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RESEARCH ARTICLE

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A laboratory high-throughput glass chamber using dynamic headspace TD-GC/MS method for the analysis of whole *Brassica napus* L. plantlet volatiles under cadmium-related abiotic stress

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Abstract

Introduction: The dynamic headspace sampling technique using thermal desorption, gas chromatography-mass spectrometry (TD-GC/MS) is a powerful method for analysing plant emissions of volatile organic compounds (VOCs), and experiments performed in sterile and controlled conditions can be useful for VOC metabolism investigations.

Objective: The main purpose of this study was to set up a laboratory high-throughput glass chamber for whole plant volatiles analysis. *Brassica napus* L. plantlets were tested with the developed system to better understand the relationship between low emission of induced terpene and cadmium (Cd)-related abiotic stress.

Methodology: VOCs emitted by 28-day-old *Brassica napus* L. plantlets cultivated *in vitro* were trapped with our device using adsorbent cartridges that were desorbed with a thermal desorption unit before cryofocusing with a cooled injection system and programmable temperature vaporising inlet into an HP-5 ms GC column. Terpene detection and quantitation from chromatogram profiles were acquired using selected ion monitoring (SIM) mode during full scan analysis and mass spectra were obtained with a quadrupole-type mass spectrometer.

Results: The new trapping method produced reliable qualitative profiles of oilseed rape VOCs. Typical emissions of monoterpenes (myrcene, limonene) and sesquiterpenes (β-elemene, (E,E)-α-farnesene) were found for the different concentrations tested. One-way analysis of variance for quantitative results of (E,E)-α-farnesene emission rates showed a Cd concentration effect.

Conclusion: This inexpensive glass chamber has potential for wide application in laboratory sterile approach and replicated research. Moreover, the non-invasive dynamic sampling technique could also be used to analyse volatiles under both abiotic and biotic stresses.

KEYWORDS

abiotic stress, TD-GC/MS, terpenoids, VOC trapping

1 | INTRODUCTION

Volatile organic compounds (VOCs), representing about 1% of plant secondary metabolites with around 1700 substances, mediate plant semiochemistry and are involved in abiotic stress responses.[1,2] Strategies for collecting dynamically plant-emitted VOCs depend hugely on recent advances in sampling methods and commonly target plant organs such as limb, leaf or flowers. The technique of volatile analysis poses analytical challenges and must be adapted either to laboratory or field experiments. Portable gas chromatography/mass

spectrometry (GC/MS) represents the best compromise for studies investigating both biotic and abiotic stresses in field research.[3] The isoprene molecule (C_5H_8) has been extensively studied with real-time volatile collection methods using proton transfer reaction/mass spectrometry (PTR-MS) technology.[4] Conversely, *in vitro* headspace analysis in connection with abiotic stress studies is usually performed under laboratory conditions. A number of studies described in recent literature have used methods involving plant biological sample destruction such as volatile extraction under air-dried conditions at 40°C for 72 h, or hydrodistillation performed from cut plant materials.[5,6] We therefore decided to develop a glass collection chamber that would not harm the plant during its *in vitro* physiological development or during the VOC sampling procedure. Another goal for this innovative *in vitro* system was to use the plant's clonal regeneration ability, leading to genetically stable propagation. Another advantage of this method is that it enables VOCs' putative phenotype biomarkers to be detected as soon as possible during plant growth and under abiotic stress without hurting the plant. Our sampling method is used over a 24-h period in order to take account of plant's circadian emission rhythms.[7]

There are many techniques for studying leaf reservoirs of stored VOCs, and practical approaches to investigating plant volatiles have been reviewed by Tholl *et al*.[8] More recently, microwaves or supercritical fluid extraction (SFE), often combined with solid-phase microextraction, have been suggested for plant volatile analysis under laboratory conditions.[9] However, these techniques are highly time-consuming and destructive, and frequently depend on the equilibrium of the sample and the headspace. The system described in our study is of particular interest for the analysis of volatiles emitted in sterile conditions and without any stress disturbance. Moreover, volatiles freely emitted from plants are dynamically trapped without any other laboratory manipulation (in comparison with solvent extraction methods) and after only a few days of plant growth under controlled and defined conditions.

VOC sampling can either be static, using a solid-phase microextraction (SPME) device providing semi-quantitative information, or dynamic, using continuous airstream flows within glass cuvette chambers coupled with adsorbent material. As the major purpose of the present study was to create an innovative design for the analysis of whole plantlet volatiles, a dynamic headspace thermal desorption, gas chromatography-mass spectrometry (TD-GC/MS) method using Tenax® TA adsorbent cartridges was developed. Adsorbent material selection is always one of the critical steps of the procedure. Large numbers of molecules of different chemical natures, molecular weights and polarities have to be adsorbed jointly, quantitatively (i.e. without any breakthrough) and reversibly, if possible in a single "sampling run". GC/MS can then be used to separate and detect volatile compounds trapped dynamically on adsorbent material, which makes it a suitable technique to study whole plant terpene emissions under controlled conditions.[10,11] Moreover, the use of a thermal desorption unit (TDU) avoids any use of solvents to extract volatiles from the sorbent, while the cooled injection system (CIS) concentrates samples by cryofocusing before their injection into the GC column.[12,13] Finally, selected ion monitoring allows analyses with higher sensitivity and improved signal-to-noise ratios as it removes much of the matrix noise.[14]

