

ORIGINAL ARTICLE

The effects of biotic and abiotic stress on the emission of volatile organic compounds by sugar beet plants

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Vol. 65, No.3: 413–423, 2025

DOI: 10.24425/jppr.2025.155780

Received: February 26, 2024

Accepted: April 29, 2024

Online publication: September 01, 2025

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Responsible Editor:
Opender Koul

Abstract

Tetranychus urticae Koch (the two-spotted spider mite, TSSM) is a major pest of sugar beet plants (*Beta vulgaris* L.), which quickly develops resistance to miticides. Volatile organic compounds (VOCs) have the potential of providing an environmentally friendly alternative to currently used insecticides. The main goal of this study was to evaluate the changes in the qualitative and quantitative composition of the VOCs released by sugar beet plants under drought conditions, TSSM infestation, or subjected to combined types of stress. Volatiles were collected over a 2 h period on days 2, 3 and 6 following TSSM feeding and/or drought and, following elution, were analyzed by gas chromatography with mass spectrometric detection. In particular, plants that were subjected to combined abiotic and biotic stress resulted in even higher levels of VOCs being released than from plants subjected to a single stress. Ten key volatiles were identified, namely: (Z)-3-hexenal, (Z)-3-hexen-1-ol, (E)-2-hexen-1-ol, (Z)-3-hexen-1-yl acetate, (Z)-ocimene, linalool, β-pinene, (E)-β-farnesene, β-caryophyllene and benzyl acetate. Of these 10, three were emitted with significantly higher quantities than the other seven: β-pinene, β-caryophyllene and benzyl acetate. This suggests that these three volatiles are potentially the most useful as natural alternatives to synthetic miticides to protect sugar beet crops from TSSM. Further research is needed to assess this hypothesis and to determine their activity against the mites.

Keywords: drought, GC-MS, miticides, sugar beet, *Tetranychus urticae*, volatile organic compounds

Introduction

Owing to its high sucrose concentration, sugar beet (*Beta vulgaris* L.) is an important crop for use in sugar production (Stevanato *et al.* 2019; Zicari *et al.* 2019). During the years 2021/2022, the European Union

produced 16.2 million tons of sugar beet (CEFS statistics, 2021). However, decreases in yields of sugar beet have been observed due to droughts (Alavilli *et al.* 2023) and to local infestation of *Tetranychus urticae*

Koch (two-spotted spider mite, TSSM for short) (Neher and Barbour 2019). It is claimed that intensive feeding by the spider mites can cause losses of up to 25–30% or more (Neher and Barbour 2019), which is considered to be a result of decreased sugar beet photosynthesis and transpiration (Kumar *et al.* 2015). Sugar beet crop losses resulted in decreased biomass and a reduction in sucrose content (Neher and Barbour 2019).

TSSM infestation is generally manifested by the yellowing of leaves, defoliation, and leaf burning (Asouguem *et al.* 2022; Shaabow *et al.* 2019). One generation of TSSM (from egg to adult stage) can be completed within 7 days when the air temperature is above 30°C (Tehri 2014). Such a short life cycle can result in 20–25 generations of this pest per year (Lucini *et al.* 2015), and, under the climatic conditions in Poland, with 4–6 generations during the growing season (Jakubowska *et al.* 2018). Such a developmental cycle significantly increases the damage that can be achieved by this pest.

Management of TSSM is primarily based on chemical control (Marcic 2012; Van Leeuwen *et al.* 2013). An important trait that makes the control of TSSM difficult is their high excretive metabolism, which results in a realistic possibility of the TSSM developing miticide resistance (Rioja *et al.* 2017; Jakubowska *et al.* 2022). Thus, there is an urgent need to look for new alternatives for TSSM control, especially environmentally friendly (namely biological control), which would result in a much limited impact on the environment and on human health (Gay 2012; Ayelo *et al.* 2021).

Biological control of TSSM can be initiated by attracting predatory insects that locate their prey by plant-induced volatile organic compound (VOC) emissions in response to TSSM feeding. The use of plant-derived volatiles that enhance the effectiveness of attracting the natural enemies of an attacking pest (or that act to deter feeding insects) can help reduce the extent of plant damage, as well as improve plant condition and yield (Dicke and Baldwin 2010). Therefore, VOCs are an important potential alternative to synthetic chemical insecticides for crop protection.

The emission of VOCs induced in response to a herbivorous insect attack can be altered by numerous abiotic factors (Ninkovic *et al.* 2021), including drought (Salerno *et al.* 2017). Water deficit stress is arguably one of the most common and important environmental factors associated with crop production. Drought affects the physiological and biochemical processes of plants (Loreto and Schnitzler 2010; Truong *et al.* 2014), and results in significant decreases in the growth and yield of most plants (Chandrasekaran *et al.* 2022; dos Santos *et al.* 2022). Drought conditions also alter plant-pest-predator and parasitoid-plant interactions (Rahman 2023).

The main goal of this study was to evaluate changes in the quantity and quality of the blend of VOCs released by sugar beet plants under drought conditions (abiotic stress), TSSM infestation (biotic stress), or subjected to combined types of stress simultaneously. The investigation's main objective was to identify emitted VOCs that could be of potential use in Integrated Pest Management. It was expected that from the investigation the following would be found: (i) that biotic and abiotic stress elicit distinct blends of VOCs released from sugar beet plants; (ii) VOC synthesis will vary over the days following a biotic stress and/or a continuing abiotic stress; and (iii) that combined biotic and abiotic stresses will result in even greater quantities of volatile emissions than one type of applied stress. The importance of this study is that it provided an improved understanding of the effects of drought on the release of VOCs from TSSM-infested sugar beet plants, and thereby contributes to the knowledge required to facilitate the use of VOCs as a part of an Integrated Pest Management protocol.

Materials and Methods

Spider mite and plant materials

Sugar beet plants of the Kujavia variety (Kutnowska Hodowla Buraka Cukrowego Sp.z o.o.) were grown in a glasshouse under conditions of $20 \pm 1^\circ\text{C}$ and day and night lengths of 16 h and 8 h (PPFD = $238.91089 \mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$), respectively, until they reached the 12–14 growth phase of the BBCH-scale (Biologische Bundesanstalt, Bundessortenamt und Chemische Industrie). The plants were then placed in plastic trays ($30 \text{ cm} \times 50 \text{ cm} \times 5 \text{ cm} = 7500 \text{ cm}^3$) in glasshouse cabins maintained at a temperature of $23 \pm 1^\circ\text{C}$, 50–55% relative humidity and applying the same photoperiod (16/8 h) for a further 2 weeks. These sugar beet plants were then placed in one of four groups: (1): control plants, i.e., those left unstressed and watered twice a week with 1400 ml (per tray containing eight plants) of water throughout the study; (2): plants watered twice a week with 1400 ml of water, but subjected to TSSM feeding for 24 hours at the end of the 2 week period (day 0); (3): plants watered twice a week with 800 ml of water, which will be referred to as a drought stress; and Group (4): plants subjected to the drought stress throughout the study (provided with 800 ml of water twice a week), but also subjected to 24 hours of TSSM feeding at the end of the 2 week period (day 0). Plants placed in groups 3 and 4 had continued water stress throughout the sampling period covering 6 days. Each of the above-mentioned groups of plants (1–4) consisted of eight plants.

