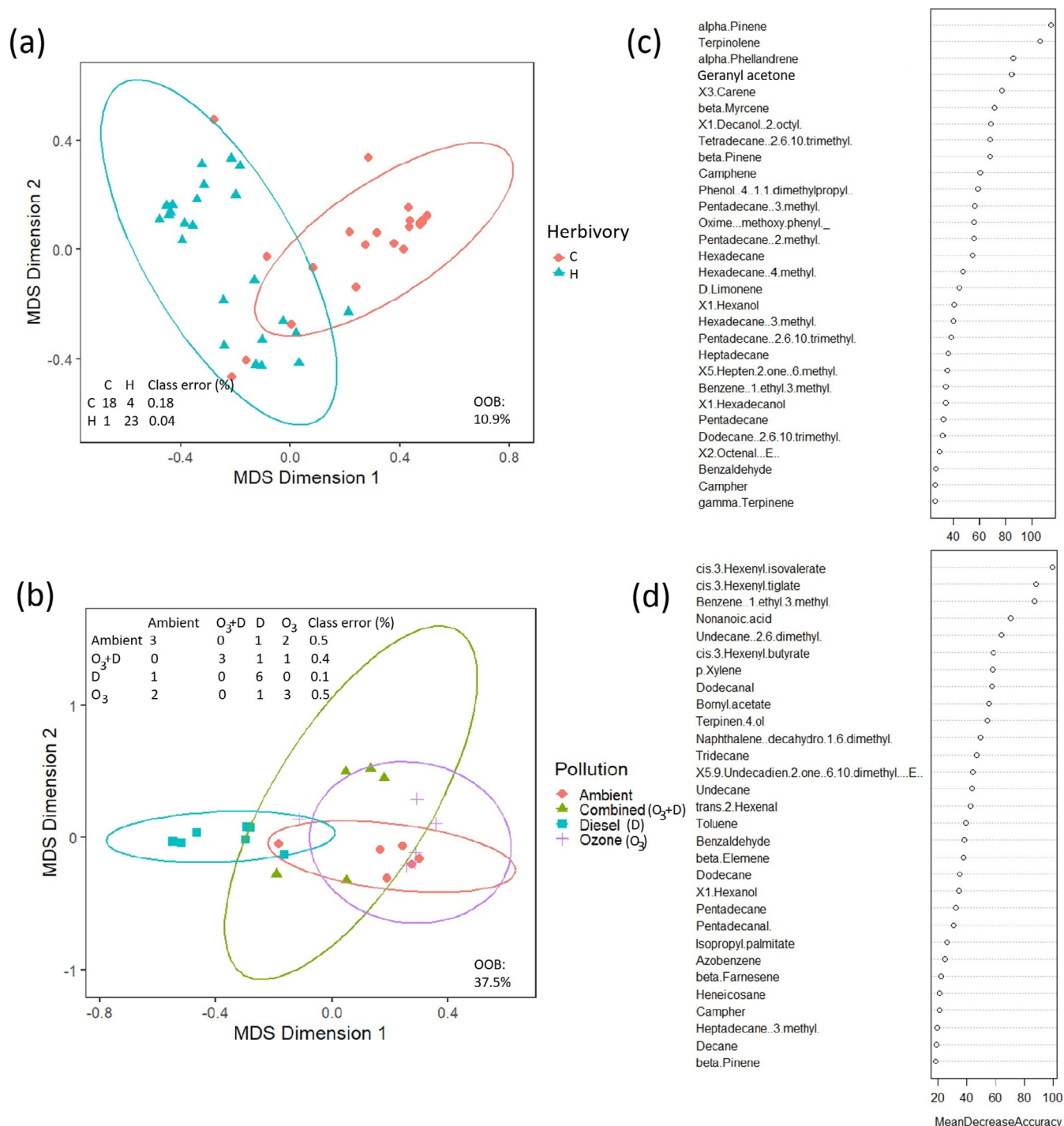


FIGURE 3 Supervised random forests (RF) analysis of belowground volatile organic compound (VOC) blends. Multidimensional scaling plots for RF on the belowground VOC blends before (C) and after damage by herbivores (H) are shown across all air pollution treatments (a), and separately between the pollution treatments after herbivory (b). The out-of-bag (OOB) classification error is in the bottom right corner. A RF confusion matrix is presented in the table within each plot. The confusion matrix shows the number of correctly and incorrectly assigned branches and the corresponding class error. VOCs with the highest mean decrease accuracy (MDA) from supervised random forests (RF) analysis of belowground VOC blends before and after herbivory across all of the air pollution treatments (c) and separately between the pollution treatments after herbivory (d).

