ORIGINAL ARTICLE

Synergistic use of iron nanofertilizers and biotic elicitors to induce defensive volatile organic compound emissions from *Brassica napus*

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Abstract

In light of the increasing world's population and progressing climate changes, novel visions for agricultural practices are needed. In recent years nanofertilizers and elicitors have been investigated as methods to provide improved crop yield and quality. The potential of foliar application of iron nanofertilizers, elicitors (methyl jasmonate (MeJA) or methyl salicylate (MeSa)) and their combinations on the emission of volatile organic compounds (VOCs), have been evaluated for *Brassica napus*. The combined application of nanofertilizers and elicitors was found to result in an increase of VOC emissions by *B. napus* in comparison to their individual usage. The highest VOC emissions were observed at the time point 24 hours after the application of a 10 μg · ml^{-1} concentration of nanofertilizers and MeJa. To our knowledge, this is the first time that combinations of nanofertilizers and elicitors have been applied to plants to determine their response on the emission of plant defense volatiles.

Keywords: GC-MS, nanofertilizers, nanoparticles, methyl jasmonate, methyl salicylate, plant defense, rapeseed, semiochemicals, VOCs

Introduction

With our growing world population, which is estimated to reach 9.8 billion by 2050 (Islam and Karim 2020), assured future food security requires the development and application of advanced agricultural practices to improve crop production (i.e., yield and quality) with minimal environmental impact. Current agricultural activities are harmful, both to humans and to the environment (Donley 2019), and have a large climate impact. Therefore, less harmful agricultural practices are needed. These could include novel foliar application of nanofertilizers or substituting chemical pesticides with compounds (e.g., elicitors) to induce natural plant defense mechanisms or to promote stress tolerance in plants. For example, Semida *et al.* (2021) demonstrated that the foliar application of zinc oxide nanoparticles promotes drought stress tolerance in eggplants thereby overcoming nutrient deficiencies and increasing crop yield. With regards to pest and pathogen control to improve crop yield, the application of elicitors [e.g., methyl jasmonate (MeJA) or methyl salicylate (MeSa)] has been demonstrated to enhance plant protection volatiles against pathogens and pest attack (Sobhy *et al.* 2014).

The use of nanomaterials (e.g., nanofertilizers and nanopesticides) is a particularly exciting new development in agriculture owing to their high potential to increase plant tolerance to biotic and abiotic stresses leading to more sustainable agriculture, especially with the predicted global warming (Wu and Li 2022). This is possible because plants possess receptors for nanoparticles (Jośko *et al.* 2017). Importantly, these nanoparticles directly regulate the biosynthesis of signal plant molecules, facilitate the transport of nutrients and expression of a plant's defense genes, in addition to controlling various secondary metabolic pathways (Wang *et al.* 2020). A particularly good use of nanoparticles is associated with nanofertilizers which contain micronutrients that can be delivered in a controlled way to a plant's rhizosphere (Nongbet *et al.* 2022). The ability of nanofertilizers to increase crop yield has been investigated for many years, for crops such as wheat (Abdel-Aziz *et al.* 2018) and cotton (Sohair *et al.* 2018), with promising results on crop yield and nutritional value. Positive effects of nanofertilizers on crop growth, yield, quality, and nutrients can be related to the reduction of abiotic stress and heavy metal toxicity (Nongbet *et al.* 2022). Additionally, nanoparticles might also accelerate phytohormone biosynthesis, differential gene expression for elemental transporters (Tripathi *et al.* 2022), regulate synthesis of carbohydrates, amino acids, and fatty acids (Hatami *et al.* 2016) and increase the production of antioxidants (Wang *et al.* 2020).