For those groups of plants subjected to TSSM feeding (groups 2 and 4), five adult female mites were applied to just one leaf (third young leaf) per plant. All females used in the experiment were 1–2 days old and developed from nymphs selected 1 day earlier from a population reared on sugar beet plants under controlled conditions ($25 \pm 2^\circ\text{C}$, 16L:8D, 50 – 55% relative humidity). The newly emerged females were not fasted before the experiment and were not gravid. After 24 h of application of the females to the plants, the mites were gently removed from the plants' leaves using a brush. Signs of TSSM feeding were visible only on the leaf on which the mites were applied. As expected, sugar beets exposed to drought stress were characterized by fewer leaves than the well-watered plants (Fig. S1).

Volatile collection of samples

For volatile collection, the plants to be analyzed were individually placed in Nalophan sleeves (MediSense, NL) of volume 6000 cm³. Only the plants were enclosed by these bags. The pot containing the soil was not enclosed in order to avoid volatiles coming from the soil. The air containing the VOCs was drawn through Super-Q traps consisting of glass tubes 76 mm in length and having an outside diameter of 6.35 mm (Analytical Research System, Inc., Gainesville, FL, USA). These glass tubes contained 30 mg of Super-Q as the VOC adsorbent (Altech Associates, Inc., Deerfield, IL, USA). To sample, a glass tube was connected to one side of the sleeve and to a flexible tube, which was in turn connected to a suction pump (Thermo Fisher Scientific, Waltham, MA, USA). Positive pressure was maintained within the Nalophan sleeves to limit volatiles entering the sleeves from the outside. This was achieved by using an inlet flow of a purified and humidified air stream being delivered at a rate of $1.0 \text{ l} \cdot \text{min}^{-1}$ and having a suction pump maintaining an exhaust airstream at $0.8 \text{ l} \cdot \text{min}^{-1}$. The VOCs were collected and trapped over a period of 2 h (the same time was used in the study by Cao *et al.* 2022) on sampling days 2, 3 and 6 following female TSSM feeding on plant groups 2 and 4. For the group of plants subjected to drought stress (group 3), VOCs were collected on a date close to that on which VOCs were collected from TSSM-damaged plants. VOCs were collected from unstressed and well-watered sugar beet plants (control) at the beginning and at the end of the sampling period. Five clean air samples (laboratory blanks) were also gathered from Nalophan sleeves. Trapped volatiles were eluted from the Super-Q collection traps using 225 μL of hexane, with 7 ng of decane (CAS Number 204-686-4, $\geq 99\%$) being used to provide an internal standard. The quantity of hexane used has been found from previous studies to

be sufficient to extract all of the trapped VOCs (Piesik *et al.* 2011a, b).

Analytical methods

The total number of samples analyzed was 88 (plus 5 blank samples). Individual samples, each of 1 μL , were injected into and analyzed by a GC-MS using an Auto System XL/Turbomass instrument (Perkin Elmer, Shelton, CT, USA) with a capillary column (30 m Rtx-5MS, 0.25 mm ID, 0.25 μm film thickness, Restek, USA). The temperature program used during analysis increased from an initial 40°C up to 200°C at a rate of $5^\circ\text{C} \cdot \text{min}^{-1}$. The identification of the plant volatiles was verified using standards purchased from Sigma-Aldrich (USA), with the β -ocimene standard solution containing both *Z* and *E* isomers. The identification of the GLVs was achieved through a comparison of the experimentally obtained mass spectra and the linear retention indices calculated against the C8–C20 n-alkane mixture (Sigma-Aldrich, Steinheim, Germany) with those available in NIST 17 Mass Spectral and Retention Index Libraries (The National Institute of Standards and Technology, Gaithersburg, MD, USA). The emission rate of each VOC (in units of $\text{ng} \cdot \text{h}^{-1}$) was determined by comparing its associated peak area relative to the peak area of the internal decane standard. Only those VOCs that were consistently emitted at rates of greater than $0.1 \text{ ng} \cdot \text{h}^{-1}$ are reported in this paper.

Statistical analysis

The normality of the distributions of the observed VOCs was tested using Shapiro-Wilk's normality test (Shapiro and Wilk 1965). Multivariate analysis of variance (MANOVA) was carried out to determine the multivariate effects of stress, day and stress $\times$ day interaction (or unstressed for control). Two-way analyses of variance (ANOVA) were carried out to determine the effects of stress type and sampling day as well as stress type $\times$ sampling day interaction on the variability of the observed VOCs. The mean values of the individual VOCs were calculated. Moreover, Fisher's least significant differences (LSDs), at the 0.05 level ($\text{LSD}_{0.05}$), were calculated. On this basis, homogeneous groups were determined for stress type, day and stress type $\times$ day sampling interaction. Relationships between observed VOCs were assessed using Pearson's linear correlation coefficients, which are presented later in the results section as a heatmap. A canonical variate analysis (CVA) was applied to present a multi-VOC assessment of similarities for the combinations of the various stress type and sampling day in a lower number of dimensions with the least possible loss of information. CVA is a statistical tool making it possible to solve the problem of multivariate relationships

between combinations of stress type and sampling day for all VOCs jointly (Wrońska-Pilarek, Szkudlarz and Bocianowski 2018; Bocianowski and Majchrzak 2019). Mahalanobis distances (Mahalanobis 2018) were used as a measure of “multi-VOC” similarity of combinations of stress type and sampling day (Seidler-Łożykowska and Bocianowski 2012), the significance of which was verified using the critical value D_α , the least significant distance (Bocianowski and Liersch 2022). Mahalanobis distances were calculated for all pairs of combinations of stress type and sampling day. The differences between the analyzed combinations of stress type and sampling day as well as between VOCs were verified by cluster analysis using the nearest neighbor method and presented in dendrograms. The GenStat v. 23 statistical software package (2023) was used for the analyses.

Results

Identified VOCs

In response to water deficit stress and/or TSSM feeding, 10 VOCs that had emission rates > 0.1 ng/h from the sugar beet plants were identified from the gas chromatography with mass spectrometric detection (GC-MS) analysis, namely: four green leaf volatiles (GLVs): (Z)-3-hexenal = (Z)-3-HAL; (Z)-3-hexen-1-ol = (Z)-3-HOL; (E)-2-hexen-1-ol = (E)-2-HOL; (Z)-3-hexen-1-yl acetate = (Z)-3-HAC; three monoterpenes: (Z)-ocimene = (Z)-OCI; linalool = LIN; β -pinene = β -PIN; two sesquiterpenes: (E)- β -farnesene = (E)- β -FAR; β -caryophyllene = β -CAR; and one aromatic benzenoid benzyl acetate = BAC. Blank measurements did not show any background contributions to these volatiles. Raw data from the GC-MS analyses are provided in the supplementary information (Table S1).

Effects of stress type and sampling day on VOC emissions

All of the 10 VOCs had normal distribution. MANOVA results indicate that the main effects of stress type (Wilk's $l = 0.18668$; $F_{20;108} = 7.1$), sampling day (Wilk's $l = 0.01918$; $F_{20;108} = 33.59$) and stress type sampling day interaction (Wilk's $l = 0.06604$; $F_{40;207} = 5.41$) were significant ($p < 0.0001$) for all 10 VOCs jointly. ANOVA indicates that the main effects of stress type ($p < 0.001$), the sampling day ($p < 0.001$) of the volatile, effect of stress type sampling day interaction ($p < 0.001$ for (Z)-3-HOL, (E)-2-HOL, (Z)-3-HAC, (Z)-OCI, LIN, β -PIN, (E)- β -FAR, BAC; $p < 0.05$ for (Z)-3-HAL and β -CAR) were significant for all of the identified VOCs, with one exception

being for (E)- β -FAR for effects of stress type and stress type × sampling day interaction (Table S2). These observations and their associated results are discussed in more detail further below.