to GLVs, such as cis-3-hexenyl isovalerate (193-fold higher in D than ambient; $t_{26} = 3.56$, $p = 0.003$, Dunnett's test), cis-3-hexenyl tiglate (sixfold higher in D than ambient; $t_{26} = 6.92$, $p < 0.001$, Dunnett's

test) and BZs, such as benzene, 1-ethyl-3-methyl- (also known as m-ethyltoluene, which was not recorded in the ambient rings, but present in D rings, $25 \text{ ng m}^{-2} \text{ h}^{-1}$ on average [Table S2]; $t_{26} = 4.81$,

$p < 0.001$, Dunnett's test) or ONIs, such as nonanoic acid (threefold higher in D than Ambient, $t_{26} = 2.52$, $p = 0.047$, Dunnett's test; Figure 3d).

3.2 | Comparison of two VOC collection methods

GCxGC-TOF-MS VOC chromatograms of VOC-mole™ samples are presented in Figure S4 and the ones taken from the soil collar are presented in Figure S5. These samples have been taken once following the interactions experiment and therefore do not reflect the treatments of direct herbivory or exposure to the damaged emitters. Due to the lack of full experimental replication, formal statistical comparison between the two sampling methods was not possible. However, both methods extracted similar compound profiles and the principal component analysis of the top discriminating ions still clearly separated the pollution treatments. Moreover, there was a log-fold higher abundance of compound peaks in samples collected using the VOC-mole™. This also included the extraction and retention of background analytes found within samples, irrespective of their experimental pollution treatment (Figures S4 and S5). Compounds such as various aldehydes (octanal, nonanal and decanal) and a ketone (1-octen-3-one) were tentatively identified using the NIST 23 spectral library. Ion abundances associated with these compounds were higher in VOC-mole™ extracts, and this was traced to background environmental effects, as most of the compounds were also found in the blank samples taken with each method (Figure S6 and Figure S7). Conversely, a high abundance of ions associated with carbon tetrachloride was found in soil collar extracts (Figure S6). This was likely the result of VOCs emitted from the plastic soil collar itself.

Ion data for each sample were processed and filtered for the top 20 discriminating ions between pollution treatments. Each replicate (one from VOC-mole™ sampling and one from soil collar sampling methods) clustered and separated clearly for these discriminating ions (Figure 4), despite differences observed in their respective Total Ion Chromatogram (TIC) intensities (Figure S8). This indicates that each respective sampling method is still able to extract these distinguishing ions in sufficient abundance. PC1 accounted for 58.5% of the variance, and PC2 explained 23.3% of the variance. The O₃ treatment showed 20 discriminating ions, separating to the left of the plot and away from the other treatments in the PCA. The combined O₃ + D treatment was most similar in the VOC blend to the control, rather than either of the single pollutant treatments.

Of the 20 discriminating ions identified, nine could be reliably associated with chromatogram peaks. Of these nine compounds, seven were tentatively identified, and two were designated as 'unknown'. Discriminating ion abundances associated with these peaks and compounds are presented in Figure S8 with their corresponding mass spectra in comparison to the top library match. Samples from rings with ambient air had a higher abundance of *p*-cymene and terpinolene ions, as well as an unknown compound (ion m/z 132). In the diesel exhaust addition rings (D) showed low abundance of the discriminating ions with the exception of β -pinene, where a greater amount was observed compared to the other treatments and the ambient air. In the O₃ rings, higher ion abundances were associated with an unknown compound (m/z 54), norbornane 2,2-dimethyl-5-methylene, γ -terpinene and styrene. Lower abundance of ions associated with *p*-cymene was also observed for this treatment. In the samples from O₃ + D rings, we did not find any significant differences amongst the identified discriminating ions.

