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Abstract	Epoxiconazole is a broad-spectrum fungicide described as highly persistent in soil and as such can be considered as an abiotic agent like other problematic agrochemicals. Furthermore, the plant phenotyping tool involving non-invasive monitoring of plant-emitted volatile organic compounds (VOCs) may be useful in the identification of metabolic markers for abiotic stress. We therefore decided to profile the VOCs from secondary metabolism of oilseed rape through a dose-response experiment under several epoxiconazole concentrations (0, 0.01, 0.1 and 1 mg L^{-1}). VOC collections of 35-day-old whole plantlets were performed through a dynamic headspace sampling technique under defined and controlled conditions. The plantlets grew freely within a home-made, laboratory and high-throughput glass chamber without any disturbance. Putative metabolic markers were analysed using a targeted metabolomic approach based on TD-GC-MS method coupled with data acquisition in SIM mode in order to focus on terpenes and sulphur-containing volatiles. Chromatograms of emitted terpenes were achieved accurately for the 35-day-old oilseed rape plantlets. We also analysed the presence of sulphur-containing volatiles in samples of shoot and root tissues using an innovative DHS-TD-GC-MS method, but no difference was found between qualitative profiles. Nevertheless, we demonstrated through this experiment that sesquiterpenes such as β-elemene and (E,E)-α-farnesene are involved in epoxiconazole dose-response. In particular, (E,E)-α-farnesene could serve as a metabolic marker of fungicide exposure for oilseed rape plantlets.

Keywords separated by ' - '	Epoxiconazole - Oilseed rape - VOCs - Metabolic markers - Terpenes - Sulphur-containing volatiles
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RESEARCH ARTICLE

Epoxiconazole exposure affects terpenoid profiles of oilseed rape plantlets based on a targeted metabolomic approach

Bastien Durenne[1] · Alodie Blondel[1] · Philippe Druart[1] · Marie-Laure Fauconnier[2]

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Abstract

Epoxiconazole is a broad-spectrum fungicide described as highly persistent in soil and as such can be considered as an abiotic agent like other problematic agrochemicals. Furthermore, the plant phenotyping tool involving non-invasive monitoring of plant-emitted volatile organic compounds (VOCs) may be useful in the identification of metabolic markers for abiotic stress. We therefore decided to profile the VOCs from secondary metabolism of oilseed rape through a dose-response experiment under several epoxiconazole concentrations (0, 0.01, 0.1 and 1 mg L^{-1}). VOC collections of 35-day-old whole plantlets were performed through a dynamic headspace sampling technique under defined and controlled conditions. The plantlets grew freely within a home-made, laboratory and high-throughput glass chamber without any disturbance. Putative metabolic markers were analysed using a targeted metabolomic approach based on TD-GC-MS method coupled with data acquisition in SIM mode in order to focus on terpenes and sulphur-containing volatiles. Chromatograms of emitted terpenes were achieved accurately for the 35-day-old oilseed rape plantlets. We also analysed the presence of sulphur-containing volatiles in samples of shoot and root tissues using an innovative DHS-TD-GC-MS method, but no difference was found between qualitative profiles. Nevertheless, we demonstrated through this experiment that sesquiterpenes such as β-elemene and (E,E)-α-farnesene are involved in epoxiconazole dose-response. In particular, (E,E)-α-farnesene could serve as a metabolic marker of fungicide exposure for oilseed rape plantlets.

Keywords Epoxiconazole · Oilseed rape · VOCs · Metabolic markers · Terpenes · Sulphur-containing volatiles

Abbreviations

CIS/PTV	Cooled injection system and programmable temperature vaporising inlet
DHS	Dynamic headspace
EDTA	Ethylenediaminetetraacetic acid
GC	Gas chromatography
GLVs	Green leaf volatiles
GSH	Glutathione
GSLs	Glucosinolates
IS	Internal standard
ITCs	Isothiocyanates
LC	Liquid chromatography
LED	Light-emitting diode
MRLs	Maximum residue levels
MS	Mass spectrometry
NMR	Nuclear magnetic resonance
PAR	Photosynthetically active radiation
RPM	Revolutions per minute
SE	Standard error of the mean
SIM	Selected-ion monitoring
TD-GC-MS	Thermal desorption and gas chromatography-mass spectrometry
TDU	Thermal desorption unit
VOCs	Volatile organic compounds

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Introduction

Pesticides are compounds widely used in farming and can reach the soil through rain, irrigation water and wind when they are applied to crops (Marican and Durán-Lara 2018). Some pesticides such as triazole fungicides persist in soil

and sediments due to low bioavailability. This is especially true of epoxiconazole which has a half-life time of more than 2 years (at 10 °C and 80% of field capacity; Bromilow et al. 1999). Epoxiconazole is a synthetic broad-spectrum fungicide interfering with the biosynthesis of the steroid ergosterol, an essential membrane component of yeast and fungi, by competitively inhibiting the enzyme lanosterol 14 α-demethylase (Chambers et al. 2014). Low application rates of 25–125 g ha^{-1} of epoxiconazole's active substance are highly effective for the control of diseases caused by Ascomycetes, Basidiomycetes and Deuteromycetes through foliar application in cereals, rice, grapes and other crops worldwide such as oilseed rape (Liang et al. 2012). This curative and preventive fungicide is therefore extensively used, but strict requirements in line with good agricultural practices must be adhered to respect maximum residue levels (MRLs) in plants and soil (Yan et al. 2015). One of the most important metabolites of this fungicide in soil is 1,2,4 triazole, which is rapidly degraded by soil micro-organisms with low persistence (EFSA (European Food Safety Authority) 2008; Blondel et al. 2018).