Emissions of green leaf volatiles (GLVs) or terpenoids from plants provide a communication method to repel herbivores, to attract natural enemies of attacking pests, to provide defense against pathogen attack, and to warn other plants of potential herbivore or pathogen attack (Ameye *et al.* 2018). The release of VOCs classified as GLVs is caused by mechanical damage (Piesik *et al.* 2011) done by herbivores (Allmann *et al.* 2013), fungal or bacterial infections (Piesik *et al.* 2011; Ponzio *et al.* 2013), or by abiotic stress, such as drought (Wenda-Piesik 2011), and heat (Copolovici *et al.* 2012) which provokes internal and external plant cell response (Sharifi and Ryu 2021). In agricultural systems, this phenomenon has been used by the artificial use of VOCs such as GLVs or semiochemicals (sex pheromones, aggregation pheromones, and plant volatile compounds used as attractants as well as repellents) for integrated pest management (Gaffke *et al.* 2021). Similarly to physical damage, the exposure of plants to exogenous volatiles such as MeJA or MeSa can induce stress related production of VOCs (Rahnamaie-Tajadod *et al.* 2019; Brosset *et al.* 2021). Briefly, stimulation by artificial elicitors, such as the ones mentioned above, triggers the defense response not only of a particular plant, but also releases a chain reaction, during which VOCs released by one plant reach other plants and stimulate them to release similar compounds (Mithöfer and Maffei 2016). This approach may be named conservation biological control, and is used in New Zealand and Australia for *Sorghum bicolor* or *Zea mays* (Chidawanyika *et al.* 2012).

The key objectives of this study were to demonstrate (i) that iron-nanoparticles, MeSa, and MeJa can be used to activate the natural defense system of *B. napus* L. (Brassicaceae) and (ii) to elucidate how the emission of VOCs depends on duration and concentration of exposure. Here, we studied VOCs' production following foliar application of iron nanoparticles separately and in combination with MeSa and MeJa. The type and amount of VOCs induced were examined 24 h and 72 h after foliar spraying.

Materials and Methods

Brassica napus cultivation

The experiments were performed at the Plant Growth Center at Bydgoszcz University of Science and Technology, Bydgoszcz, Poland. Winter oilseed rape, *B. napus*, cv. 'Tommy' plants were planted and grown in a greenhouse (even span greenhouse) with supplemental light (16 h of light and 8 h of darkness) and an ambient humidity of 75–85%. Day and night temperatures were maintained at 22 ± 2°C and 18 ± 2°C, respectively. Plants were grown with one individual per pot (diameter 20 cm, height 20 cm) in sterilized soil (consisting of 60% peat-type organic matter and 40% mineral fraction (Profi Substrate, Gramoflor GmbH & Co. KG). The substrate was sterile, homogeneous, free of any odors, and used as an inert mixture for control growing. It was watered four times weekly (four irrigations were provided per week, with each application at a dose of 80–100 ml per pot) , and fertilized twice weekly (20-20-20 NPK fertilizer, NPK ChemiRol, Poland) – balanced fertilizer, i.e., total nitrogen 20% (including nitrate nitrogen $N\text{-}NO_3$ – 4.3%, ammonium nitrogen – $N\text{-}NH_4$ – 2.4% and amide nitrogen – $N\text{-}NH_2$ – 13.3%); phosphorus pentoxide P_2O_5 – 20% and potassium oxide K_2O – 20%. Twice weekly fertilization was prepared along with irrigation at the amount of 100 ml per pot (fertilization concentrate was 0.63 $g \cdot l^{-1}$ water). All plants were used at BBCH 19 (Biologische Bundesanstalt, Bundessortenamt und Chemische Industrie – a scale used to identify the phenological development stages. A total of 160 individual plants were used separately in this study and VOCs were collected separately from each *B. napus* plant.

Application of nanoparticles and plant elicitors and spraying procedure

The plants were subjected to iron nanoparticles and artificial stress induction procedures using MeSa and

MeJa. The iron nanoparticles, diameter <30 nm, were purchased from American Elements (Northern Trust, FL, USA, (https://www.americanelements.com/iron-nanoparticles-7439-89-6), product code: Fe-M-03-NP, average Particle Size 100–250 nm, specific Surface Area 3–7 m^2/g, morphology spherical.