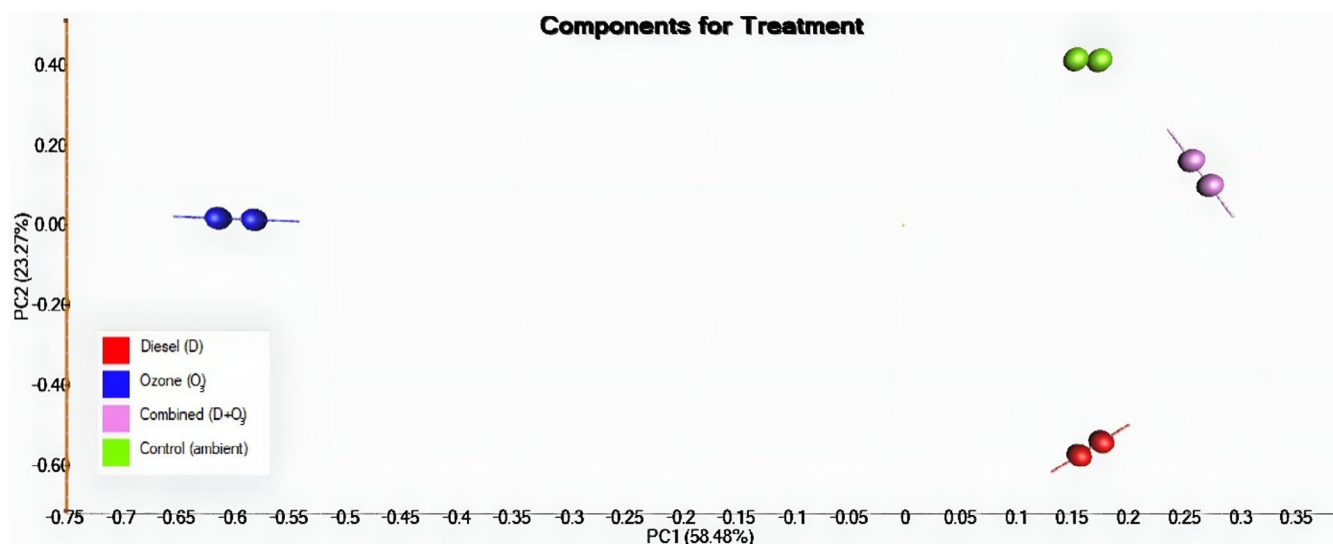


FIGURE 4 Principal component analysis (PCA) of top 20 discriminating ions between plant soil volumes under four treatment conditions: control (ambient atmosphere; green), diesel exhaust addition (red), ozone addition (blue) and combined diesel + ozone addition (pink). Each of the two samples per treatment represent one VOC-mole™ sample and one collar sample.

4 | DISCUSSION

4.1 | Effect of aboveground herbivory

In the first part of our study, we demonstrated that aboveground feeding by the large pine weevil substantially increased belowground VOC concentration in Scots pine. The increase was significant for total VOC concentration and individual VOC groups.

This finding is in contrast to the field-based work of Rasheed et al. who found no significant effect of aboveground pine weevil herbivory on pine rhizosphere VOC emission (Rasheed et al., 2017, 2021). This difference could be due to the use of different methods to collect the VOCs. While Rasheed et al. collected VOCs by removing the seedling from the pot, gently removing the soil and enclosing the whole rhizosphere in a PET bag, we placed the bags around the plastic soil collar previously installed next to the seedlings in the field, a method previously used by (Maja et al., 2014) to collect rhizosphere (belowground) VOCs of birch trees, which is inherently less invasive than removing the seedlings from the soil.