TSSM feeding (biotic stress) induced higher emission of VOCs

As expected, the analysis of the emitted VOCs from unstressed plants (the controls) revealed differences in their quantity compared to those VOCs emitted by plants that underwent any type of stress. The plants exposed to female TSSM feeding released significantly higher amounts of the 10 volatile compounds than the unstressed plants, regardless of the sample day (Fig. 1 and Table 1). The highest emission levels for all 10 VOCs were recorded on the second day of sampling, with the highest emissions being found for β -PIN ($1468 \text{ ng} \cdot \text{h}^{-1}$), β -CAR ($2959 \text{ ng} \cdot \text{h}^{-1}$), and BAC ($2400 \text{ ng} \cdot \text{h}^{-1}$). The emission of VOCs by sugar beet plants in response to TSSM feeding decreased over time. For sampling day 6 significantly lower emissions of all 10 VOCs than for the other two-time sampling points were observed. Based on these results, it can be concluded that the strongest response of sugar beet plants to TSSM damage occurred 48 hours after treatment and that the high emission rates of β -PIN, β -CAR and BAC over all of the sampling days suggest that they play a key role in the early sugar beet plant response to TSSM feeding.

Drought (abiotic stress) induced higher VOCs emission

The emission levels of individual volatile compounds were found to be higher in plants exposed to drought stress than in unstressed and well-watered plants, but lower than in plants exposed to TSSM infestation (Fig. 1 and Table 1). For all 10 VOCs, the greatest release from the plants was recorded on sampling day 2, with β -CAR ($2754 \text{ ng} \cdot \text{h}^{-1}$), BAC ($1558 \text{ ng} \cdot \text{h}^{-1}$) and β -PIN ($920 \text{ ng} \cdot \text{h}^{-1}$) being released in the greatest amounts. As found for biotic stress, there was a decrease in the amount of each volatile compound released with increasing time.

Combination of drought and TSSM infestation on the release of VOCs

The application of both biotic and abiotic stresses resulted in an additive effect on the release rate of VOCs, with the emission rates of the VOCs being increased when the water-stressed sugar beet plants were additionally subjected to TSSM infestation. With respect to the individual compounds, the emission rates of β -CAR, BAC and β -PIN were found to be the highest

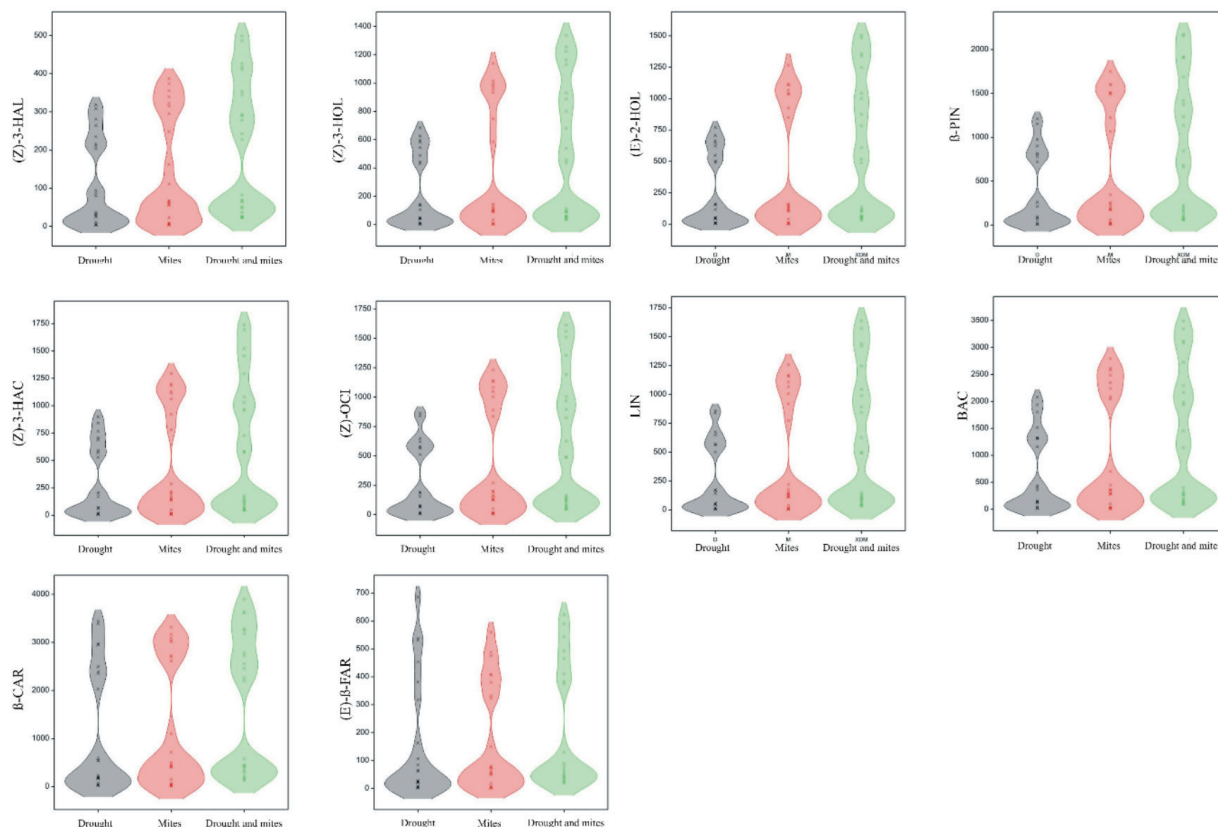


Fig. 1. The density of the 10 VOC distributions that were consistently found to be emitted at rates greater than 0.1 ng/h from the sugar beet plants exposed to drought, mites, and combined drought and mite. (Z)-3-hexenal = (Z)-3-HAL, (Z)-3-hexen-1-ol = (Z)-3-HOL, (E)-2-hexen-1-ol = (E)-2-HOL, (Z)-3-hexen-1-yl acetate = (Z)-3-HAC, (Z)-ocimene = (Z)-OCI, linalool = LIN, β-pinene = β-PIN, (E)-β-farnesene = (E)-β-FAR, β-caryophyllene = β-CAR and benzyl acetate = BAC.

Table 1. Mean values and Fisher's least significant differences (LSDs) at the 0.05 level of the 10 VOCs depending on the type of stress applied (drought, mites, or combined drought and mites) for sugar beet plants on sampling days 2, 3, and 6. The control plants were unstressed, well-watered sugar beet plants from which VOCs were collected at the beginning and at the end of the sampling period. Only those VOCs that were consistently detected at rates of greater than 0.1 ng · h⁻¹ are reported: (Z)-3-hexenal = (Z)-3-HAL, (Z)-3-hexen-1-ol = (Z)-3-HOL, (E)-2-hexen-1-ol = (E)-2-HOL, (Z)-3-hexen-1-yl acetate = (Z)-3-HAC, (Z)-ocimene = (Z)-OCI, linalool = LIN, β-pinene = β-PIN, (E)-β-farnesene = (E)-β-FAR, β-caryophyllene = β-CAR and benzyl acetate = BAC