The iron nanostructures were provided in the form of a powder coated with carbon with a stated purity of >99%. To achieve specific nano-Fe concentrations, a stock solution with a concentration of 100 ppm was prepared (0.01 g of powder was added to 100 ml of bi-distilled water). The flask with the solution was placed in an Elma S 80H Elmasonic ultrasonic cleaner for 3.5 minutes, which created a suspension that was applied to the leaves. Two doses of nanoparticles, i.e., 5 and 10 $\mu g \cdot ml^{-1}$, were applied. To achieve the given amounts, a 100 $\mu g \cdot ml^{-1}$ solution was prepared (0.01 g of the powder was added to 100 ml of distilled water).

MeSa and MeJa were purchased from Sigma-Aldrich (purity 95%). Separate solutions were prepared with each containing 250 μg of the respective compound per 100 ml of distilled water.

Four different treatment groups were set up; each of them consisting of 32 *B. napus* plants (Table 1). The first group was sprayed with liquids containing only iron nanoparticles using the concentration levels mentioned above. Thus, 16 plants had 50 $\mu g \cdot ml^{-1}$ (concentration 1) applied and 16 plants had 100 $\mu g \cdot ml^{-1}$ (concentration 2) applied. The second group was exposed to MeSa (16 plants) and MeJa (16 plants) with the same concentrations (50 $\mu g \cdot ml^{-1}$ or 100 $\mu g \cdot ml^{-1}$). The third group received a combination of nanoparticle-solution and MeSa solution (32 plants) with the same concentrations (50 $\mu g \cdot ml^{-1}$ or 100 $\mu g \cdot ml^{-1}$). The fourth group was subjected to both iron nanoparticles and MeJa solution (32 plants) with the same concentrations (50 $\mu g \cdot ml^{-1}$ or 100 $\mu g \cdot ml^{-1}$). The surfaces of the plants were directly sprayed with 1 mL of each application mixture. A fifth group of 32 plants received no treatments, i.e., they were not subjected to any artificial stress apart from being placed in Nalophan bags during volatile collection, and hence acted as controls.

Collection of volatile organic compounds (VOCs)

Sampling of the VOCs emitted from *B. napus* was carried out according to the method described by Piesik *et al.* (2011) using adsorption tubes (diameter of 6.35 mm and a length of 76 mm, Analytical Research System, Inc., Gainesville, FL, USA), containing 30 mg Super-Q polymer (Divinylbenzene/Ethylvinylbenzene) adsorbent (Alltech Associates, Inc., Deerfield, IL, USA). Briefly, the plants were placed in Nalophan bags, which were connected with flexible silicone tubes to a pump (Thermo Fisher Scientific, Waltham, MA, USA) used to transfer air. In this way the VOCs were sent to the adsorption tubes and hence the VOCs contained therein. Purified and humidified air was introduced into the bottom of the Nalophan bag at a rate of 1.0 $l \cdot min^{-1}$, while the flow rate for the collection of the VOCs was set at 0.8 L min^{-1} in order to maintain a positive pressure. For all groups (1–5) of plants, VOCs were collected using the following exposure procedures:

1. Iron nanoparticle application using two concentrations, with the VOCs collected after 72 h;
2. MeSa and MeJa application, with the VOCs collected after 24 and 72 h;
3. A combination of iron nanoparticles and MeSa or iron nanoparticles and MeJa on the first day of application of nanoparticles, on the second day of application of MeSa or MeJa, with the VOCs collected after 24 and 72 h.