Volatile terpenes are the most important compounds in plant VOC emissions in terms of functional diversity and plant protection. They can be constitutive and highly controlled by genetic and environmental factors. Emission rates vary with genotype (variety for example), season and physiological development of the plant.[15] Moreover, emissions can be induced by abiotic stresses (temperature, light, drought, salt, ozone and UV-B radiation). Plant's constitutive VOCs are well known to be stored within internal structures such as resin ducts (pines) and glandular cells (Lamiaceae) or within external structures such as glandular trichomes (Lamiaceae, Solanaceae).[7] These compounds are synthesised via two interconnected isoprenoid pathways within the plant cell: the formation of homoterpenes, sesquiterpenes and triterpenes from cytosolic mevalonic acid (MVA) and the formation of hemiterpenes, monoterpenes, diterpenes and tetraterpenes from chloroplastic 2-C-methyl-D-erythritol 4-phosphate (MEP).[16,33] Flowers produce large numbers of terpene volatiles involved in the attraction of pollinators[17] and of seed-disperser.[18]

However, many plant species also emit constitutive VOCs from their foliage without any storage pool structure, depending on factors such as the plant's phenological stage, ambient temperature and light intensity. Our system allows VOCs to be trapped at a wide range of plant development stages, e.g. flowering. VOCs such as monoterpenes and sesquiterpenes are lipophilic compounds with high vapour pressures which cross membranes freely. They can therefore be directly emitted in the plant's biosphere depending on abiotic or biotic factors. Their release is greatly restrained in normal conditions due to the plant's carbon balance, with the apparent exception of sesquiterpenes.[19] The optimisation of the sample chamber, the design of the sampling lines and the adsorbent properties of the cartridges used can improve the experimental recovery of these sesquiterpenes.[9] However, leaf stomatal closure resulting from abiotic stresses such as temperature may prevent volatile transmission. Emitted constitutive monoterpene and sesquiterpene VOCs have been shown to serve as non-destructive markers of phenotypic abiotic stress using measurements under sterile and controlled conditions.[15] The present study was therefore designed to develop a sensitive sampling-analysis technique that would make two things possible: firstly, the investigation of large numbers of *in vitro* replicates with the guarantee of genetic stability, thus overcoming any response plasticity, and secondly, the measurement of minute amounts of secondary volatile emitted products such as putative abiotic stress biomarkers. The system developed was tested and applied to clonal oilseed rape *Brassica napus* L. regenerated plants at the vegetative stage using *in vitro* axillary shoot branching method.

Brassica napus L. is a worldwide crop of high interest as one of the main sources of oil and protein for food and feed or biofuel production.[20] Monoterpenes and sesquiterpenes emitted from oilseed rape in the field were first investigated as potential causal irritation agents leading to human allergic reaction.[21,22] Higher emissions of monoterpenes (α-thujene, sabinene, myrcene and limonene) have also been observed during the flowering stage of oilseed rape[23] and the effect of nitrogen (N) fertilisation on bud and flower volatile bouquets has been demonstrated under laboratory conditions with higher emission of several monoterpenes at increased N dosages.[24] A multivariate study demonstrated that high concentrations of carbon dioxide (CO_2) related stress (720 µl/L) lead to enhanced emissions of α-thujene, sabinene, limonene, 1,8 cineole and γ-terpinene for *Brassica napus spp. oleifera* at the vegetative stage.[25] Most previous investigations of *Brassica napus* L. VOCs have involved the collection of the floral

scent. Floral volatile plasticity may be environmentally and genetically based and any evolution may be mediated by pollinators.[17] Push–pull or multi-chamber cuvette systems described in the literature in connection with investigations of oilseed rape at the plant–vegetative stage only take parts of the plant into account for VOC capture. For example, several abiotic stress studies have been performed under field conditions using the panicle of the main raceme,[24] using foliage in order to compare VOC emission in relation to different soil types,[26] or using foliage of CO_2-stressed oilseed rape plants.[25] The initial step of our research was therefore to set up an innovative glass VOC trapping system in which whole oilseed rape plantlets could grow under sterile, controlled and strictly defined conditions. When experiments are performed effectively using whole plantlets or plants, all constitutive or induced VOCs emitted can be trapped. The TD-GC/MS method was optimised so that volatiles could be actively trapped on adsorbent material at any moment.

The impact of abiotic stresses such as temperature, drought, ozone and UV-B radiation on terpene emission in oilseed rape is poorly understood. Winter *et al*.[27] reported that heavy metal stress has a strong influence on terpene emissions. Moreover, diffuse cadmium (Cd) sources, notably phosphorus (P)-fertilisers and atmospheric deposits, can contribute to the concentration of Cd in agricultural soils,[28] and Cd as a hazardous pollutant can lead to inhibition of plant growth processes and to chlorosis (decreasing of photosynthetic activity).[29] Cadmium stress-induced VOC profiles were therefore investigated in order to study the relationship between this specific stress and emissions from winter oilseed rape plantlets. Finally, this sterile laboratory high-throughput system revealed an easy-to-use method for *in vitro* plants regeneration ensuring genetic stability with advantages for studying the impact of one stress at a time without other disturbances to the plant's secondary VOC metabolism.