It is well known that aboveground VOC emission increases after herbivory as a result of mechanical damage (Laotawornkitkul et al., 2009; Ninkovic et al., 2019). Indeed, herbivory has been shown to significantly increase the aboveground VOC emissions in field experiments on tundra plants (Li, Holst, et al., 2019; Rieksta et al., 2020, 2021), boreal evergreen trees (Ghimire et al., 2016; Heijari et al., 2011; Kivimäenpää et al., 2016) and others and play important roles in biosphere–atmosphere interactions (Holopainen & Blande, 2013). Higher shoot VOC emission was consistently reported for Scots pine after feeding by the large pine weevil (Heijari et al., 2011; Simin et al., 2026; Tiiva, Häikiö, and Kasurinen, 2018; Yu et al., 2022, 2024). In addition to the bark, the large pine weevil readily feeds on roots of pine seedlings (Yu et al., 2024), and the results of our study suggest that changes in concentration of VOCs extend to the rhizosphere, as part of the systemic approach for protection against herbivory.

As the second most-emitted VOC group, MTs played an important part of the total belowground VOC profile in our study, which is not surprising considering that MTs are the main VOC group emitted from the aboveground part of the plants (Simin et al., 2026; Yu et al., 2022, 2024; Yu & Blande, 2021). Notably, the belowground (rhizosphere) VOC profile of Scots pine is dominated by MTs in studies where authors removed the whole root system of the seedlings, enclosed it and left the VOCs to accumulate before collection (Rasheed et al., 2017, 2021; Yu & Blande, 2021). Therefore, it is possible that the pine roots are the main contributors to MTs in the belowground VOC profile in our study, an idea generally supported by the work of Lin et al. (2007) on the Mediterranean stone pine and Scots pine and the work of Mäki et al. (2019) who concluded that belowground VOCs from Scots pine forest floor (rich in MTs and SQTs) collected using a soil collar method similar to ours were likely derived from roots. Previous studies have shown that aboveground herbivory by large pine weevils increases the relative proportions of thicker roots in the Scots pine root system (Rasheed

et al., 2017; Tiiva, Julkunen-Tiitto, et al., 2018). Indeed, following herbivory, we have observed a 246% increase in total MT concentrations and a shift in the VOC blend towards higher concentrations of MTs, such as α -pinene, terpinolene, α -phellandrene, 3-carene and β -myrcene, seen in our RF analysis (Figure 3c), which suggests rapid root growth in response to herbivory and implies a shift of carbon resources to belowground VOC concentrations sustained at least 36 h after herbivory (Jiao et al., 2023; Yang et al., 2024).

Conversely, MTs serve as an important carbon source for soil microorganisms, such as *Pseudomonas* or *Rhodococcus* bacteria, which can mineralise almost all isoprenoids synthesised belowground (Marmulla & Harder, 2014). *Pseudomonas* are common endobacteria of ectomycorrhizal root tips in Scots pine (Izumi et al., 2006). This bacterial consumption in the soil, along with physical and chemical abiotic properties of the soil may explain why we observed much lower proportion of isoprenoids (17% MTs, 2.5% oMTs and SQTs) compared to non-isoprenoid compounds (4% BZs, 3.5% GLVs and 74% ONIs) in the total VOC blend in our interactions experiment.

Tiiva, Häikiö, and Kasurinen (2018) also show that short-term herbivory increases mycorrhizal root colonization rates of Scots pine seedlings, a result generally supported by a review paper on the topic (Barto & Rillig, 2010). Fungal VOCs are composed mostly of carbonyl compounds and open-chain hydrocarbons (Guo et al., 2021), which fit our ONI group and indicate that at least some of the increase we observed could be attributed to fungal VOCs. For example, we have detected 2-ethylhexanol in our VOC blend, a compound released by *Tomentellopsis zygodesmoides* mycorrhizal fungus of Scots pine that improves plant defense and growth (Guo et al., 2021). Moreover, geranyl acetone, an ONI, had one of the highest MDA scores in our RF analysis (Figure 3c), meaning that, following herbivory, the total VOC blend contained more of this fungal compound, which was found to have anti-attractant properties for *Ips typographus* (Kännaste et al., 2026), the European spruce bark beetle, another herbivore of Scots pine, so it could have a similar effect on the large pine beetle. VOCs from mycorrhizal fungi can promote plant development, protection and tolerance to biotic and abiotic stress and promote the beneficial soil microbiome (Miñambres et al., 2026).