Oilseed rape (*Brassica napus* L.), belonging to the *Brassicaceae* family, is an allotetraploid crop species resulting from a natural hybridisation of the diploid species *B. oleracea* and *B. rapa* (Chalhoub et al. 2014). Global oilseed rape production has tremendously increased in the last decade and these oil-rich seeds are processed into edible oil, biodiesel and high-quality animal feed (Derbyshire and Denton-Giles 2016). In addition to its use against *Sclerotinia sclerotorium* to prevent the annual oilseed rape yield losses caused by this fungus, epoxiconazole can also regulate plant growth (Bertelsen et al. 2001; Li et al. 2015). It is well known that triazole compounds are involved in the inhibition of gibberellin biosynthesis at the stage of conversion of *ent*-kaurene to *ent*-kaurenoic acid (Rademacher 2000; Yamaguchi 2008). Several publications have also described this plant growth regulatory effect on oilseed rape crops after foliar application or uptake by roots (Bruns et al. 1990; Berry and Spink 2009; Durenne et al. 2018b). The metabolism of epoxiconazole in plants using foliar application is limited but a significant uptake of some triazole derivative metabolites (triazole alanine and triazole acetic acid) has been demonstrated for cereals (EFSA (European Food Safety Authority) 2008). Although detoxification mechanisms of pesticide residues have been widely studied in mammalian cells, the regulation network in plants remains elusive (Zhou et al. 2015). Recent research described that crop plants seem to be able to detoxify absorbed pesticide residues through a system including enzymes, glutathione (GSH) and sequestration in the vacuole (Coleman et al. 1997; Shahzad et al. 2018).

Agrochemical products commonly used in agriculture could be investigated as such an abiotic factor with metabolomic study purposes (Kráľová et al. 2012). Metabolites profiling related to plants' pesticide response is also becoming increasingly common in ecotoxicological risk assessment, as a means of investigating the modes-of-action of bioactive substances and discovering new compounds (Aliferis and Chrysayi-Tokousbalides 2011). Petersen et al. (2011) have also discussed the putative use of environmental metabolomics to detect oilseed rape exposure to glyphosate. From an analytical point of view, gas and liquid chromatography coupled with mass spectrometry (GC/LC-MS) through a non-targeted full-scan approach or a targeted approach using selected-ion monitoring (SIM) are both cutting-edge technologies, not to mention nuclear magnetic resonance (NRM) spectrometry. NMR-based methods have been used to describe some changes in plant metabolite content and composition for *Agrostis capillaris* and *Arabidopsis thaliana* after epoxiconazole exposure (Strandberg et al. 2013) and have recently be used to monitor plant metabolic changes in association with pesticide exposure in major crops such as maize (Blondel et al. 2016). However, NMR-screening is expensive and time-consuming. Principal component analysis (PCA) is frequently needed to obtain response patterns as putative indicators. Methods based on GC-MS and derivatisation were recently used to study the metabolomic profile of rice under pesticide stress through a pseudotargeted approach (Zhao et al. 2015) and to show the integration of *Lolium perenne* metabolic responses after exposure to glyphosate and tebuconazole (Serra et al. 2015). Derivatisation protocols remain also time-consuming involving additional sample handling and chemical steps (Jorge et al. 2016). In order to identify specialised metabolites as markers of abiotic stress response as accurately as possible, technologies such as GC or LC-MS must be used with a strong emphasis on secondary metabolism (Nakabayashi and Saito 2015). Finally, the SIM mode available with mass spectrometry can be performed at the same time as a full-scan and can greatly help to target metabolites of interest (Delory et al. 2016).

Secondary metabolism, especially terpenoids and glucosinolates within *Brassica* spp., is clearly identified to play a crucial role in tolerance to environmental stresses resulting from agriculture challenges (Rodziewicz et al. 2014). Some secondary metabolites such as volatile organic compounds (VOCs) are emitted from plants and, represent a specific and non-invasive way to phenotype plants' response to abiotic stress (Niederbacher et al. 2015). This is particularly true if the data can be obtained from a high-throughput system with high repeatability in order to describe the most representative state of the plant's metabolism in response to environmental stressors. Volatile isoprenoids are well known to be involved in abiotic stress (Vickers et al. 2009), and terpenes in particular can represent an attractive target as a marker of

adaptive response to abiotic stress (Loreto and Schnitzler 2010; Durenne et al. 2018a).

Specialised terpenoids have fundamental functions in plants' growth and development, coupled with roles in their environmental interaction (Tholl 2015). In addition, sulphur-containing volatiles such as nitriles, epithionitriles and isothiocyanates (ITCs), representing well-known breakdown products of glucosinolates (GSLs), are frequently mentioned in investigations of oilseed rape's volatile response to biotic stress (van Dam et al. 2012). They are not normally emitted by oilseed rape in response to abiotic factors, but can be analysed using the GC-MS method and dynamic headspace (DHS) sampling after flash-freezing of the plant tissue with liquid nitrogen. Such a technique can be used to profile the metabolites in plant tissues by stopping metabolic processes in cells through the use of very low temperatures (Jorge et al. 2016; Delory et al. 2016; Gemperline et al. 2016). We therefore decided to investigate the volatile response of oilseed rape plantlets under several concentrations of epoxiconazole using a thermal desorption and gas chromatography-mass spectrometry (TD-GC-MS) method, with a targeted approach based on selected-ion monitoring (SIM) mode acquisition of data. Analysis was focused on volatile terpenes and ITCs such as sulphur-containing but non-volatile compounds due to their role in environmental stress responses. The dose-response experiment was performed under controlled and defined conditions using perlite substrate in order to highlight putative metabolic markers for oilseed rape as indicators of fungicide exposure.