Table 1. List of treatments used in the experiments under consideration

Methyl jasmonate – MeJa [$\mu g \cdot ml^{-1}$]		Methyl salicylate – MeSa [$\mu g \cdot ml^{-1}$]	
treatment	symbol	treatment	symbol
after iron-NPs application (+ 4 days), concentration 1	A	after iron-NPs application (+ 4 days), concentration 1	I
after iron-NPs application (+ 4 days), concentration 2	B	after iron-NPs application (+ 4 days), concentration 2	J
after MeJa application (+ 24h), without iron-NPs	C	after MeSa application (+ 24h), without iron-NPs	K
after MeJa application (+ 72h), without iron-NPs	D	after MeSa application (+ 72h), without iron-NPs	L
after MeJa application, (+24h), with iron-NPs, concentration 1	E	after MeSa application, (+24h), with iron-NPs, concentration 1	M
after MeJa application, (+72h), with iron-NPs, concentration 1	F	after MeSa application, (+72h), with iron-NPs, concentration 1	N
after MeJa application, (+24h), with iron-NPs, concentration 2	G	after MeSa application, (+24h), with iron-NPs, concentration 2	O

For each kind of exposure and for each collection, eight samples were taken.

Extraction of volatile organic compounds and subsequent GC/MS analysis

For the extraction of the VOCs (*Z*-3-hexenal= *Z*-3-HAL, *E*-2-hexenal=*E*-2-HAL, *Z*-3-hexenol=*Z*-3-HOL, *Z*-3-hexenyl acetate=*Z*-3-HAC, *Z*-β-ocimene= *Z*-OCI, linalool=LIN, benzyl acetate=BAC, methyl salicylate=MeSa, indole=IND, β-caryophyllene= β-CAR, and *E*-β-farnesene=*E*-β-FAR) from the "Super-Q trap", the adsorbent was extracted three times using 250 µl of *n*-hexane (Sigma-Aldrich, Steinheim, Germany). Thereafter, 7 ng of *n*-decane (Sigma--Aldrich, Steinheim, Germany) was added to the combined extract as an internal standard. Then the extracts were concentrated using a rotary-vacuum evaporator to approximately 200 µl and placed in 1.5 ml chromatographical vials with 300 µl glass inserts (Sigma-Aldrich, Steinheim, Germany). For the analysis of the extracted VOCs, a gas chromatograph coupled to mass spectrometer (GC-MS) AutoSystem XL system (Perkin Elmer, Shelton, CT, USA), equipped with a Restek (Bellefonte, PA, USA) DB-5MS column (30 m, 0.25 mm internal diameter, 0.25 µm film thickness) was used. The injection conditions were as follows: 250°C, sample volume 1 µl, helium with flow 1.0 ml $\cdot$ min^{-1} as a carrier gas, and split ratio 1 : 10. Separation of the analytes was obtained using the following GC program: initial temperature 40°C, which was then raised to 200°C at a rate of 5°C $\cdot$ min^{-1}. The ion source of the mass spectrometer was maintained at 250°C and the interface temperature was set to 200°C. The scan range was set to 35–350 *m/z*, using a scan rate of 1250 amu $\cdot$ s^{-1}.

Identification of analytes was performed by comparing experimentally obtained mass spectra with those available in the NIST 17 Mass Spectral and Retention Index Libraries (National Institute of Standards and Technology, Gaithersburg, MD, USA); as potential targets only compounds with similarity score ≥ 90% were considered (a lower similarity score was not taken into consideration). A semi-quantitative analysis was performed. Thereby the peak areas of the identified 11 volatile compounds [confirmed by synthetic standards (Sigma-Aldrich, Steinheim, Germany)] were calculated, and the total of the values was divided by the peak area of the *n*-decane.

Statistical analysis

All analyses of VOCs were conducted separately for MeJa and MeSa applications. A Shapiro-Wilk's normality test was used to test the normality of the distribution of 11 VOCs. A one-way multivariate analysis of variance (MANOVA) test was performed. One--way analyses of variance (ANOVA) were carried out to determine the main effects of applications. Differences between applications were compared by Fisher's least significant differences (LSDs). The correlations between all pairs of observed VOCs were calculated using Pearson's linear correlation coefficients. Correlation coefficients were tested, which are presented later as heatmaps. A canonical variance analysis (CVA) and Mahalanobis distances were used for the multivariate comparison of applications. Discriminant analysis was carried out to determine the relative share of each original VOC in the multivariate variation of the treatments using Pearson's correlation coefficients.