Sampling day	Stress type	Green leaf volatiles (GLVs)				Monoterpenes		Sesquiterpenes		Aromatic benzenoid
		(Z)-3-HAL	(Z)-3-HOL	(E)-2-HOL	(Z)-3-HAC	(Z)-OCI	LIN	β-PIN	(E)-β-FAR	
2	drought	254.9 c	549 c	619.1 c	700.3 c	650.7 c	653 c	920.1 c	397.2 b	2754 a
	mites	329.8 b	919.4 b	1051.9 b	1086.2 b	1046.2 b	1056.7 b	1468.1 b	422.1 ab	2399.6 b
	drought and mites	397.4 a	1091.9 a	1232.4 a	1347.6 a	1263.5 a	1280.8 a	1734.2 a	485.5 a	3124 a
3	drought	51.6 ef	76.2 e	84.3 e	112.8 ef	110 ef	92.3 e	144.3 ef	41.7 c	331 cd
	mites	80.7 e	113.1 e	129.3 e	185.5 e	171.8 e	145.7 e	231.2 e	75.1 c	566 c
	drought and mites	169.9 d	312.9 d	357.1 d	433.3 d	374.2 d	369.6 d	512 d	70.2 c	1590 b
6	drought	5 f	5.9 e	7 e	11.2 f	10.2 f	7.9 e	17.9 f	4.2 c	35 d
	mites	8.3 f	10.5 e	11.3 e	17.6 f	16.9 f	12.8 e	21.9 f	6.2 c	50 d
	drought and mites	37.4 ef	48.2 e	51.4 e	75.8 ef	81 ef	62.7 e	97.6 ef	30.8 c	240 cd
LSD _{0.05}		54.26	123.2	132.7	165.7	147.7	151.5	200.2	79.24	510.9
Drought		103.8 C	210.4 C	236.8 C	274.8 C	257 C	251.1 C	360.8 C	147.7 B	605.8 C

Table 1. Mean values and Fisher's least significant differences (LSDs) at the 0.05 level of the 10 VOCs depending on the type of stress applied (drought, mites, or combined drought and mites) for sugar beet plants on sampling days 2, 3, and 6. The control plants were unstressed, well-watered sugar beet plants from which VOCs were collected at the beginning and at the end of the sampling period. Only those VOCs that were consistently detected at rates of greater than $0.1 \text{ ng} \cdot \text{h}^{-1}$ are reported: (Z)-3-hexenal = (Z)-3-HAL, (Z)-3-hexen-1-ol = (Z)-3-HOL, (E)-2-hexen-1-ol = (E)-2-HOL, (Z)-3-hexen-1-yl acetate = (Z)-3-HAC, (Z)-ocimene = (Z)-OCI, linalool = LIN, β -pinene = β -PIN, (E)- β -farnesene = (E)- β -FAR, β -caryophyllene = β -CAR and benzyl acetate = BAC – continuation

Sampling day	Stress type	Green leaf volatiles (GLVs)				Monoterpenes		Sesquiterpenes		Aromatic benzenoid	
		(Z)-3-HAL	(Z)-3-HOL	(E)-2-HOL	(Z)-3-HAC	(Z)-OCI	LIN	β-PIN	(E)-β-FAR	β-CAR	BAC
Mites		139.6 B	347.7 B	397.5 B	429.7 B	411.6 B	405.1 B	573.7 B	167.8 AB	1192 B	941 B
Drought and mites		201.6 A	484.3 A	547 A	618.9 A	572.9 A	571 A	781.3 A	195.5 A	1651 A	1268.8 A
LSD _{0.05}		31.33	71.1	76.6	95.7	85.3	87.5	115.6	45.75	295	188.6
2 days		327.4 X	853.4 X	967.8 X	1044.7 X	986.8 X	996.8 X	1374.1 X	434.9 X	2946 X	2245.3 X
3 days		100.7 Y	167.4 Y	190.2 Y	243.9 Y	218.7 Y	202.5 Y	295.8 Y	62.3 Y	829 Y	497.8 Y
6 days		16.9 Z	21.5 Z	23.2 Z	34.8 Z	36 Z	27.8 Z	45.8 Z	13.7 Z	108 Z	72.6 Z
LSD _{0.05}		31.33	71.1	76.6	95.7	85.3	87.5	115.6	45.75	295	188.6
Control before the experiment		3.3	1.3	1.5	4.5	0.7	1.2	0.8	0.9	0.9	1.0
Control after the experiment		2.2	1.5	1.1	4.5	0.7	0.8	0.4	1.3	1.6	0.7

a, b, c, d, e, f, g, h – in columns, mean values denoted by the same letters are not statistically significantly different

A, B, C – in columns, mean values denoted by the same letters are not statistically significantly different

X, Y, Z – in columns, mean values denoted by the same letters are not statistically significantly different

for the infested and water stressed sugar beet plants for each of the sampling days (Fig. 1 and Table 1). The emission rates of these three VOCs were recorded to be the greatest on the second day of sampling (β -CAR – $3124 \text{ ng} \cdot \text{h}^{-1}$, BAC – $2778 \text{ ng} \cdot \text{h}^{-1}$ and β -PIN – $1734 \text{ ng} \cdot \text{h}^{-1}$).

Correlation between monitored VOCs

All pairs of VOCs were found to have statistically significant linear correlations (all coefficients are significant at the 0.001 level), except for pairs with (E)- β -FAR, for which no significant differences were noted for sampling days 2 and 3, being directly correlated positive (Fig. 2).

Differences between plant stresses

The first and second canonical variates provided about 80% and 15%, respectively, of the total variation between the type of stress to which the sugar beet plants were subjected and sampling days. A clear clustering of the samples for sampling days 3 and 6 is observed. In contrast to the sampling day variable, no clear clustering of samples is evident for stress type (Fig. 3).

Differences between volatile compounds

For all analyzed volatile compounds, no statistically significant correlation with the first (V_1) and second (V_2) canonical variate was noted (Table 2). The greatest

variation in terms of all of the 10 VOCs was found between plants subjected to drought on sampling day 6 and drought plus mites feeding on sampling day 2 (Mahalanobis distance of 13.55). The greatest similarity was found between plants subjected to mites and drought on sampling day 6 (Mahalanobis distance of 0.22).

Clustering analyses combinations of stress type, sample days, and VOCs

All of the examined combinations of stress type and sample days were divided into two groups. The first group included well-watered plants exposed to TSSM feeding (sampling days 3 and 6 of the VOC collection) and plants subjected to drought stress (sampling days 3 and 6 of the VOC collection) or drought stress and TSSM infestation (sampling day 6 of the VOC collection). The second group included drought plus mites on sampling days 2 and 3 as well as mites, drought on sampling day 2, which is presented in a heatmap (Fig. 4). The 10 VOCs were also divided into two groups. The first group included β -CAR and BAC. The second group included all of the other VOCs (Fig. 4).

Discussion

Previous studies have shown that abiotic stress can modulate the blend of VOCs emitted by plants by inducing or suppressing the release of pre-existing VOCs and/or by inducing *de novo* synthesis of other VOCs