2 | MATERIAL AND METHODS

2.1 | Plants

Winter oilseed rape (*Brassica napus* L. var. *Es Astrid*) shoots grown from germinated seeds were propagated *in vitro* using genetically very stable axillary branching proliferation and voucher specimens (n°0312) held at the Walloon Agricultural Research Centre (Belgium). Twenty-eight-day-old plantlets were obtained after 14 days of a hermetic *in vitro* preliminary rooting phase and 14 days acclimation growth of shoots under sterile culture conditions. After the plantlets' growth and leaf development had been tested, two standardised shoots were cultivated in each glass chamber. The culture medium consisted of 400 mg/L NH_4NO_3; 800 mg/L KNO_3; 300 mg/L $Ca(NO_3)_2$; 180 mg/L $MgSO_4$; 150 mg/L KH_2PO_4; 1.5 mg/L $MnSO_4$; 0.5 mg/L $ZnSO_4$; 3 mg/L H_3BO_3; 0.5 mg/L KI; 0.25 mg/L Na_2MoO_4; supplemented with 20 mg/L Na_2EDTA and 15 mg/L $FeSO_4$. Sucrose (3%) and 0.5% dehydrated powdered agar Pastagar B (Biorad, Temse, Belgium) as a strong gelling agent were added to the culture medium. In parallel to three controls (0 μM of Cd), cadmium abiotic stress ($CdCl_2$) was added (Sigma-Aldrich, Diegem, Belgium) to three culture media at 5 μM (low stress corresponding to 0.56 ppm in soil), three at 15 μM (mild stress corresponding to 1.68 ppm in soil) and three at 45 μM (severe stress corresponding to 5.04 ppm in soil). The oilseed rape was cultivated under controlled environmental conditions at 23°C:18°C (day:night), a long-day photoperiod of 16 h, 45% relative humidity and 10 μmol/m^2/s PAR (photosynthetically active radiation). The entire experiment was repeated five times. Phenotyping consisted of leaf symptom observation, and phenotypic results consisted of the two plantlets' growth (in millimetres) and fresh weight biomass (in grams) data recorded after VOC collection.

2.2 | Volatiles trapping system

An open enclosure glass system was developed based on existing gas wash Drechsel® bottles without filter disc of 500 mL capacity [borosilicate 3.3 glass bottle and head, overall height of 275 mm, standard ground joint neck size of 29/32, hose nozzles with outer diameter of 11 mm (Brand, Wertheim, Germany)]. These bottles were manually modified by cutting away the glass tube in order to allow the growth of two oilseed rape plantlets under Cd-related abiotic stress and the collection of volatile terpenes (Figure 1). Clean sterile air (filtered via activated charcoal and Acro™ 37 TF 0.2 μm PTFE) was supplied continuously to the homemade system by a portable diaphragm pump with 2.4 bar operating pressure and 6 L/min delivery (N86 KN.18, KNF, Neuberger, Germany) connected with Teflon® and connection tubes. Blank tests (bottles with culture medium only) and the trapping of VOCs from 28-day-old plantlets were performed using Tenax® TA (Supelco, Bellefonte, PA, USA) adsorbent cartridges (Gerstel, Mülheim an der Ruhr, Germany) in order to minimise water vapour collection (Figure 2). Tenax TA is a porous polymer (2,6-diphenylene oxide) commonly used for trapping volatile and semi-volatile compounds from C_7 to C_{26}. The cartridges were connected to the cuvette system with swageloks® stainless steel links for a sampling time of 24 h at a flow of approximately 0.200 L/min (calibrated using air flow calibrator TSI™ model 4199). No volatile compound breakthrough was observed using sampling conditions after analysis of a second cartridge placed after the first one. All cartridges were preliminarily conditioned at an elevated temperature of 280°C for 10 h under a flow of N (ultra-high purity grade, Air Liquide, Liège, Belgium) independently of the analytical system using a tube conditioner TC 2® (Gerstel). The cartridge cleaning protocol was checked with blank analyses on these conditioned cartridges. Before use and after all conditioning procedures, the cartridges were stored directly in a Gerstel Twister® rack suitable for manual operations and capable of storing up to 15 cartridges. Five repetitions of volatile collection were performed successively using micropropagated oilseed rape clones and in triplicate for each Cd concentration (0 μM, 5 μM, 15 μM and 45 μM).

2.3 | TDU/CIS coupled to GC-MS profile analysis

Each sample was loaded and injected using a MultiPurpose Sampler (MPS). The VOCs were firstly thermally desorbed from Tenax TA cartridges with a TDU (Gerstel) running in splitless mode from 40°C to 120°C (110°C/min) in order to prevent thermal degradation and for 2 min, and then at 280°C (200°C/min) for 5 min. Cryofocusing with a programmable temperature vaporising inlet (CIS4/PTV inlet) was performed at −10°C before injection into the GC column by heating the CIS/PTV inlet to 260°C for 5 min at a rate of 12°C/s. VOC separation was