The elementary comparisons between particular levels of the analyzed applications for MeJa and MeSa were tested using the two-sample *t*-test for equal means for all the observed VOCs. To account for multiple testing, we used the Bonferroni correction.

The data were analyzed using GenStat v. 23.1 software (VSN International; Hemel Hempstead, UK).

Results

VOCs identification

Eleven volatile compounds could be identified in the plants' headspace namely: *Z*-3-HAL, *E*-2-HAL, *Z*-3-HOL, *Z*-3-HAC, *Z*-OCI, LIN, BAC, MeSa, IND, β-CAR and E-β-FAR. These identified volatiles may be classified as Green Leaf Volatiles.

Influence of elicitors and iron nanoparticles on *Brassica napus* VOCs emission

All the VOCs were found to have a normal distribution. MANOVA results (Wilks' λ = 0.00003; $F_{77;283}$ = 17.70) indicated statistically significant ($p < 0.0001$) differences between applications for all VOCs taken together. Analysis of variance indicated that the main effect of application was significant for all the VOCs (Table 2). Mean values and standard deviations of observed VOCs for applications of MeSa are also presented in Table 2. The highest VOC emissions were found for the samples taken at the time point 24 h associated with exposure of the third group treated with a combination of MeJa and iron nanoparticles (Table 3).

Statistically significant positive correlations for MeJa were observed between all pairs of VOCs except *Z*-3-HAL and *E*-β-FAR, *E*-2-HAL and *E*- β-FAR, *Z*-3-HOL and *E*-β-FAR, *Z*-3-HAC and *E*-β-FAR, as well as *E*-2-HAL and MeSa, as indicated in the heatmap shown in Figure 1 (see also Table S1, supplementary material).

Table 2. Mean values and standard deviations (s.d) for VOC concentrations that are emitted after a plant is subjected to MeJA and /or iron nanoparticles. Fisher's least significant differences (LSDs) and *F*-statistics from one-way analysis of variance for MeJa