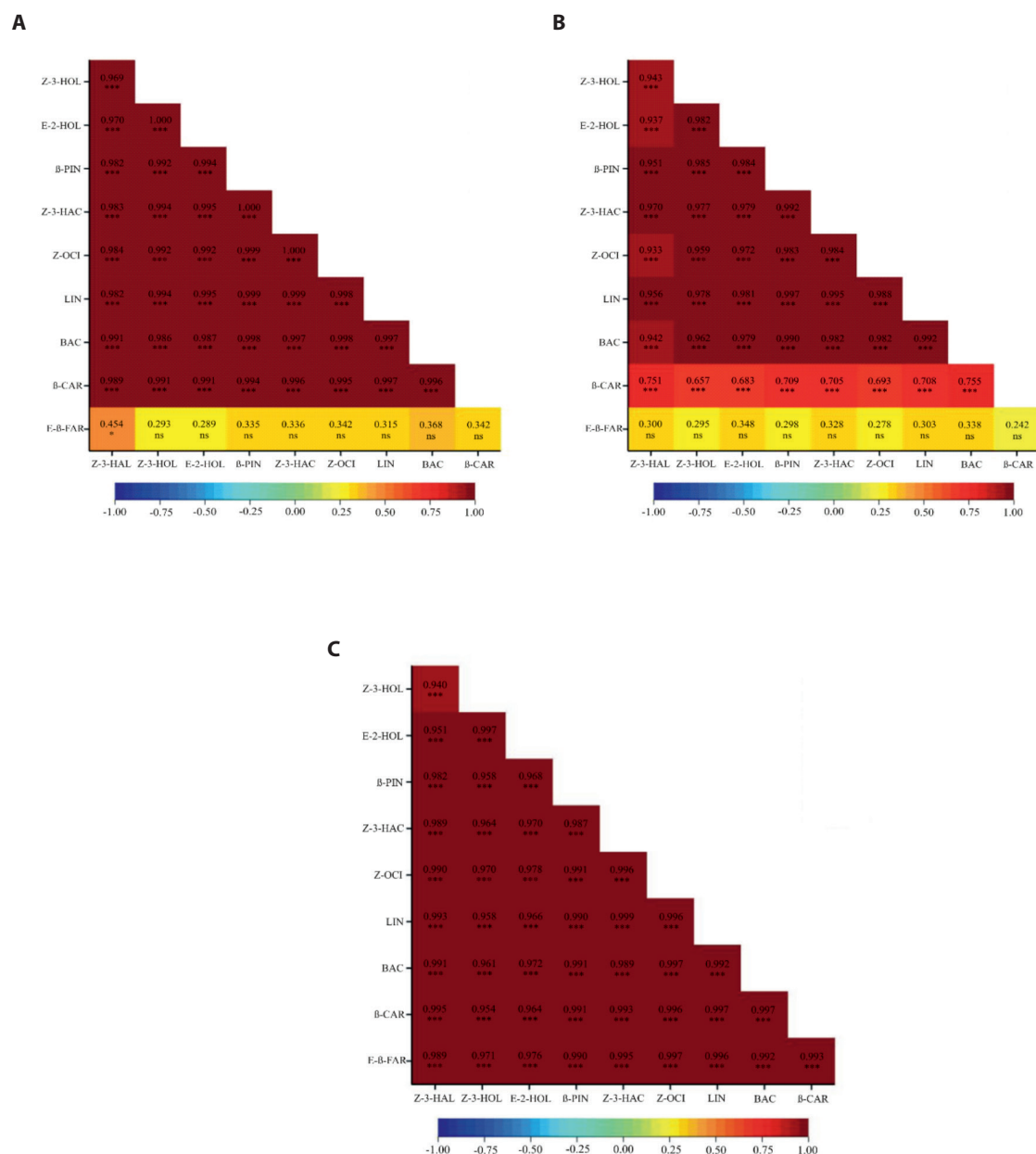


Fig. 2. Heatmaps showing the correlation coefficients between the 10 VOCs collected from sugar beet plants on sampling days 2 (A), 3 (B), and 6 (C). VOCs that were consistently detected at rates of greater than 0.1 ng/h are reported: (Z)-3-hexenal = (Z)-3-HAL, (Z)-3-hexen-1-ol = (Z)-3-HOL, (E)-2-hexen-1-ol = (E)-2-HOL, (Z)-3-hexen-1-yl acetate = (Z)-3-HAC, (Z)-ocimene = (Z)-OCI, linalool = LIN, β -pinene = β -PIN, (E)- β -farnesene = (E)- β -FAR, β -caryophyllene = β -CAR and benzyl acetate = BAC. Dark blue depicts the lowest and red the highest mean emission rates. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

(Sharifi and Ryu 2021; Lazazzara *et al.* 2022). In this study, the effect of drought, TSSM infestation and a combination of these two stresses on the quantity of VOCs released by sugar beet plants were examined.

The emission rates of 10 VOCs from sugar beet plants were found to be higher for plants subjected to drought conditions than for well-watered plants (the controls). These findings are consistent with other studies that have examined the effects of drought stress on the release of VOCs by other plants (Vallat *et al.* 2005; Wei *et al.* 2007), but inconsistent with another study dealing with sugar beet (Rahman 2023).

Vallat *et al.* (2005) observed that the emissions of four monoterpenes (α -pinene, β -pinene, limonene, and camphene, with the latter only detected in the summer of 2023) and two GLVs [(E)-2-hexenal and (Z)-3-hexen-1-ol] emitted from apple trees were negatively correlated to rainfall. The authors suggested that their results support the hypothesis that drought leads to a higher formation of secondary metabolites (several individual monoterpenes and resin acids). A similar finding was reported by Turtola *et al.* (2003), who analyzed monoterpenes in the main stem wood of Scots pine (*Pinus silvestris* L.) and Norway spruce

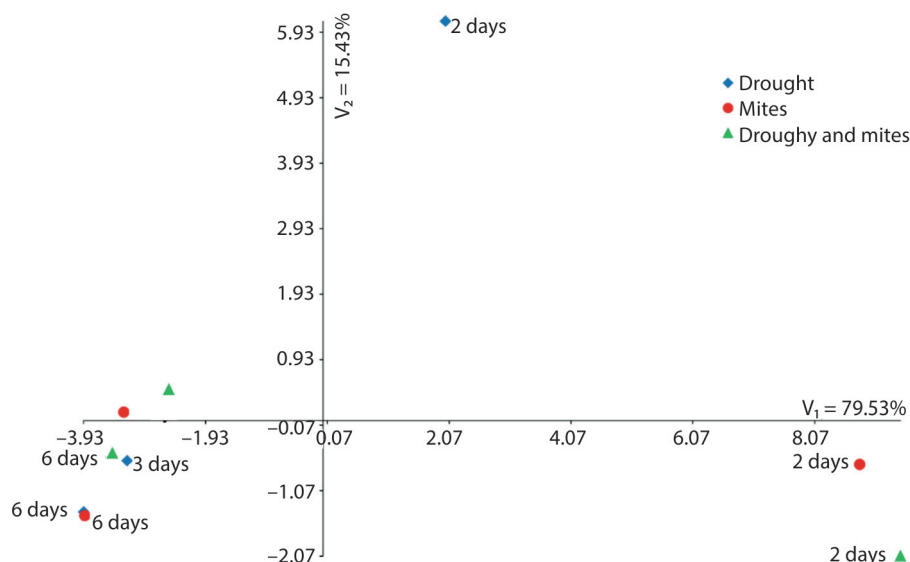


Fig. 3. Effects of the type of stress (drought, mites, and drought plus mites) on the distribution of the 10 VOCs emitted by sugar beet plants on the three sampling days (days 2, 3 and 6) in terms of the first two canonical variates, V_1 and V_2

Table 2. Results of discriminant analysis of the 10 VOCs detected at emission rates of greater than 0.1 ng/h from sugar beet plants in terms of the first two canonical variates (V_1 and V_2). (Z)-3-hexenal = (Z)-3-HAL, (Z)-3-hexen-1-ol = (Z)-3-HOL, (E)-2-hexen-1-ol = (E)-2-HOL, (Z)-3-hexen-1-yl acetate = (Z)-3-HAC, (Z)-ocimene = (Z)-OCI, linalool = LIN, β -pinene = β -PIN, (E)- β -farnesene = (E)- β -FAR, β -caryophyllene = β -CAR and benzyl acetate = BAC