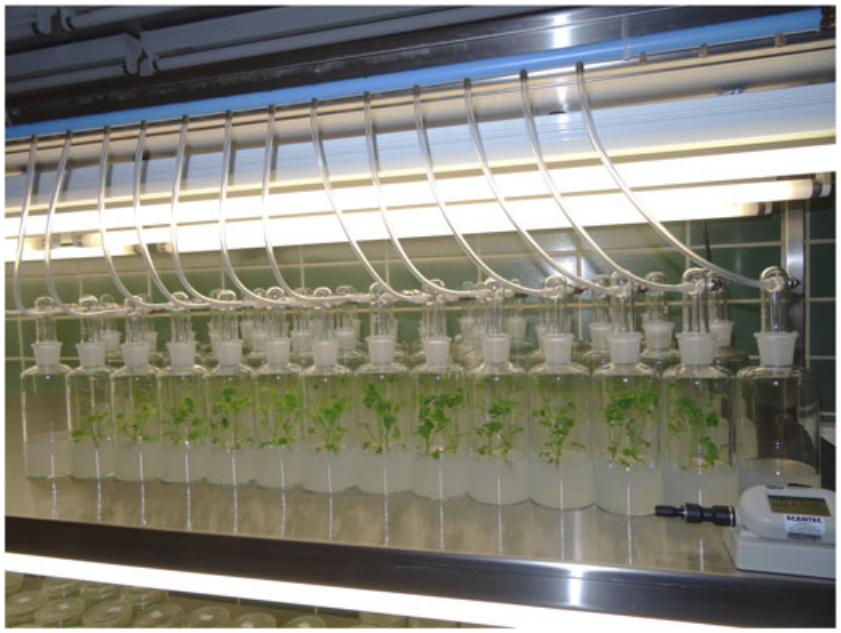

FIGURE 1 Picture of the laboratory glass chamber system allowing the *in vitro* growth of *Brassica napus* L. plantlets and the trapping of VOCs under cadmium-related abiotic stress [Colour figure can be viewed at wileyonlinelibrary.com]

performed using GC (7890A; Agilent Technologies, Palo Alto, CA, USA), with an HP-5 ms capillary column (30 m length × 0.25 mm internal diameter × 0.25 μm film thickness; Agilent Technologies). High-purity helium (Air Liquide) was used as the carrier gas at a constant flow of 1.6 mL/min. The oven temperature programme started at 40°C with an heating rate of 4°C/min to 90°C and followed by a heating rate programme at 20°C/min to 300°C with a final hold for 5 min at this temperature.

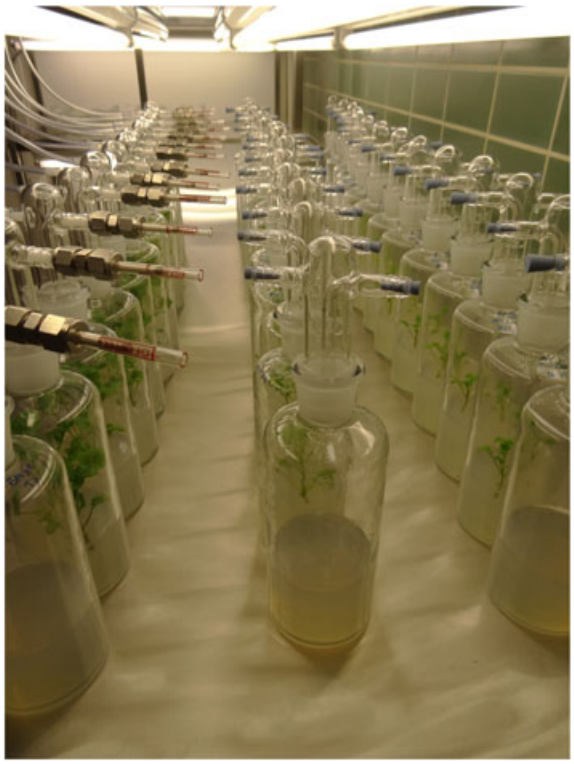

FIGURE 2 Picture of the high-throughput dynamic headspace sampling technique using Tenax® TA cartridges connected to the cuvette system with swageloks® stainless steel links for a sampling time of 24 h [Colour figure can be viewed at wileyonlinelibrary.com]

2.4 | Identification of volatile organic compounds (VOCs)

VOC detection was performed using a quadrupole-type mass spectrometer (MS 5975C; Agilent Technologies). Mass spectra were obtained using electron impact mode (70 eV) and operated in SCAN mode with a range of 35 to 350 amu for *m/z* ratios. Both selected ion monitoring (SIM) (with only ion 93 recorded) and full-scan modes were used in the same run of 28 min. MS was performed with a source temperature of 230°C and a quadrupole temperature of 150°C. GC/MS data were analysed using the Agilent MSD Chemstation E 02.00.493 (Agilent Technologies). Identification of emitted terpenes was performed by comparing the data with a Wiley 275 mass spectral database and further confirmed by comparison to retention times and fragmentation patterns of commercially available analytical standards for myrcene, β-elemene and (E,E)-α-farnesene (Sigma-Aldrich, Diegem, Belgium). Kovats indices were also calculated using saturated n-alkanes (C7–C30) standard solution (Sigma-Aldrich) on the HP-5 ms column. SIM mode was used for quantification, based on terpene's most representative *m/z* 93 ion response with a dwell time set at 100 ms. In this way representative single-ion peaks with respective relative abundance of myrcene (23.03%), β-elemene (7.29%) and (E,E)-α-farnesene (9.45%) were integrated and compared with the equivalent single-ion response of 1 μL of hexane solution containing an internal standard of octylbenzene (0.5 mg/mL) (2.69%) (Sigma-Aldrich). The internal standard was injected directly on Tenax TA cartridges with a 10 μL Hamilton gastight syringe, and adjusted chromatograms with a 3-min solvent delay were used to remove hexane. The terpenoid emission rate was calculated as pg/g/L of fresh weight plantlet and air extracted.

2.5 | Statistical analysis

Statistical analysis was carried out with Minitab® package version 17 and all data sets were tested for normality and equality of variances. Phenotypic results for growth and fresh weight biomass for 28-day-

old oilseed rape plantlets were analysed using one-way analysis of variance (ANOVA) followed by a *post hoc* Dunnett's 95% confidence intervals test comparing the mean of the control group (0 μM of Cd) with the mean of every other group (5 μM, 15 μM and 45 μM of Cd). The values are reported as means with standard error for all results. One-way ANOVA was also used to test the impact of the Cd concentration factor on (E,E)-α-farnesene emission rates followed by a *post hoc* Tukey range test to find significant difference among pairwise means.