VOCs		Treatment								LSD0.05	Average	Control	Control + 7 days	*F*-ANOVA
		A	B	C	D	E	F	G	H					
		[ng]								[ng]	[ng]	[ng]		
Z-3-HAL	mean	29.5 e	63.7 e	707.5 b	175.5 de	845.4 b	239.8 cd	1155.9 a	382.2 c	154.2	499.9	2.5	3.3	56.48***
	s.d.	11	16.6	92.4	40.8	142.8	71.6	365.3	141.5		412.0	1.8	2.3	
E-2-HAL	mean	3.09 c	5.11 c	26.38 b	2.93 c	34.18 b	7.97 c	64.45 a	9.05 c	9.4	19.14	0.9	2.5	42.46***
	s.d.	1.375	1.596	16.094	1.319	16.646	4.619	11.741	1.961		22.23	1.3	2.3	
Z-3-HOL	mean	5.27 e	15.83 de	31.32 c	19.64 cd	61.98 b	24.14 cd	153.15 a	28.77 cd	13.26	42.51	1.1	0.3	103.59***
	s.d.	5.99	7.26	10	3.2	17.67	15.85	24.19	7.44		46.63	0.9	0.7	
Z-3-HAC	mean	48.5 f	67.7 f	835.1 c	185.1 ef	1310.2 b	259.1 de	1544.7 a	434.4 d	181.3	585.6	2.2	2.3	82.15***
	s.d.	21	13	94	52.1	373.3	58.1	298.2	134.8		572.7	0.7	1.2	
(*Z*)-OCI	mean	21.8 e	19.8 e	285.8 d	331.4 d	407.5 cd	465 bc	540.8 ab	646.5 a	131.9	339.8	1.2	1.8	23.79***
	s.d.	8.5	4.2	101.2	77	83.1	110.9	172.9	271		247.6	1.0	0.4	
LIN	mean	19.1 e	25.8 e	260.3 d	541.9 b	401.3 c	558.1 b	617.2 ab	686 a	77.57	388.7	2.5	0.8	91.05***
	s.d.	4.25	8.61	75.06	96.66	103.19	80.01	100.24	76.21		256.9	2.6	0.5	
BAC	mean	43.88 e	52.06 de	71.81 cd	46.4 e	104.26 b	76 c	177.24 a	108.21 b	22.41	84.98	1.1	1.1	31.83***
	s.d.	15.33	17.81	12.35	10.28	22.08	36.17	25.42	27.41		47.06	1.8	0.4	
MeSa	mean	44.08 d	42.91 d	93.7 bc	88.36 c	126.16 b	111.78 bc	121.98 bc	169.96 a	34.93	99.87	1.6	0.8	11.99***
	s.d.	20.53	19.14	15.23	11.86	59.73	33.57	35.36	51.23		51.98	1.1	0.7	
IND	mean	3.3 e	3.01 e	14.06 de	32.22 c	25.85 cd	50.69 b	74.72 a	51.04 b	15.19	31.86	1.2	0.9	22.67***
	s.d.	2.66	1.29	4.76	6.03	5.78	16.61	14.13	35.53		27.99	0.9	1.0	
β-CAR	mean	30.7 e	34.8 e	894.9 b	341 d	98.6 e	472.9 d	1232.6 a	656.2 c	158.1	470.2	3.4	4.8	61.22***
	s.d.	14.7	9.6	283.8	76.3	21	68.8	300.1	131.5		437.6	1.0	3.4	
β-FAR	mean	25.7 e	34.2 e	658.8 d	1237.5 c	119.8 e	1491.5 b	1015.3 c	1813.5 a	234.1	799.5	2.8	3.3	71.35***
	s.d.	11	21.5	69.8	382.3	19.5	376.6	286.3	247.8		694.1	1.5	1.3	

A – after iron-NPs application (+ 4 days)a concentration 1; B – after iron-NPs application (+ 4 days), concentration 2; C – after MeJa application (+ 24h), without iron-NPs; D – after MeJa application (+ 72h), without iron-NPs; E – after MeJa application, (+24h), with iron-NPs, concentration 1; F – after MeJa application, (+72h), with iron-NPs, concentration 1; G – after MeJa application, (+24h), with iron-NPs, concentration 2; H – after MeJa application, (+72h), with iron-NPs, concentration 2; *Z*-3-hexenal=*Z*-3-HAL, *E*-2-hexenal=*E*-2-HAL, *Z*-3-hexenol=*Z*-3-HOL, *Z*-3-hexenyl acetate=*Z*-3-HAC, *Z*-β-ocimene=*Z*-OCI, linalool=LIN, benzyl acetate=BAC, methyl salicylate=MeSa, indole=IND, β-caryophyllene=β-CAR, and *E*-β-farnesene=*E*-β-FAR; *** $p < 0.001$. In rows, average values marked with the same letter are not statistically differentiated

Table 3. Mean values and standard deviations (s.d.) for VOC concentrations that are emitted after a plant is subjected to MeSA and /or iron nanoparticles, Fisher's least significant differences (LSDs) and *F*-statistics from one-way analysis of variance for MeSa