VOCs	First canonical variate (V_1)	Second canonical variate (V_2)
(Z)-3-HAL	-0.4149	0.1758
(Z)-3-HOL	-0.3954	0.0918
(E)-2-HOL	-0.3964	0.0895
(Z)-3-HAC	-0.4065	0.1026
(Z)-OCI	-0.4022	0.1004
LIN	-0.3973	0.0992
β -PIN	-0.3966	0.1108
(E)- β -FAR	-0.3052	0.345
β -CAR	-0.3779	0.3114
BAC	-0.3972	0.1297

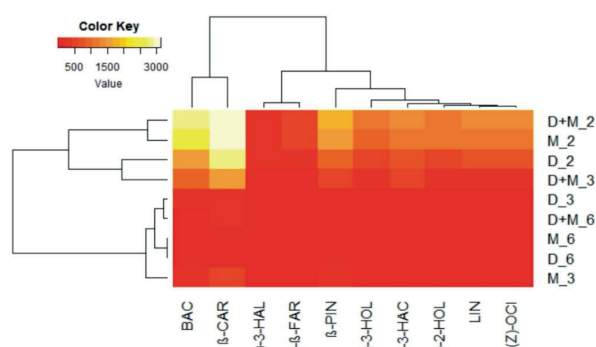


Fig. 4. Heatmap showing the quantitative occurrence of individual VOCs according to the stress type (M – mites, D – drought) applied or combined stresses (D + M – drought plus mites) and day of the sample collection (i.e., on sampling days 2, 3, and 6 following TSSM feeding). A dendrogram of clustering analyzed combinations of stress type and sample days, as well as the 10 VOCs that were emitted by the sugar beet plants consistently at rates of greater than 0.1 ng/h: (Z)-3-hexenal = (Z)-3-HAL, (Z)-3-hexen-1-ol = (Z)-3-HOL, (E)-2-hexen-1-ol = (E)-2-HOL, (Z)-3-hexen-1-yl acetate = (Z)-3-HAC, (Z)-ocimene = (Z)-OCI, linalool = LIN, β -pinene = β -PIN, (E)- β -farnesene = (E)- β -FAR, β -caryophyllene = β -CAR and benzyl acetate = BAC

[*Picea abies* (L.) Karst] seedlings subjected to medium and severe drought stress for two growing seasons. They found that medium drought stress had statistically no significant effect on the concentration of monoterpenes in both conifer species, whereas under severe drought stress an increase in the concentrations of several individual monoterpenes and the total amount of monoterpenes were noted. In contrast to these findings Rahman (2023) and Rahman *et al.* (2024) noted that sugar beet plants exposed to drought

stress emitted fewer volatiles and at lower concentrations than well-watered plants, with decreasing emissions being observed with increasing drought. The different outcomes in these three studies could imply that the amount of VOC emissions from plants under water stress depends not only on the species of the plants, but also on the severity and duration of drought stress.

Compared to a single stress, the combined stress of drought and TSSM infestation resulted in higher levels

of VOCs being released from the sugar beet plants than obtained from plants under one type of stress. This agrees with a study by Truong *et al.* (2014) dealing with *Arabidopsis* plants exposed to water stress and *Myzus persicae* (Sulzer) feeding. In their study, more terpenes were found to be emitted under double stress conditions than emitted by plants only subjected to a single stress. A similar finding was obtained by Copolovici *et al.* (2014). They reported significantly higher emissions of monoterpenes (α -pinene, β -pinene, camphene, limonene, 3-carene, *p*-cymene, β -phellandrene and (*E*)- β -ocimene) from drought stressed larvae-infested *Alnus glutinosa* plants (on days 2 to 7) than from those released from control plants and those exposed to only drought stress or larval feeding. Further, Gouinguéné and Turlings (2002) indicated that soil humidity had a considerable effect on the amount of volatiles emitted from mechanically damaged corn plants, where caterpillar *Spodoptera littoralis* (Boisduval) - derived regurgitant was applied to the damage. Significantly higher emissions of indole and (3*E*)-4,8-dimethyl-1,3,7-nonatriene were detected when the soil was relatively dry (20–40% relative humidity).

The problem of combined abiotic and biotic stress is complicated by the fact that the response of sugar beet plants to an abiotic stress can alter its ability to respond to biotic attack. Drought affects not only the physiological and biochemical processes of plants (Loreto and Schnitzler 2010; Truong *et al.* 2014), but it may also alter the plants' interactions with herbivorous pests by altering the blend of VOCs emitted (Orlovskis *et al.* 2015). Previous investigations have indicated that different abiotic stresses can have antagonistic or synergistic effects on a plant's resistance response to herbivorous insects (Suzuki *et al.* 2014). For example, Rahman (2023) and Rahman *et al.* (2024) found that drought altered the interaction between sugar beet plants, an aphid (*Aphis fabae*) and the attraction of its parasitoid (*Aphidius colemani*), owing to reduced plant volatile emissions, which benefited *Aphis fabae* with faster development and hence higher reproduction rates. In contrast to this, Copolovici *et al.* (2014) found that larvae of the green alder sawfly (*Monsoma pulveratum*) mostly preferred well-watered *Alnus glutinosa* plants to those plants subjected to drought stress. The leaf area eaten by this pest was significantly higher for well-watered plants than for drought-stressed plants. The authors suggested that plants subjected to drought were probably less palatable to the larvae. In turn, Takabayashi *et al.* (1994) noted that water-stressed Lima bean plants released greater amounts of linalool, (*E*)- β -ocimene, (3*E*)-4,8-dimethyl-1,3,7-nonatriene, and methyl salicylate than non-water-stressed plants. Thanks to these compounds, plants subjected to water stress were probably more attractive to predators than unstressed plants.

Of the 10 volatiles which were monitored, three, namely β -CAR (a sesquiterpene), β -PIN (a monoterpene) and BAC (an aromatic benzenoid), are considered to be useful as natural alternatives to synthetic miticides to protect sugar beet plants from TSSM. Surprisingly, BAC was recorded from green leaves not flowers, which is typical for this compound. However, Piesik *et al.* (2022, 2023, 2024) and Sendel *et al.* (2022) also found this component following the injury of green parts of the plant. This is because of their significant increase in their emissions from the sugar beet plants subjected to biotic/abiotic stresses compared to the unstressed plants. Various other studies suggest that most of the vegetative volatiles that have been identified in repelling herbivores or attracting herbivore enemies are either terpenes ((*E*)- β -farnesene, (*E*)- β -ocimene, and (*E*)- β -caryophyllene) or GLVs (e.g., isoprene) (Unsicker *et al.* 2009). For instance, the effect of constitutive overexpression of (*E*)- β -caryophyllene synthases increased the attraction of parasitoids to rice plants (Xiao *et al.* 2012) or improved the defense response of maize plants by attracting entomopathogenic nematodes that infect and kill voracious root pests (Degenhardt *et al.* 2009). The function these volatiles play in sugar beet plant-TSSM interactions is unknown. Hence, further research focusing on these three compounds will be undertaken.

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ORIGINAL ARTICLE

The effects of biotic and abiotic stress on the emission of volatile organic compounds by sugar beet plants

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Vol. 65, No. 3: 424–428, 2025

DOI: 10.24425/jppr.2025.155780

Received: February 26, 2024

Accepted: April 29, 2024

Online publication: September 01, 2025

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Responsible Editor:

Opendar Koul

SUPPLEMENTARY MATERIAL

The authors are fully responsible for both the content and the formal aspects of the supplementary material. No editorial adjustments were made.