Materials and methods

Plant material and growth conditions

The winter oilseed rape plantlets were grown from germinated seeds of *Brassica napus* L. var. *Es Astrid* (Euralis semences, France). Seeds were surface-sterilised in 70% ethanol for 1 min, followed by immersion in calcium hypochlorite (7% *W/V*) for 45 min, rinsed two times with sterile water for 15 min and sown in Petri dishes (100 × 15 mm) with distilled water in order to germinate. Two standardised seedlings with well-developed cotyledons were transferred after 10 days to a home-made glass cuvette system previously described by Durenne et al. (2018a) containing sterile perlite substrate with the addition of 40 mL of a modified Hoagland's nutrient solution (590 mg L^{-1} $Ca(NO_3)_2$; 70 mg L^{-1} KH_2PO_4; 250 mg L^{-1} KNO_3; 750 mg L^{-1} $MgSO_4$; 0.1 mg L^{-1} $ZnSO_4$; 0.8 mg L^{-1} $MnSO_4$; 1.5 mg L^{-1} H_3BO_3; 0.1 mg L^{-1} $CuSO_4$ and 65 mg L^{-1} Fe-EDTA). The plantlets were cultivated for 35 days in a climate room equipped with LED lighting (Valoya L28 Spectrum NS12 Clear), at 23/18 °C (day/night), with a photoperiod of 16 h, 45% relative humidity and 130 μmol m^{-2} s^{-1} of PAR. The plantlets were watered every 3 days with 5 mL of the nutrient solution under sterile conditions.

Epoxiconazole dose-response experiment

Analytical standard epoxiconazole corresponding to a LC-MS grade of 99% (Sigma-Aldrich, Darmstadt, Germany) was diluted in purified water Milli-Q (Millipore, Bedford, USA) to obtain a stock solution (6 mg L^{-1}) that was stored in the dark at 6 °C. For the dose-response experiment, the stock solution was diluted in the nutrient solution to reach precisely 4 mg L^{-1} and in order to achieve final epoxiconazole concentrations of 0, 0.01, 0.1 and 1 mg L^{-1} in a volume of 40 mL. Each concentration of epoxiconazole was tested in triplicate on two oilseed rape plantlets per cuvette system (Fig. 1). A blank consisting of a plant-free glass cuvette (containing only perlite substrate and nutrient solution) and an empty cuvette was also included in the dose-response experiment.

Phenotyping of plantlets

At the end of the experiment, oilseed rape plantlets were gently harvested from the glass chamber system for physiological and biochemical analysis. The roots were carefully immersed in tap water to remove perlite substrate, rinsed with distilled water and wiped with tissues. Phenotyping consisted of plant observation at each concentration of epoxiconazole, and a picture of each plantlet was taken using the DSC-HX50™ (Sony, Belgium). The fresh weight biomass (g), the length of the shoot (cm) and the length of greatest root (cm) of each 35-day-old oilseed rape plantlet were measured and recorded. Shoot and root samples were obtained by cutting plantlets with a scalpel and were carefully stored at − 25 °C before further analysis.

Collection and quantitation of terpenes emission

Volatile terpenes from the 35-day-old oilseed rape plantlets were analysed and quantitated according to the non-

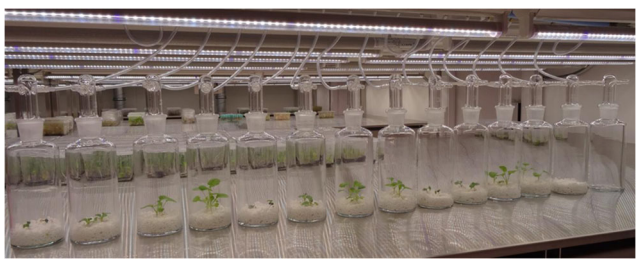

Fig. 1 Epoxiconazole dose-response experimental set-up with two oilseed rape plantlets (at the 21-day-old stage) with home-made cuvette system using perlite substrate Q3

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destructive TD-GC-MS method, fully described in Durenne et al. (2018a). Terpenes were trapped for 24 h on Tenax® TA adsorbent cartridges that were thermally desorbed before cryofocusing with a CIS/PTV into an HP-5 ms GC column. The terpene detection and quantitation from chromatogram profiles were acquired with SIM mode using the most representative ion (m/z 93) during full-scan analysis. The mass spectra were obtained with a quadrupole-type mass spectrometer. 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 sabinene, myrcene, β-elemene and (E,E)-α-farnesene (Sigma-Aldrich, Diegem, Belgium). Retention indices were also calculated using a saturated n-alkanes (C7–C30) standard solution (Sigma-Aldrich, Diegem, Belgium). Single-ion peaks of m/z 93 with relative abundance of sabinene (25.44%), myrcene (23.03%), β-elemene (7.29%) and (E,E)-α-farnesene (9.45%) were respectively integrated and compared with the equivalent single-ion response of 1 μL of hexane solution containing an internal standard of octylbenzene (0.58 mg mL^{-1}) (2.69%) (Sigma-Aldrich, Diegem, Belgium). Terpenoid emission rates were calculated as pg g^{-1} L^{-1} of fresh weight plantlet and air extracted.