VOCs		Treatment								LSD0.05	Average	Control	Control + 7 days	*F*-ANOVA
		I	J	K	L	M	N	O	P					
		[ng]								[ng]	[ng]	[ng]		
Z-3-HAL	mean	31.4 f	55.7 ef	347.6 c	71.9 ef	434.4 b	107.7 de	612.3 a	163.d	76.25	228.0	2.4	2.7	62.31***
	s.d.	7.5	20.02	86.07	13.67	108.8	16.7	151.94	55.74		212.8	1.2	1.4	
E-2-HAL	mean	3.91 c	5.44 c	23.24 b	5.6 c	27.35 b	7.51 c	50.11a	9.55 c	10.53	16.59	0.8	0.7	18.85***
	s.d.	3.25	2.454	13.441	2.595	20.337	5.883	13.882	6.223		18.15	0.4	0.5	
Z-3-HOL	mean	5.81 d	10.25 cd	35.05 bc	16.34 cd	47.54 b	24.91 bcd	150.99 a	27.24 bcd	26.39	39.77	0.7	1.4	25.36***
	s.d.	5.71	4.82	11.25	4.27	21.8	8.16	68.41	11.42		50.72	0.5	2.0	
Z-3-HAC	mean	56.2 e	70.4 e	464.7 c	88.1 e	641 b	115.6 de	821 a	225 d	127.6	310.2	4.0	5.9	41.98***
	s.d.	25.84	16.76	130.52	17.86	239.1	20.62	201.12	116.47		303.3	1.2	1.5	
(*Z*)-OCI	mean	24.8 c	17.4 c	160.2 b	156.9 b	195.7 b	264.8 a	268.7 a	281.6 a	65.07	171.3	0.8	1.4	20.70***
	s.d.	18.62	8.11	41.33	63.61	96.97	101.64	46.71	75.37		116.0	0.4	1.4	
LIN	mean	11.6 e	24.6 e	128.6 d	253 bc	188.6 cd	264.9 b	268.1 b	366.6 a	74.87	188.3	1.5	4.4	22.45***
	s.d.	5.4	9.63	23.91	95.23	34.11	63.03	42.96	167.19		137.5	1.1	2.7	
BAC	mean	36 e	46 e	306.4 c	178.6 d	413.8 bc	313.5 bc	777.5 a	422 d	114.6	311.7	0.8	0.1	35.26***
	s.d.	16.65	12.01	42.59	55.37	78.65	84.59	184.92	227.79		250.9	1.0	0.2	
MeSa	mean	41 e	43.4 e	384.6 cd	339.3 cd	479.3 b	382.4 cd	448.2 bc	621.1 a	83.8	342.4	0.9	2.9	47.57***
	s.d.	13.12	14.43	63.42	108.94	67.49	74.21	85.31	149.55		207.8	0.7	1.6	
IND	mean	2.76 c	4. 99 c	11.16 c	34.27 b	30.79 b	43.3 b	66.62 a	62.61 a	12.57	32.06	0.4	3.2	31.12***
	s.d.	3.96	3.27	3.52	9.45	16.29	11.14	20.53	17.91		26.17	0.8	1.8	
β-CAR	mean	23.4 f	26.9 f	261.7 b	99.5 e	355.8 a	152.7 d	392.1 a	207.2	48.24	189.9	3.7	4.4	67.85***
	s.d.	10.33	12.21	78.23	26.67	52.16	53.28	61.46	46.19		139.8	1.5	1.7	
β-FAR	mean	20.8 f	29.3 f	174.3 e	432 bc	246.9 de	518.6 b	344.2 cd	730.1 a	116	312.0	2.6	2.4	35.89***
	s.d.	8.7	17.69	35.91	199 83	46.77	130.44	91.79	194.93		255.7	0.7	1.1	

I – after iron-NPs application (+ 4 days), concentration 1; J – after iron-NPs application (+ 4 days), concentration 2; K – after MeSa application (+ 24h), without iron-NPs; L – after MeSa application (+ 72h), without iron-NPs; M – after MeSa application, (+24h), with iron-NPs, concentration 1; N – after MeSa application, (+72h), with iron-NPs, concentration 1; O – after MeSa application, (+24h), with iron-NPs, concentration 2; P – after MeSa application, (+72h), with iron-NPs, concentration 2; *Z*-3-hexenal=*Z*-3-HAL, *E*-2-hexenal=*E*-2-HAL, *Z*-3-hexenol=*Z*-3-HOL, *Z*-3-hexenyl acetate=*Z*-3-HAC, *Z*-β-ocimene=*Z*-OCI, linalool=LIN, benzyl acetate=BAC, methyl salicylate=MeSa, indole=IND, β-caryophyllene=β-CAR, and *E*-β-farnesene=*E*-β-FAR; *** $p < 0.001$. In rows, average values marked with the same letter are not statistically differentiated