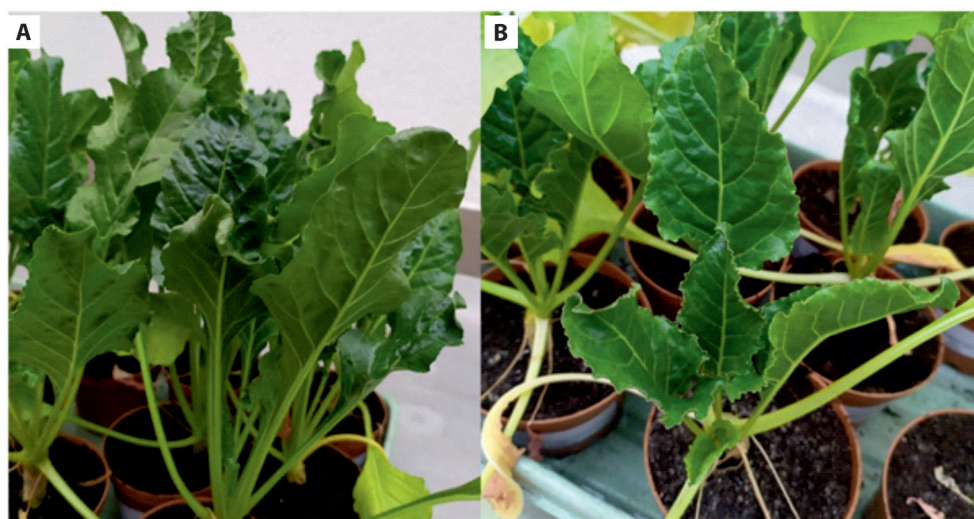


Fig. S1. Sugar beet plants: A – unstressed (control, watered twice a week with 1400 ml of water), and B – subjected to drought stress (watered twice a week with 800 ml of water) over a period of 2 weeks

Table S1. Raw data obtained from the GC-MS analyses of volatile samples collected from sugar beet plants depending on the stress type (i.e., drought conditions (800 mL of water twice a week), mite attack, and drought plus mite attack) for samples collected on sampling days 2, 3, and 6 following mite feeding. The controls were unstressed sugar beet plants (1400 mL of water twice a week). (Z)-3-HAL = (Z)-3-hexenal, (Z)-3-HOL = (Z)-3-hexen-1-ol, (E)-2-HOL = (E)-2-hexen-1-ol, β -PIN = β -pinene, (Z)-3-HAC = (Z)-3-hexen-1-yl acetate, (Z)-OCI = (Z)-ocimene, LIN = linalool, BAC = benzyl acetate, β -CAR = β -caryophyllene, and (E)- β -FAR = (E)- β -farnesene

Plant treatment/volatile compounds	(Z)-3-HAL	(Z)-3-HOL	(E)-2-HOL	β -PIN	(Z)-3-HAC	(Z)-OCI	LIN	BAC	β -CAR	(E)- β -FAR
Drought and mites – day 2										
1	499.25	1256	1486.25	2173.25	1738.75	1560.5	1638.75	3493.25	3282.25	622.75
2	486.25	1339	1504.25	2159.25	1694.75	1511.25	1573.75	3348.25	3902.75	590
3	411.75	1163.5	1341.25	1914	1524.25	1613.75	1441	3119.5	3183.75	465.75
4	418.25	1131.25	1248.25	1689.5	1294.25	1193.5	1247.75	2725	3259.75	492.75
5	427	1224.25	1356.5	1908.25	1456.25	1357	1418.75	3089.25	3615.25	543.5
6	353.5	932	1043.75	1416.75	1079.5	1004.5	1044	2296	2728	410.75
7	290	800.25	878	1238	962	898.25	892.5	1985.75	2457	374.75
8	293	889	1001.25	1374.5	1031	969	989.5	2170.25	2559.25	384
Mites – day 2										
1	315.5	977.75	1068.75	1494.75	1115.25	1047.5	1066.75	2347.25	2695.25	488.25
2	339.5	995	1108.5	1601	1198.5	1139	1167.75	2579	3036.5	476
3	355.75	935.25	1034.75	1508	1131.75	1083.25	1106.5	2487.25	3020.5	409.75
4	374.25	1014.75	1117.25	1598	1183.75	1134.75	1156.25	2613.25	3171	406
5	322.5	959.25	1043	1504.75	1062.75	1004	1006.75	2240	2727	332.75
6	387	1139.5	1267.25	1752	1294.25	1233.75	1257.25	2793.5	3316.75	380
7	295.5	747.25	850	1220.75	923	890.25	918.5	2086	2614.5	324
8	248.5	586.5	925.5	1065.25	780.5	837.25	773.75	2050.25	3094	560
Drought – day 2										
1	235	582	646.5	977	707.25	621	673.5	1804.75	2949.5	685.5
2	215.75	488.5	549.5	812.25	592	578.25	570	1323.75	2387.75	537.75
3	281.25	625.5	706.75	1152.5	842.5	840.5	840.75	1939.75	3393.5	106.75
4	211.5	430.75	491.75	776.5	572.75	564.5	561.5	1310.25	2356.25	453.75

Table S1. Raw data obtained from the GC-MS analyses of volatile samples collected from sugar beet plants depending on the stress type (i.e., drought conditions (800 mL of water twice a week), mite attack, and drought plus mite attack) for samples collected on sampling days 2, 3, and 6 following mite feeding. The controls were unstressed sugar beet plants (1400 mL of water twice a week). (Z)-3-HAL = (Z)-3-hexenal, (Z)-3-HOL = (Z)-3-hexen-1-ol, (E)-2-HOL = (E)-2-hexen-1-ol, β -PIN = β -pinene, (Z)-3-HAC = (Z)-3-hexen-1-yl acetate, (Z)-OCI = (Z)-ocimene, LIN = linalool, BAC = benzyl acetate, β -CAR = β -caryophyllene, and (E)- β -FAR = (E)- β -farnesene – continuation