3 | RESULTS AND DISCUSSION

3.1 | Volatile collection system set-up

The developed open enclosure glass system successfully enabled two whole winter oilseed rape plantlets to be grown *in vitro* in sterile and controlled conditions. The plantlet growth was achieved inside the glass chamber without biotic stresses such as moisture, as was confirmed by clean culture media at the end of all experiments. The main advantages of the system are that it allows whole plant VOCs to be trapped and is a simple yet high-throughput system. The dynamic headspace sampling method using TD-GC/MS produced reliable qualitative data for each triplicate of the control and of each Cd concentration tested (5 μM, 15 μM and 45 μM). This very inexpensive volatile sampling cuvette is a useful tool for collecting complete whole plant VOCs at defined and repeated intervals under sterile and controlled conditions. The plant choice can be adapted to the purpose of the study, suggesting that this new system represents, for example, a non-invasive opportunity for quality checking of aromatic plants on the basis of the terpenes typically emitted.

This innovative method can also be used in order to screen putative abiotic or biotic phenotypic plant indicators. The capacity of the bottle (using several volumes) can also be adapted to a large numbers of plants or to further *in vitro* culture research. It can also be used to follow VOC emission at different developmental stages in numerous plant species, or, for example, to study the considerable quantities of volatiles emitted by flowering plants. Finally, other substrates such as soil (inside the bottles) could be used as an alternative model of plant growth, leading to new possibilities for investigation. The most recent literature describes *in vitro* studies of VOCs using SPME techniques leading to difficult quantitative interpretations[6] or using destructive methods such as incubation of the harvested tissue at a non-biological temperature followed by headspace analysis.[5] The glass chamber set-up was based on preliminary tests focusing on the size of the cuvette system, the development-age pattern of the plantlets (i.e. their ability to emit VOCs), the optimal number of plantlets to use (showing a perfect foliage development without disturbance) and the flow rate applied into the developed system.

All these points can be crucial for accurate recording of plant VOC emissions. Unappropriated gas flow inside the cuvette due to the use of an incorrect volume can lead to air stagnation, CO_2 depletion and condensation that disrupt the trapping of VOCs on adsorbent material.[8,15] It was also decided to push and not pump the filtered air across our system between experiment repetitions to avoid any memory effects of Teflon lines and silicone tubing. Our expertise in trapping plant VOCs was experimentally based, and we tested numerous combinations in terms of the number and the physiological age of the oilseed rape plantlets as this affects the emission of volatile terpene. The most relevant profiles were obtained when analysis was performed for 24 h using two 28-day-old plantlets with well-developed foliage. The extent of plant community growth and associated specific microclimate, the foliage atmosphere and circadian rhythms could have a significant impact on plant-discrete VOC emission detection.[7,30] We therefore used the CIS, as part of our study with the objective of detecting discrete amounts of plant VOCs. Ultimately, our system thus

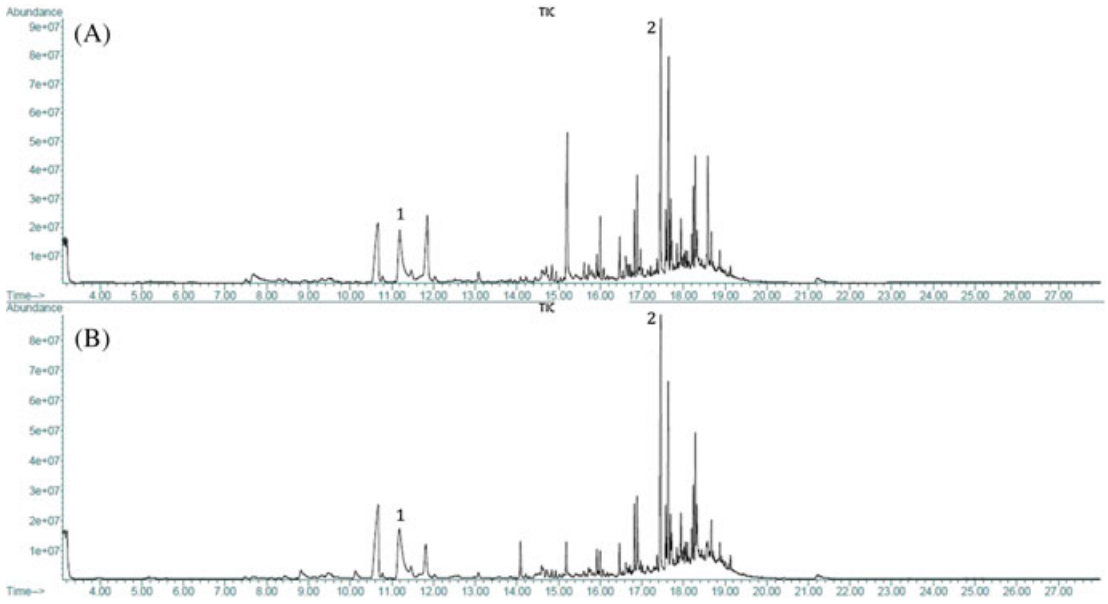

FIGURE 3 Total ion chromatogram (TIC) for (A) whole profile of blank test performed on growing medium and (B) whole profile of 28-day-old plantlets of *Brassica napus* L. var. *Es Astrid* volatiles. Peak identification: 1: *n*-butyl benzene internal standard (IS) not used, 2: octylbenzene (IS) [Colour figure can be viewed at wileyonlinelibrary.com]