Analysis of sulphur-containing volatiles in plantlet tissues

Sulphur-containing volatiles contained in plant organs (not emitted) were analysed in the shoot and root tissues respectively at the end of the dose-response experiment. Shoot and root samples of 35-day-old oilseed rape plantlets were frozen in liquid nitrogen before being pulverised in a mortar. The root and shoot powders were placed in a 20-mL glass vial supplied with a silicone/PTFE septum (FilterService, Eupen, Belgium), and stored at − 80 °C before automated DHS-TD-GC-MS analysis. The sulphur-containing volatiles were collected using a DHS system (Gerstel, Mülheim an der Ruhr, Germany) during an incubation time of 2 min at 23 °C under constant agitation (500 rpm). They were trapped on Tenax TA cartridges with a 500-mL volume of trapping phase and using a helium flow rate of 20 mL min^{-1}. Finally, VOCs were thermally desorbed with a TDU (Gerstel, Mülheim an der Ruhr, Germany) running in splitless mode from 40 to 120 °C (110 °C min^{-1}) for 2 min in order to prevent thermal degradation, and then at 280 °C (200 °C min^{-1}) for 5 min. Cryofocusing with a programmable temperature vaporising inlet was performed at − 30 °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^{-1}. VOC separation was performed using gas chromatography (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, Palo Alto, CA, USA). High-purity helium (Air Liquide, Liège, Belgium) was used as the carrier gas at a constant flow of 1.6 ml/min. The oven temperature programme started at 40 °C with increasing at a rate of 10 °C min^{-1} to 65 °C, then of 5 °C min^{-1} to 90 °C and then 20 °C min^{-1} to 300 °C with finally, 5 min at this temperature. VOC detection was performed using a quadrupole-type mass spectrometer (MS 5975C; Agilent Technologies, Palo Alto, CA, USA). Mass spectra were obtained using electron impact mode (70 eV) and operated in SCAN mode with a range of 35 to 450 amu for m/z ratios. Accurate profiles of sulphur-containing volatiles were obtained using SIM mode targeting the most representative 72 m/z ion in the same full-scan run of 23 min. GC-MS data were analysed using the Agilent MSD Chemstation E 02.00.493 (Agilent Technologies, Palo Alto, CA, USA). Because no commercial analytical standard was available, a tentative compound identification was performed by comparing the data with a Wiley 275 mass spectral database, with the database of the National Institute Standard and Technology (NIST08) and with previously published mass spectral data (m/z and relative abundance) (Al-Gendy and Lockwood 2003; Taveira et al. 2009; Hong and Kim 2013).

Statistical analysis

All statistical analyses were carried out with Minitab® package version 17 and all data sets were tested for normality and equality of variances. Phenotypic results of shoot and root growth (cm) for 35-day-old oilseed rape plantlets were analysed using one-way analysis of variance (ANOVA). One-way ANOVA was also used to test the impact of the epoxiconazole concentration factor on sabinene, myrcene, β-elemene and (E,E)-α-farnesene emission rates. This analysis was followed by a post hoc Tukey's range test to find significant differences among pairwise means at a 0.05 level of probability. The values are reported as means with standard error for all results.

Fig. 2 35-day-old plantlets at the end of the epoxiconazole dose-response experiment with each concentration tested in triplicate—0, 0.01, 0.1 and 1 mg L^{-1}

Results and discussion

Phenotypic results of 35-day-old oilseed rape plantlets

It was apparent that a progressive plantlet growth inhibition was found at the end of the dose-response experiment along the range of epoxiconazole concentrations (0, 0.01, 0.1 and 1 mg L^{-1}) which was tested in triplicate using perlite substrate (Fig. 2). Boxplots showing the mean, median, outliers and 25th and 75th percentiles of shoot and root growth have confirmed the morphological responses of growth inhibition with a dose-dependent pattern (Fig. 3a, b). In addition, one-way ANOVA showed that epoxiconazole significantly affects shoot growth (cm) ($F_{(3.23)} = 249.18$, $P < 0.001$) and significantly affects root growth (cm) ($F_{(3.23)} = 17.43$, $P < 0.001$) measured from the 35-day-old oilseed rape plantlets. In our experimental conditions, the concentration of 0.1 mg L^{-1} corresponds to a subtoxic condition test and, the concentration of 1 mg L^{-1} corresponds to the dose which any plantlet can normally grow. The concentration of 1 mg L^{-1} was therefore disregarded for the further analysis of volatiles induced by