Plant treatment/volatile compounds	(Z)-3-HAL	(Z)-3-HOL	(E)-2-HOL	β -PIN	(Z)-3-HAC	(Z)-OCI	LIN	BAC	β -CAR	(E)- β -FAR
5	204	442.25	502.5	722	530	512.5	500.25	1159.5	2030.75	163.5
6	318.75	684.75	769.75	1210.25	899.5	864.75	859	2079.25	3445.5	317.5
7	264.25	543.5	622.25	809.5	689.5	576.5	570.5	1327.75	2499.5	531.25
8	308.75	594.75	664	901	768.75	647.75	648.25	1518	2972	381.5
Drought and mites – day 3										
1	279	539	609.75	845	728	627.25	628	1455	2790.25	67
2	243.25	436.75	489	661	574.75	487.25	492	1142.5	2265.5	131
3	345	680.75	784.5	1138	964.25	824.25	845.25	1944	3639.25	39.5
4	228	458	518.25	681	584.5	494	498	1141.75	2198.25	89.25
5	81.75	92	110	223.75	176.75	163.5	147.75	401	579.25	78.5
6	51.75	88.75	97.5	162	133	119.25	102.75	261.25	365.5	48.25
7	64.25	113	135	206.75	164.5	148.5	130.25	328.5	452	58.75
8	66.25	95.25	113	178.75	141	130	112.75	300	433.25	49.25
Mites – day 3										
1	65.25	124.75	142	236.5	185.75	164.75	142	364.75	498.25	72.5
2	111	113.75	133	257.25	204.75	192	176.25	447.5	718.5	80
3	67.25	102.25	123.75	215.75	163.5	157.75	134	361.5	497.5	76
4	66.75	90.25	106.25	169.5	135.75	124.5	110.25	283	427.5	49.25
5	57.25	92	104.75	186	146.75	133.75	118.75	295.75	416.75	56.5
6	55.25	142	155	256.75	216.25	200.75	149.75	354	460.25	62.75
7	61	97	110.75	182.25	143	129.75	115.25	289	406.75	53.5
8	162.25	142.75	158.75	345.25	288	270.75	219.5	702.75	1100.25	150
Drought – day 3										
1	94.75	106	118	211.25	170.75	156.25	144.25	360.25	549.5	85
2	87.75	136	159.5	260.75	204	190.75	173.75	435.5	595.75	64.75
3	80.5	143.5	154.75	259.5	202.75	185	167.25	407.25	540.25	61.75
4	30	45	50.25	80.5	63	65.75	48.75	129	187	24.75
5	25.75	41.5	45.75	81.75	62.5	67	48.5	125.75	178.5	24
6	36.75	48.75	52.5	95.25	72.5	79.5	58.25	157	228	28.5
7	31.5	43.25	45	83	64	69.25	50.5	135.25	200.25	23.5
8	25.5	45.25	48.75	82.25	63.25	66.25	47.25	126	171	21.5
Drought and mites – day 6										
1	47.75	53	56	121.5	107	101.5	87.5	183.25	311	38.75
2	26	47.5	51.5	88	63.75	75.25	51.75	137.75	191.75	27
3	69.25	58.75	68	161	119.75	134	104.75	269	418.5	49.25
4	48	63	67	122.5	93.75	101.25	82	195.25	316	40.25
5	22	35.25	33	59.25	45.75	47.5	35.25	92.5	138	20.5
6	37	53.5	58.75	96	73.25	79.5	59.5	156.5	231.5	29
7	24.25	37	39	64	49.5	52.5	39.25	104.25	155.25	20.25
8	25	37.5	38	68.25	53.25	56.25	41.75	111.5	159.75	21

Table S1. Raw data obtained from the GC-MS analyses of volatile samples collected from sugar beet plants depending on the stress type (i.e., drought conditions (800 mL of water twice a week), mite attack, and drought plus mite attack) for samples collected on sampling days 2, 3, and 6 following mite feeding. The controls were unstressed sugar beet plants (1400 mL of water twice a week). (Z)-3-HAL = (Z)-3-hexenal, (Z)-3-HOL = (Z)-3-hexen-1-ol, (E)-2-HOL = (E)-2-hexen-1-ol, β -PIN = β -pinene, (Z)-3-HAC = (Z)-3-hexen-1-yl acetate, (Z)-OCI = (Z)-ocimene, LIN = linalool, BAC = benzyl acetate, β -CAR = β -caryophyllene, and (E)- β -FAR = (E)- β -farnesene – continuation

Plant treatment/volatile compounds	(Z)-3-HAL	(Z)-3-HOL	(E)-2-HOL	β -PIN	(Z)-3-HAC	(Z)-OCI	LIN	BAC	β -CAR	(E)- β -FAR
Mites – day 6										
1	22.75	35.5	40.25	62.5	48.25	51.75	37.5	103	149.5	18
2	8.25	7.25	8.5	19.25	15.25	15.5	11.5	37.25	50	6.75
3	4.25	8	7	15	12	10.75	8	25	31.75	3
4	3.25	3	1.75	9.25	7.25	6.25	4.5	16	22	2
5	3.75	4	5	10.5	8.5	7.5	5.5	19	25.75	2.5
6	8.75	8.75	8.75	20.25	16.5	15.75	11.75	40.25	53.25	4.5
7	7.25	9.5	10.75	24	18	17.25	13.25	38.75	57.25	6.5
8	8	7.75	8.5	14.5	14.75	10.25	10	10.75	11.75	6.5
Drought – day 6										
1	4.5	5	5.5	12.5	10	8.75	6.25	21.25	29.75	3.5
2	4	6.5	7.5	15	12	10.5	7.75	24.75	33.25	5
3	3.5	4.25	4.25	10	7.75	7	5	17	24	2.5
4	3.75	3.75	5.75	11	8	7	5	19	25.25	3.25
5	5	7	8.5	16	12.5	11.5	8.75	28	36.75	4.25
6	4	4.25	4	10.25	8.25	7.25	5.5	18.75	25.75	2.5
7	10.75	11	13.25	55	20.75	20.75	17.5	48	73	9
8	4.75	5.5	7.25	13.25	10.5	9.25	7.25	24.5	31.5	3.25
Control before the experiment										
1	2.5	3.5	2.4	0.6	6.4	0.4	0.9	1.2	1.6	1.4
2	2.3	2.1	2.1	0.5	4.3	0.5	1.2	1.3	1.1	1.2
3	3.5	0.5	0.5	0.4	5.0	0.5	1.1	0.5	0.5	0.5
4	4.2	0.9	0.6	0.2	3.5	0.4	0.5	0.9	1.2	0.6
5	6.2	0.4	2.1	1.6	4.1	2.1	0.9	0.4	0.6	0.8
6	0.9	0.5	1.5	1.8	6.0	0.5	2.1	0.5	1.2	0.1
7	2.6	1.0	1.6	0.5	3.2	0.6	1.5	2.1	0.6	2.1
8	4.0	1.2	1.4	0.8	3.5	0.4	1.0	1.2	0.4	0.5
Control after the experiment										
1	3.2	2.5	0.9	0.8	6.4	0.4	0.5	0.9	0.5	0.8
2	2.1	2.1	1.2	0.2	4.3	0.5	0.6	0.5	0.6	0.6
3	2.2	1.5	1.5	0.4	5.0	0.5	0.4	0.6	0.1	2.1
4	3.6	3.2	2.1	0.5	3.5	0.4	0.5	0.2	2.3	0.6
5	2.5	0.8	0.5	0.6	4.1	2.1	0.9	1.2	5.2	0.5
6	2.4	0.4	0.4	0.1	6.0	0.5	1.0	0.8	0.0	3.2
7	0.6	0.6	0.6	0.2	3.2	0.6	2.1	0.4	0.5	1.4
8	0.8	1.0	1.2	0.5	3.5	0.4	0.0	1.0	3.2	1.3

Table S2. Two-way analyses of variance (ANOVA) carried out to determine the effects of stress type and sampling day as well as stress type $\times$ sampling day interaction on the variability of the observed volatile organic compounds emitted by sugar beet plants. (Z)-3-hexenal = (Z)-3-HAL, (Z)-3-hexen-1-ol = (Z)-3-HOL, (E)-2-hexen-1-ol = (E)-2-HOL, (Z)-3-hexen-1-yl acetate = (Z)-3-HAC, (Z)-ocimene = (Z)-OCI, linalool = LIN, β -pinene = β -PIN, (E)- β -farnesene = (E)- β -FAR, β -caryophyllene = β -CAR, and benzyl acetate = BAC

Source of variation	Stress type	Sampling day	Stress type $\times$ sampling day
d.f.	2	2	4
(Z)-3-HAL	58675***	619085***	7453*
(Z)-3-HOL	450423***	4736072***	149657***
(E)-2-HOL	577509***	6098907***	196570***
β -PIN	1060980***	11959013***	314526***
(Z)-3-HAC	712825***	6819218***	185791***
(Z)-OCI	598908***	6109460***	169147***
LIN	614567***	6401569***	187842***
BAC	2637193***	31820651***	706407***
β -CAR	2431118***	52199929***	767179*
(E)- β -FAR	13826 ns	1274556***	3560 ns

* $p < 0.05$; *** $p < 0.001$; ns – not significant