It is also important to consider that the VOCs we detected might have come from the soil itself, as a result of physicochemical processes and heterogeneous oxidation of the organic substrate (Li, Cheng, et al., 2019).

Finally, belowground VOC profiles of plants are often different from aboveground ones (Peñuelas et al., 2014). In the present experiment, we did not detect a significant effect of exposure to damaged emitters on belowground VOC concentrations. This may reflect the greater complexity of the soil environment, where physical sorption, chemical oxidation and microbial consumption can rapidly modify or attenuate VOCs, thereby reducing the detectability of potential plant–plant signals. Targeted laboratory studies examining root growth, mycorrhizal colonisation and microbial community composition would be valuable for revealing any subtler belowground effects of neighbouring plant damage.

4.2 | VOC collection methodology

The first study to investigate collecting rhizosphere VOCs from pine was published by Lin et al. (2007), which compared active methods of collection (by removal of seedlings from the soil and putting the rhizosphere in the PET bag) to passive ones (where an adsorption cartridge was buried soil wrapped in a cotton bag at differing distances from the tree). Our study used different methods and the comparison of the soil collar and VOC-mole™ methods revealed both to be effective for collecting belowground VOCs, with comparable profiles.

According to our observations, there may be some trade-offs between using the two VOC extraction methods. The VOC-mole™ offers the ability to extract compounds in greater abundance, as VOCs are more concentrated below the soil surface (compared to the air). This is beneficial for collecting greater amounts of low abundance compounds, but it also captures low abundance background environmental compounds, rather than just plant-derived metabolites. This may be useful in itself to develop environmental profiles, but it could confound interpretations between experimental locations if there is a large degree of variability between sampling sites. Awareness of such compounds will allow them to be characterised and omitted from interpretations so that they will not be confused with effects generated by the experimental treatments.

The soil-collar approach is by comparison nondestructive (in that there is no chance of root tissues being damaged in the process), but it does not achieve as high yields of VOCs compared to the VOC-mole™. It may also be prone to sampling contamination, as evidenced by the presence of significantly higher amounts of carbon tetrachloride, for example; but these can be omitted from data and interpretations if identified by collecting appropriate blanks and control samples.

The analysis of discriminatory ions between the sampling methods shows that each was capable of extracting the related compounds and provided a means of separating the experimental treatments using chemometrics. The choice of sampling method may therefore be based more on practical considerations, such as availability of resources, soil type, plant species and plant size. Researchers should select the method based on experimental needs, with the soil collar preferred for nondestructive and low-disturbance sampling in shallow soils and the VOC-mole™ for deeper, precise collections.

4.3 | The effect of air pollution on belowground VOC concentrations

We have taken blank samples from a container without Scots pine seedlings that was placed in one of the ambient air (control) rings and, therefore, pollutants originating from diesel exhaust (D) and/or ozone (O₃) addition that deposited from the air were considered in all belowground VOC profiles. Chromatograms from soil collar VOC blanks and from belowground Scots pine VOCs in different pollution rings (Figure S1) show that the ion count (and the VOC concentration on which it was calculated) was higher in our samples compared to the

blanks and higher in D and D + O₃ rings compared to ambient air and O₃ rings (Figure 2, Figure S1). During our experiment, air NO_x concentrations in all FADOE rings (and especially in D rings) exceeded the measurements from the centre of the town of Reading in England, while only in O₃ and O₃ + D rings did the O₃ exceed the local concentrations (Mofikoya et al., 2024; Simin et al., 2026). Oxidation of soil by O₃ addition has been shown to be an effective way to reduce VOC concentrations in soils contaminated with Benzene, Toluene, Ethylbenzene and Xylene (BTEX; Jousse et al., 2017). Therefore, it is possible that these processes affected our Scots pine belowground VOC concentrations.