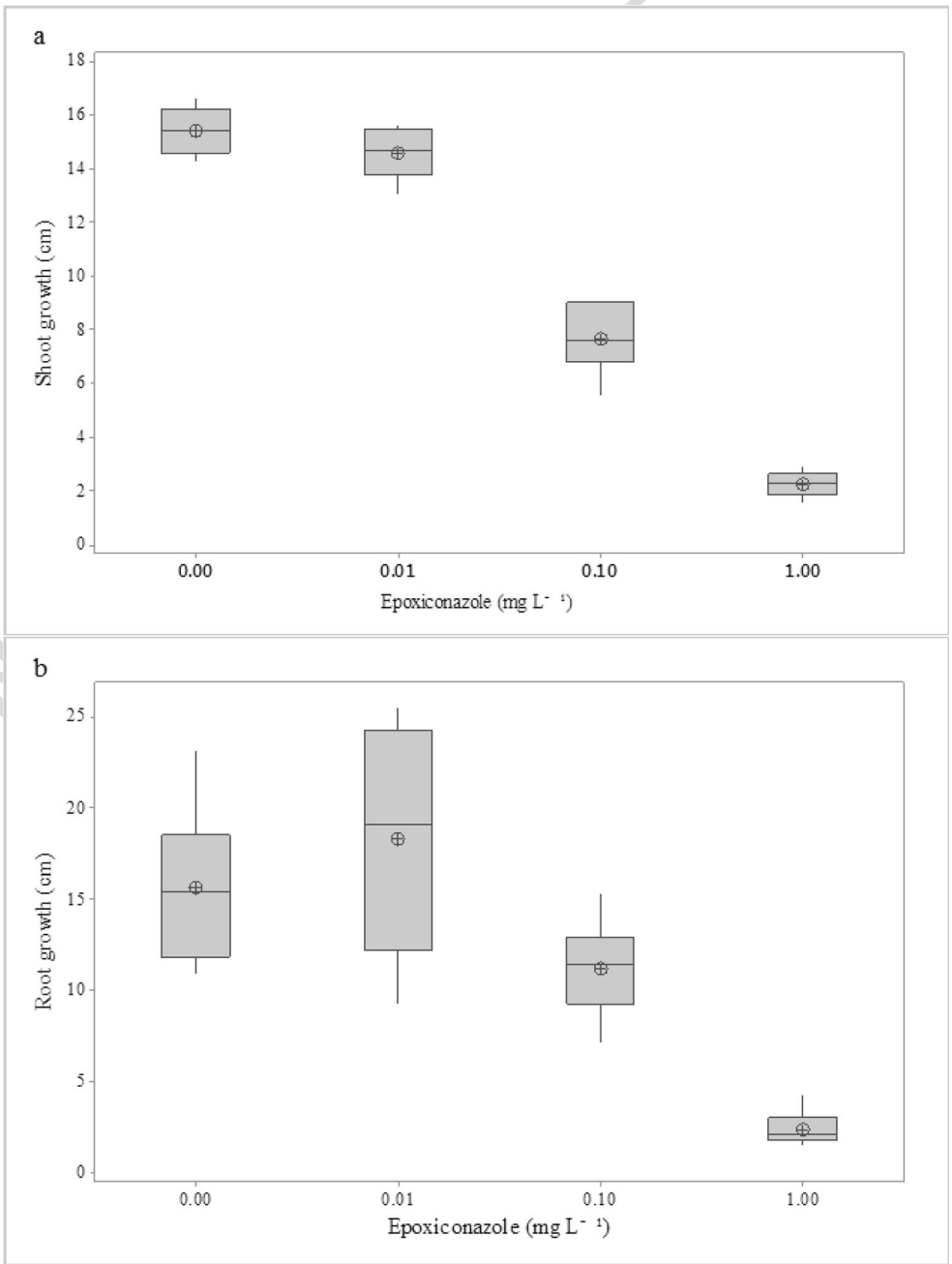

Fig. 3 Boxplots (showing mean (⊕), median (line), 25th and 75th percentiles and outliers) of **a** shoot growth and **b** root growth for 35-day-old oilseed rape plantlets under different concentrations of epoxiconazole (0, 0.01, 0.1 and 1 mg L^{-1}) ($n = 6$)

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epoxiconazole. Berry and Spink (2009) have previously described anti-gibberellin activity of triazole compounds affecting the growth of oilseed rape and these compounds can be also used for their fungicidal and regulatory properties. Recently, a field experiment showed also that nine triazole and strobilurin fungicides significantly influenced the plant height and green area index of winter oilseed rape (Ijaz and Honermeier 2012). The presence of epoxiconazole, a well-known soil-persistent systemic fungicide, in the rhizosphere of oilseed rape was also demonstrated to act as a plant growth