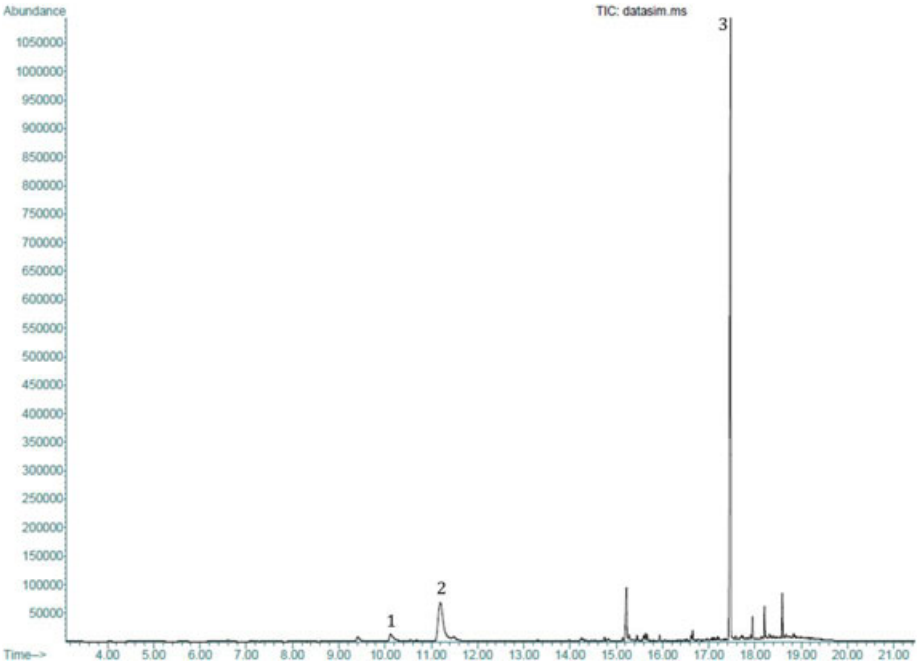

FIGURE 4 Typical chromatogram of growing medium using selected ion monitoring (SIM) mode. Peak identification: 1: limonene, 2: *n*-butyl benzene internal standard (IS) not used, 3: octylbenzene (IS) [Colour figure can be viewed at wileyonlinelibrary.com]

provides a new tool for the rapid detection of VOC emissions from very small plantlets.

3.2 | VOCs qualitative results using TD-GC/MS and Tenax TA

We firstly investigated the whole profile of emitted VOCs using the TD-GC/MS method, which is a new high-throughput approach to analysing plant volatiles within undisturbed and sterile environment. To our knowledge, no work has been reported on the potential effect of Cd in VOC metabolism cross talk. Because the results of chromatograms for the whole profile using full SCAN mode exhibited too much noise, we were unable to find any VOC qualitative differences in our investigated test conditions. This can be observed in Figure 3 for (a) total ion chromatogram (TIC) of whole profile of blank test of growing medium and (b) TIC of whole profile of plant volatiles (28-day-old plantlets of *Brassica napus* L. var. *Es Astrid*). We therefore decided to perform volatile analysis targeting terpenes

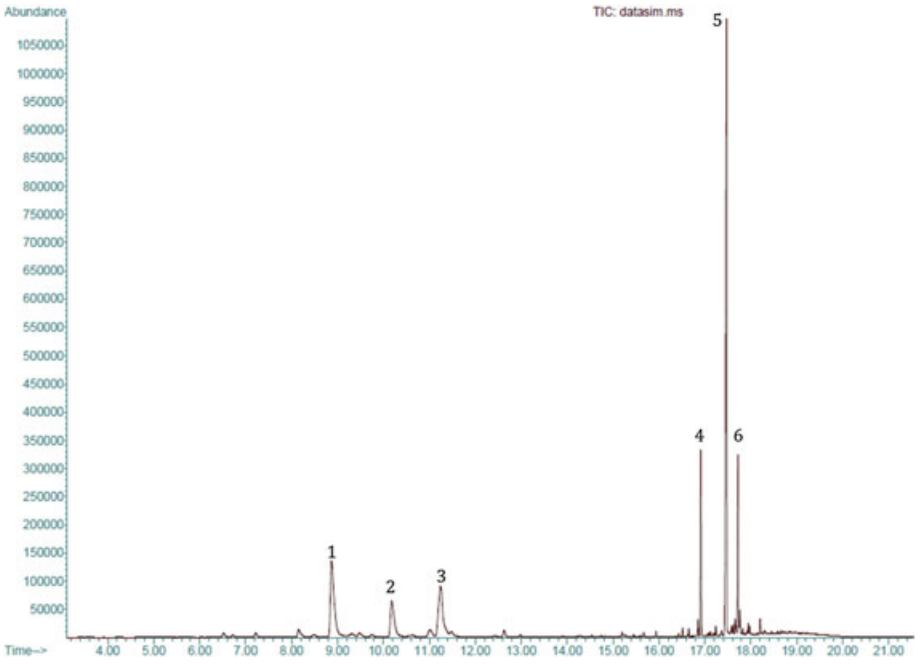

FIGURE 5 Typical chromatogram of 28-day-old plantlets of *Brassica napus* L. var. *Es Astrid* using selected ion monitoring (SIM) mode. Peak identification: 1: myrcene, 2: limonene, 3: *n*-butyl benzene internal standard (IS) not used, 4: β-elemene, 5: octylbenzene (IS), 6: (E,E)-α-farnesene [Colour figure can be viewed at wileyonlinelibrary.com]

emitted by oilseed rape using SIM mode (monitoring one single of *m/z* 93 being the most relevant ion) and Tenax TA cartridges, as this polymer is regarded as the best compromise for terpene and volatile trapping.[8]