We have observed clear effects of air pollution on BZ and oMT concentrations and an interaction between herbivory and pollution for most groups. The effect of air pollution on monoterpene synthesis in pines is known, and it has been shown that these compounds become elevated in response to different treatments. It has likewise been observed that there are inverse correlations between some monoterpene and aromatic compounds (Bakó et al., 2024). As highlighted in a recent review (Kopaczky et al., 2020), the reasons why some monoterpenes become elevated in response to certain types of pollution and not others are not well understood and that pollution may form a distinct monoterpene 'fingerprint' in affected trees.

The effects of air pollution on VOC blends presented in this study differed between the different parts of this work. While VOCs for the interactions experiment were collected before and immediately following herbivory, the collection for the method comparison occurred after the experiment and when the effect of herbivory had stabilised. It has been previously suggested that O₃ pollution mimics the effect of herbivores, such as aphids, on plant VOC blends (Vuorinen et al., 2004), which could be the reason why O₃ was the most important pollution factor in the methods comparison section using PCA. Furthermore, in the interactions experiment using RF we first see the separation of VOC blends before and after herbivory and when looking at the blends of VOCs following herbivory, diesel exhaust becomes the dominant factor that influenced the VOC emissions. This might have been the case for compounds with high MDA in our RF, such as *m*-ethyltoluene. However, the substantial increase in the emission of GLV-related VOCs, such as *cis*-3-hexenyl isovalerate and *cis*-3-hexenyl tiglate in diesel exhaust enrichment rings was likely due to damage to pine seedlings (Ameje et al., 2018) by reactive products of diesel fuel combustion, for example, nitrogen oxides (NO_x).

β -Pinene is a MT found ubiquitously in pine tree species. Such compounds are typically produced by plants as a defence mechanism against herbivory or infections (bacteria and fungi) but are also produced in response to abiotic stress (Escobar-Bravo et al., 2023). We observed that diesel pollution elicited a significant increase in *m/z* 93 ions associated with β -pinene, suggesting that this triggers an abiotic response in the plant. It is interesting to note that this effect was not observed in the combined diesel + ozone pollution treatment (Figure S8); however, another monoterpene, sabinene (*m/z* 81), was elevated in this treatment. The O₃ treatment alone elicited a significant increase in ions associated with γ -terpinene (*m/z* 80), which

indicates there is a sophisticated and modulated defense response according to the type and combination of pollutants that trees are exposed to.

5 | CONCLUSIONS

Herbivory by the large pine weevil on Scots pine increased below-ground VOC concentrations, revealing a systemic defence mechanism in the plant. This response has broader implications for carbon balance and atmospheric chemistry. Both soil collar and VOC-mole™ methods proved effective, providing practical tools for field-based studies. Moreover, diesel exhaust and ozone exposure elicited distinct changes in VOC concentrations, reflecting stressor-specific monoterpene responses. These findings emphasise the urgent need for broader rhizosphere research to understand forest responses to environmental stressors. Our study provides two practical methods for this purpose.

AUTHOR CONTRIBUTIONS

Conceptualization: Tihomir Simin, Luke Bell, James D. Blande, Robbie D. Girling and James M. W. Ryalls. **Methodology (fieldwork):** Tihomir Simin, Oliver E. I. Welling and James M. W. Ryalls. **Methodology (maintenance of the field site):** Neil J. Mullinger, James M. W. Ryalls, Robbie D. Girling and Tihomir Simin. **Methodology (data analysis and visualisation):** Tihomir Simin and Luke Bell. **Writing—original draft:** Luke Bell, James M. W. Ryalls and Tihomir Simin. **Writing—review and editing:** Tihomir Simin, Luke Bell, Muhammad Usman Rasheed, Oliver E. I. Welling, James M. W. Ryalls, Robbie D. Girling, Neil J. Mullinger and James D. Blande.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

Data obtained during fieldwork and used for statistical analysis and generating figures for this article are available in Zenodo online repository: [10.5281/zenodo.22092622](https://doi.org/10.5281/zenodo.22092622).

ORCID

Tihomir Simin  <https://orcid.org/0000-0002-3914-500X>

Robbie D. Girling  <https://orcid.org/0000-0001-8816-8075>

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