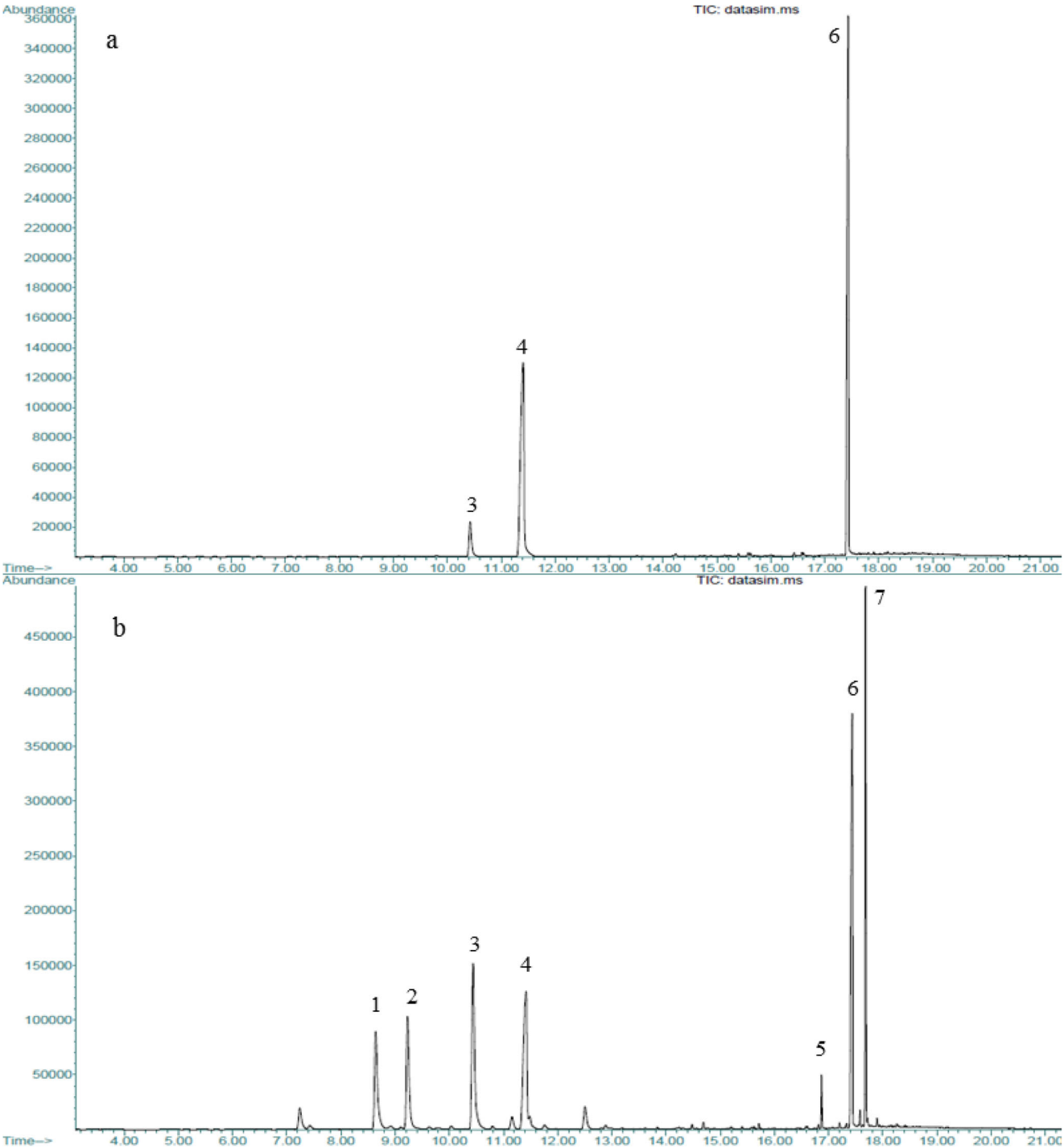

Fig. 4 Typical chromatograms achieved using SIM mode (m/z 93) of **a** blank and **b** terpenes emitted by the two 35-day-old plantlets of oilseed rape (untreated). Peak identification: 1, sabinene; 2, myrcene; 3, limonene; 4, n-butyl benzene (IS) not used; 5, β-elemene; 6, octylbenzene (IS); 7, (E,E)-α-farnesene

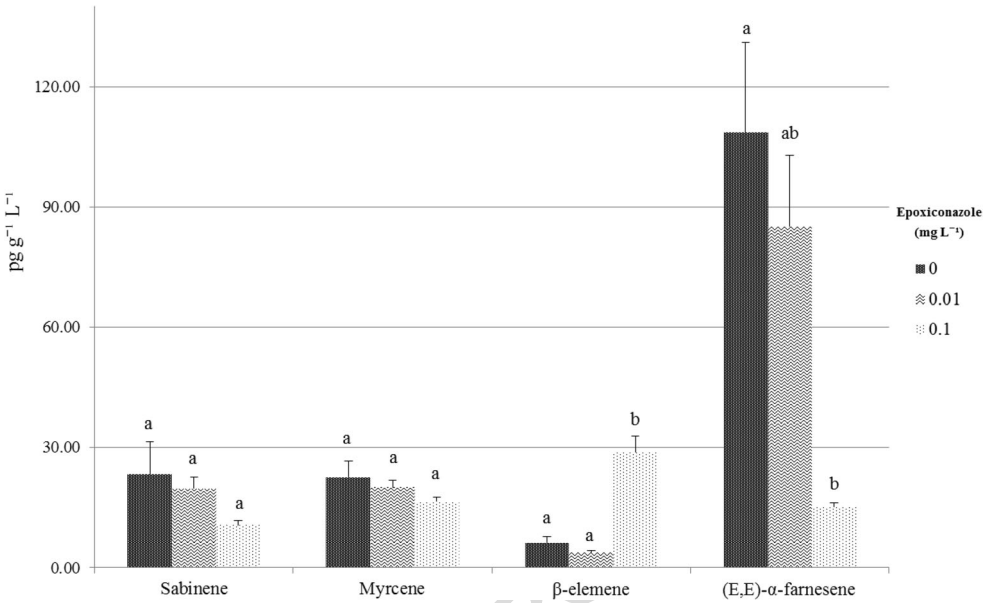

Fig. 5 Graph of means (± SE) of terpene emission rates (pg g^{-1} L^{-1}) for 35-day-old plantlets of oilseed rape and Tukey's post hoc test between means for sabinene, myrcene, β-elemene and (E,E)-α-farnesene at 0, 0.01 and 0.1 mg L^{-1} of epoxiconazole ($n = 3$)

regulator and in excess in agar medium, severe stress symptoms such as chlorosis and anthocyanosis can also occur (Durenne et al. 2018b).