Qualitative terpene profiles were specifically achieved for the control group of plantlets (0 µM of Cd) and at different Cd concentrations (5 µM, 15 µM and 45 µM). As confirmed by phenotypic results showing plantlets' physiological symptoms and analysis of plantlets' growth reduction, these concentrations can be considered as low (0.56 ppm), mild (1.68 ppm) and severe (5.04 ppm) Cd stress, respectively. In our study no significant effect of Cd was found on qualitative results of emitted terpenes from the 28-day-old plantlets of *Brassica napus* L. var. *Es Astrid*, which means that no specific compound related to Cd stress could be identified. Figure 4 shows the typical chromatogram achieved using data acquisition in SIM mode (*m/z* 93) for blank tests using growing medium, while Figure 5 shows the profiling of monoterpenes (myrcene, limonene) and sesquiterpenes (β-elemene, (E,E)-α-farnesene) emitted by the two 28-day-old oilseed rape plantlets. Kovats indices were 983.55 for myrcene, 1019.76 for limonene, 1408 for β-elemene and 1500.18 for (E,E)-α-farnesene. Terpene identifications were in line with mass spectral data and published retention indices.[31] Finally, they were consistent with previously published data on oilseed rape terpenoid emissions at the vegetative stage.[24,25] As described earlier, a plant-free glass cuvette containing only the same quantity of *in vitro* culture medium was used as a blank to determine the chemical background. Small amounts of limonene coming from these growing media were detected, as confirmed by also testing an empty glass cuvette without culture medium, and limonene was therefore disregarded for the further analysis of the results. SIM mode was very useful for focusing on *m/z* 93, which most accurately represents monoterpene and sesquiterpene emissions, excluding impurities and contaminants volatiles from the homemade system (such as plasticisers for example clearly coming from silicone connection tubes).

Tenax TA adsorbent material was selected in view of the characteristics of this polymer, which are ideal for plant VOC analysis. Preliminary experiments using Carbotrap® and Carbosieve® polymers were performed, but the results were not usable because these polymers have too strong affinity for water. Most studies have focused on developing new types of sorbents to extract a wide range of plant volatiles, but Tenax TA adsorbent can reversibly bind high-molecular-weight compounds and is particularly recommended for trapping target analytes such as volatile terpenes. Finally, the polymer material has a low affinity to water, thus avoiding water vapour troubleshooting during GC injection, especially with the CIS.

3.3 | Cadmium-related stress and induced terpenoids

Heavy metal stress related to terpenoid emission is poorly documented. We therefore decided to quantitatively investigate the concentration of myrcene, β-elemene and (E,E)-α-farnesene induced by the different levels of Cd stress (5 µM, 15 µM and 45 µM) in comparison with a control group (0 µM of Cd). It is commonly known that Cd induces reactive oxygen species (ROS) playing a multitude of signalling roles within abiotic stress response, but leading in excess to the destabilisation of thylakoid membranes.[29] Leaf chlorosis was observed in 28-day-old plantlets cultivated under 15 µM and 45 µM Cd concentrations, confirming that chlorosis represents a typical phenotypic symptom of Cd contamination. One-way ANOVA showed that Cd significantly affects the plantlets' growth ($F_{(3,111)}$ = 134.77, P < 0.001) and fresh weight biomass ($F_{(3,111)}$ = 39.39, P < 0.001). Cadmium was responsible for a significant decrease of growth and fresh weight biomass at a concentration of 15 µM and for very severe stress at the 45 µM concentration. No leaf chlorosis and no phenotypic effect were observed for plantlets cultivated under 5 µM (corresponding to 0.56 ppm), confirming that *Brassica napus* L. is one of the most tolerant species to Cd.[32] Dunnett's 95% confidence intervals tests comparing the mean from the control group (0 µM of Cd) with the mean of every other group (5 µM, 15 µM and 45 µM of Cd) for the growth and fresh weight biomass of 28-day-old plantlets are shown in Figure 6(a,b), respectively.

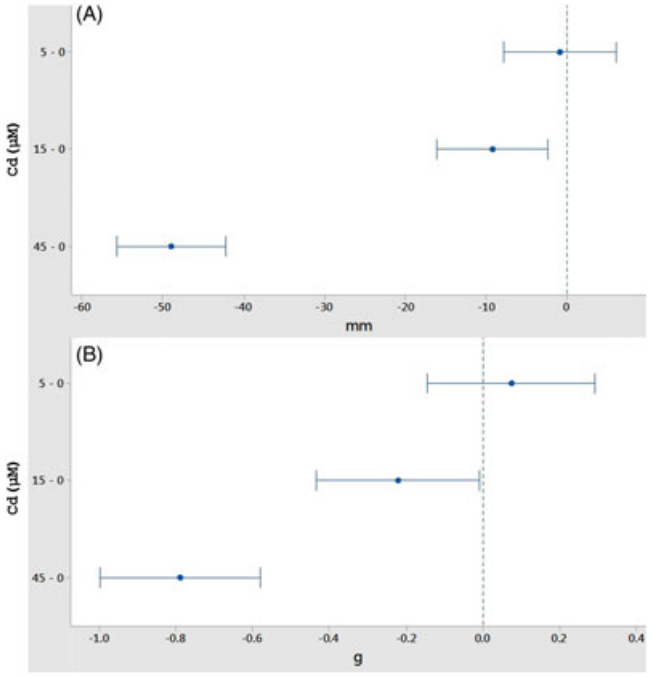

FIGURE 6 Graphs of Dunnett's 95% confidence intervals tests comparing the mean from the control group (0 µM of Cd) with the mean of every other group (5 µM, 15 µM and 45 µM of Cd) for the (A) growth (mm) and (B) fresh weight biomass (g) of 28-day-old plantlets of *Brassica napus* L. var. *Es Astrid* [Colour figure can be viewed at wileyonlinelibrary.com]