Volatile terpenes and epoxiconazole exposure

At laboratory-scale, foliar application of epoxiconazole on *Galium aparine* L. can affect phytosterol profiles and modify photosynthetic electron transport (Benton and Cobb 1997; Petit et al. 2012). To our knowledge, there is no scientific information about terpene emission related to fungicide exposure, and the influence of pesticide residues on oilseed rape plant metabolome is as yet poorly documented. We therefore used the GC-MS technique and our sampling method to compare differences in VOCs emitted by the 35-day-old oilseed rape plantlets for each concentration of epoxiconazole tested (0, 0.01 and 0.1 mg L^{-1}). We first investigated the data of the full-scan chromatogram, but this yielded no reliable evidence. Typical chromatograms of a blank (a plant-free glass cuvette containing only perlite substrate with the nutrient solution) and terpenes emitted by the 35-day-old oilseed rape plantlets were therefore achieved using selected-ion monitoring (SIM) mode (m/z 93) (Fig. 4a, b). Except obviously at 1 mg L^{-1}, the two plantlets significantly emitted three monoterpenes (sabinene, myrcene and limonene) and two sesquiterpenes (β-elemene and (E,E)-α-farnesene). These results were consistent with previously published data relating to oilseed rape terpene emission at vegetative stage and under abiotic stress (Veromann et al. 2013; Durenne et al. 2018a). No difference was found between qualitative profiles of terpenes at the different concentrations of epoxiconazole tested.

We decided to quantitatively investigate the terpene response under epoxiconazole exposure in order to identify any induced emission. Limonene results were disregarded because very small amounts were found in blank tests. One-way ANOVA followed by a post hoc Tukey's range test showed no difference between means of emission rates (pg g^{-1} L^{-1}) for the two monoterpenes sabinene and myrcene but, interestingly, showed differences between means of emission rates for the two sesquiterpenes β-elemene and (E,E)-α-farnesene ($F_{(2,8)} = 32.69$, $P < 0.001$ and $F_{(2,8)} = 8.64$, $P < 0.05$, respectively) (Fig. 5). As can be observed on the graph, the ranges of β-elemene and (E,E)-α-farnesene depended on the concentration of epoxiconazole in the perlite substrate, with a spectacular increase for β-elemene at 0.1 mg L^{-1} and a dose-dependent decrease for (E,E)-α-farnesene. Monoterpenes and sesquiterpenes are synthesised via distinct ways within the plant cell (Tholl 2015) and precisely, via two interconnected isoprenoid pathways: the formation of homoterpenes, sesquiterpenes and triterpenes come from cytosolic mevalonic acid (MVA) and the formation of hemiterpenes, monoterpenes, diterpenes and tetraterpenes come from chloroplastic 2-C-methyl-D-erythritol 4-phosphate (MEP) (Vickers et al. 2009; Lange and Ahkami 2013). It seems clear from this dose-response experiment that sesquiterpenes are more influenced by epoxiconazole exposure and that (E,E)-α-farnesene emission is particularly affected. Literature is replete with examples of studies where *Brassica* pests are responding to VOCs such as terpenoids, isothiocyanates (ITCs) and green leaf volatiles (GLVs) at some points in their life cycle (Himanen et al. 2017). Most described biological functions of sesquiterpenes are ecological as being nonspecific toxins active against a wide range of organisms (i.e. bacteria, fungi, plants and animals) (Rosenkranz and Schnitzler 2016). The response of monoterpenes and sesquiterpenes under abiotic stress needs to be yet clarified. Further investigations are

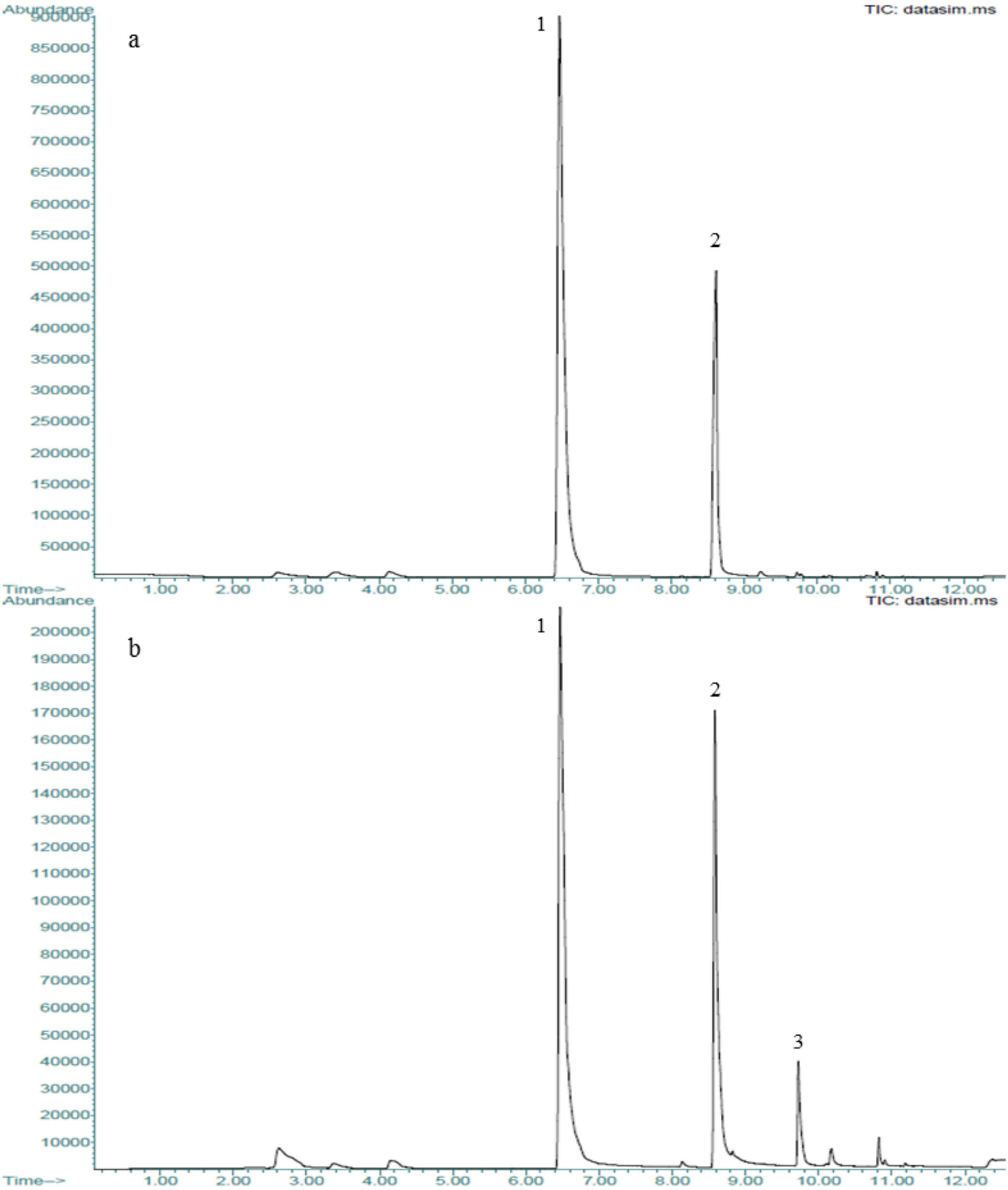

Fig. 6 Typical chromatograms achieved using SIM mode (m/z 72) of **a** sample of shoot tissue and **b** sample of root tissue of 35-day-old oilseed rape plantlets (untreated). Peak of tentatively identified compound: 1: 3-butenyl isothiocyanate, 2: 4-pentenyl isothiocyanate, 3: 4-methylpentyl isothiocyanate

needed by testing several concentrations of epoxiconazole in subtoxic conditions in order to identify any crosstalk between related pathways of (E,E)-α-farnesene synthesis and the control of elongation growth by gibberellins (Davidson et al.

2006; Yamaguchi 2008). It is known that volatile terpenes are involved in abiotic stress response (Loreto and Schnitzler 2010), and we can conclude that pesticide residues also affect the volatilome of oilseed rape plantlets through an adaptive emission. We also demonstrated that they could possibly serve as metabolic markers of fungicide exposure, but this should be confirmed in association with biotic stress.

Profiling of sulphur-containing volatiles in shoot and root samples

We tried to highlight glucosinolate breakdown products in association with epoxiconazole exposure after the flash-freezing of the 35-day-old oilseed rape plantlet tissues (roots and shoots) and analysis using an innovative DHS-TD-GC-MS method. First as expected, we found well-known green leaf volatiles (GLVs) in our full-scan chromatograms, resulting from damage to oilseed rape plantlet tissues (crushing in liquid nitrogen) and the peroxidation of polyunsaturated fatty acids. The same GLV compound profiles were found in roots and shoots of oilseed rape plantlets at the different epoxiconazole concentrations tested (0, 0.01 and 0.1 mg L^{-1}). The *Brassicaceae* family is known to contain very interesting secondary metabolites such as GSLs that are involved in abiotic stress response (Rodziewicz et al. 2014). These consist of a β-thioglucose, a sulfonated oxime and a variable aglycone side chain derived from an α-amino acid. In the cell after disruption of the vacuole, they are hydrolysed with myrosinase, resulting in the production of isothiocyanates (ITCs), thiocyanates, nitriles, goitrin and epithionitriles depending on the pH conditions (Ishida et al. 2014). Three isothiocyanates (3-butenyl ITC, 4-pentenyl ITC, 4-methylpentyl ITC), resulting from hydrolysis of GSL secondary metabolites were found by profiling ITCs using SIM mode and the most representative ion (m/z 72), and we observed that 4-methylpentyl ITC was only present in the sample of root tissues (Fig. 6). No qualitative difference in our ITC profiles was found for root and shoot samples in relation to fungicide exposure of 0.01 mg L^{-1} and in comparison to the control without epoxiconazole. The results of plantlets' physiological stress under 0.1 mg L^{-1} of epoxiconazole have been previously described and can simply be determined by visual observations. We suggest that ITC cannot be used as a metabolic marker of epoxiconazole exposure for oilseed rape plantlets, but we have demonstrated with this DHS-TD-GC-MS method targeting a single ion (m/z 72) the possibility of studying ITCs as metabolic markers for others stresses (e.g. biotic). Numerous *Brassica* species investigations relating to biotic stress have concerned GSL and their relative breakdown products such as ITCs (van Dam et al. 2012).

Concluding remarks

Plant metabolic profiling, under various subtoxic conditions of chemical stress, such as that caused by pesticide residue, can reveal complex metabolic shifts and physiological perturbations (Serra et al. 2015). VOC profiling and GC-MS studies seem to be a convenient and non-invasive approach to identifying some metabolic markers for pesticide exposure. It will be also interesting to confirm the results and observations obtained from these experimental conditions using other substrates such as soil and with other pesticide residues, for example. Finally, further research is needed to gain a more accurate understanding of crop plant pesticide detoxification, and brassinosteroids also seem to play an important role in the alleviation of pesticide physiological stress (Zhou et al. 2015; Sharma et al. 2016; Shahzad et al. 2018).

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