TABLE 1 Mean [± standard error (SE)] of emission rates (in pg/g/L) of terpene emitted (myrcene, β-elemene and (E,E)-α-farnesene) by 28-day-old plantlets of *Brassica napus* L. var. *Es Astrid* under the different cadmium stress conditions (0 µM, 5 µM, 15 µM and 45 µM)

Cadmium concentrations (µM)	Emission rates (pg/g/L) Mean (±SE)		
	Myrcene	β-elemene	(E,E)-α-farnesene
0[a]	27.72 ±5.6	37.89 ±7.5	19.94 ±3.8
5[a]	22.86 ±4.9	38.33 ±6.8	37.99 ±5.1
15[a]	27.73 ±3.8	52.26 ±6.4	29.23 ±4.3
45[b]	31.98 ±5.0	58.04 ±13.6	17.25 ±3.3

[a]n = 10.

[b]n = 11.

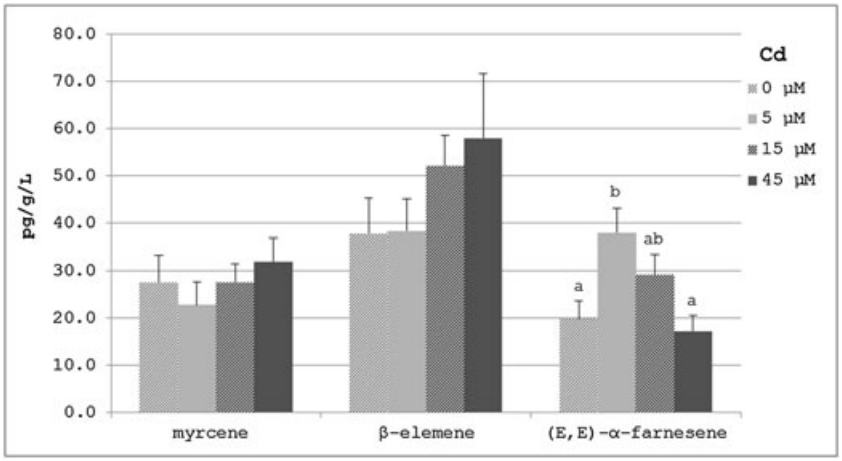

FIGURE 7 Graph of terpene emission rates (in pg/g/L) of 28-day-old plantlets of *Brassica napus* L. var. *Es Astrid* under the different cadmium (Cd) stress conditions (0 µM, 5 µM, 15 µM and 45 µM) and Tukey's *post hoc* test between means of emission rates (in pg/g/L) for (E,E)-α-farnesene at 0 µM, 5 µM, 15 µM and 45 µM of Cd

As can be observed from the graphs, the confidence intervals of both growth and fresh weight biomass means show an overlap for the control condition (0 µM) and the first Cd concentration. These results confirm the tolerance of *Brassica napus* L. var. *Es Astrid* plantlets at 5 µM of Cd.

Volatile terpene emission from plants represents more than half of the total emission of plants VOCs.[7] In addition to constitutive terpenes, abiotic stress can induce a dynamic and multifaceted response reflecting variations in volatile terpene metabolism and biological response.[15] Induced VOC emission is a *de novo* emission and is closely associated with the photosynthetic activity of the plant.[34] Table 1 summarises the data obtained from quantitative results showing the mean (± standard error) of emission rates (in pg/g/L) for myrcene, β-elemene and (E,E)-α-farnesene emitted by oilseed rape under the different Cd stress conditions (0 µM, 5 µM, 15 µM and 45 µM). Emission rates for myrcene and β-elemene showed very high plasticity and no influence of Cd abiotic stress could be found.

However, one-way ANOVA test performed on (E,E)-α-farnesene emission rates revealed a significant effect for Cd stress ($F_{3,40}$ = 5.17, P = 0.004). A significant difference was found using Tukey's *post hoc* test between means of emission rates for (E,E)-α-farnesene at 0 µM and 5 µM of Cd, as can be observed in Figure 7. This may suggest that the sesquiterpene (E,E)-α-farnesene could be implicated in an elastic and reversible response allowing tolerance to low Cd stress conditions, such as in our experiment at 5 µM of Cd, where the plantlets showed no stress symptoms. This increase of (E,E)-α-farnesene (47.5%) at 5 µM could also represents a potential biomarker of Cd presence within the culture medium without biotic stress interaction. According to the literature, the sesquiterpene (E,E)-α-farnesene is also involved with stress related to biotic damage.[11] A trend of elevation of myrcene and β-elemene emissions correlated to Cd stress concentration can also be observed in Figure 7.

Some studies have reported similar results concerning a putative variation of emitted terpene under abiotic stress conditions.[2,25,27] It seems to be clear that volatile organic compounds, especially terpenoids, mediate plants' resilience to abiotic stress related to heavy metals, but more research is needed. It would also be very interesting to perform other experiments using the developed system in order to recover plant volatiles using other substrate of plant growth such as soil.

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