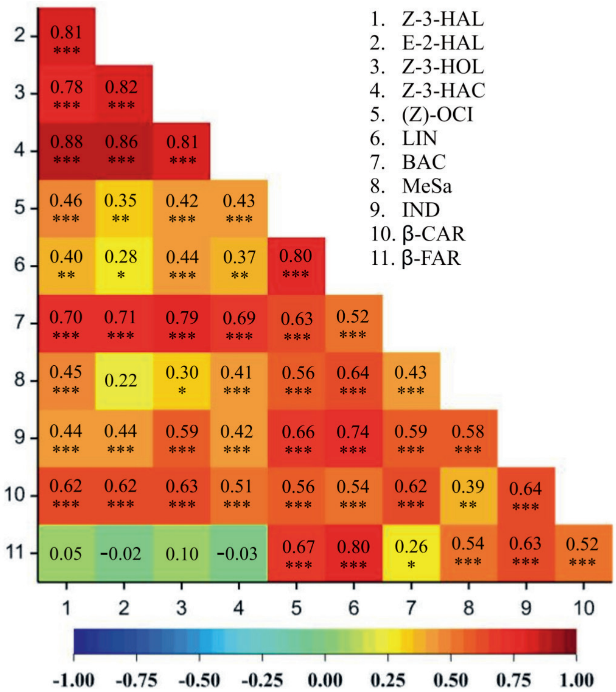

Fig. 1. Heatmaps for linear Pearson's correlation coefficients between the observed VOCs for MeJa ($r_{0.05} = 0.25$, $r_{0.01} = 0.32$, $r_{0.001} = 0.40$); * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; *Z*-3-hexenal=*Z*-3-HAL, *E*-2-hexenal=*E*-2-HAL, *Z*-3-hexenol=*Z*-3-HOL, *Z*-3-hexenyl acetate=*Z*-3-HAC, *Z*-β-ocimene=*Z*-OCI, linalool=LIN, benzyl acetate=BAC, methyl salicylate=MeSa, indole=IND, β-caryophyllene=β-CAR, and *E*-β-farnesene=*E*-β-FAR

Canonical variable analysis is a statistical tool making it possible to solve the problem of multivariate relationships between applications for all VOCs jointly (Wrońska-Pilarek *et al*. 2018; Bocianowski and Majchrzak 2019). The results of the CVA for the applications for MeJa are shown in Figure 2 (see also Table S2, supplementary material).

The first and second canonical variates provided approximately 67% and 23%, respectively, of the total variation between the applications (see Table S2, supplementary material, and Fig. 2) for MeJa. Figure 2 shows the distribution of the applications in the system of the first two canonical variates for MeJa. In the diagrams, the coordinates of a given application are values of the first and second canonical variate, respectively. A significant positive linear relationship with the first canonical variate was found for *Z*-3-HAL, *E*-2-HAL, *Z*-3-HOL, *Z*-3-HAC, BAC and β-CAR (see Table S2 supplementary material). The second canonical variate was significantly negatively correlated with (*Z*)-OCI, LIN, MeSa and β-FAR (see Table S2 supplementary material). For MeJa the greatest variation in terms of all the 11 VOCs jointly measured with Mahalanobis distances was found after iron-NPs application (+4 days), concentration 1 and after MeJa application, (+24 h), with iron-NPs, concentration 2 (distance between them amounted to 23.58). The greatest

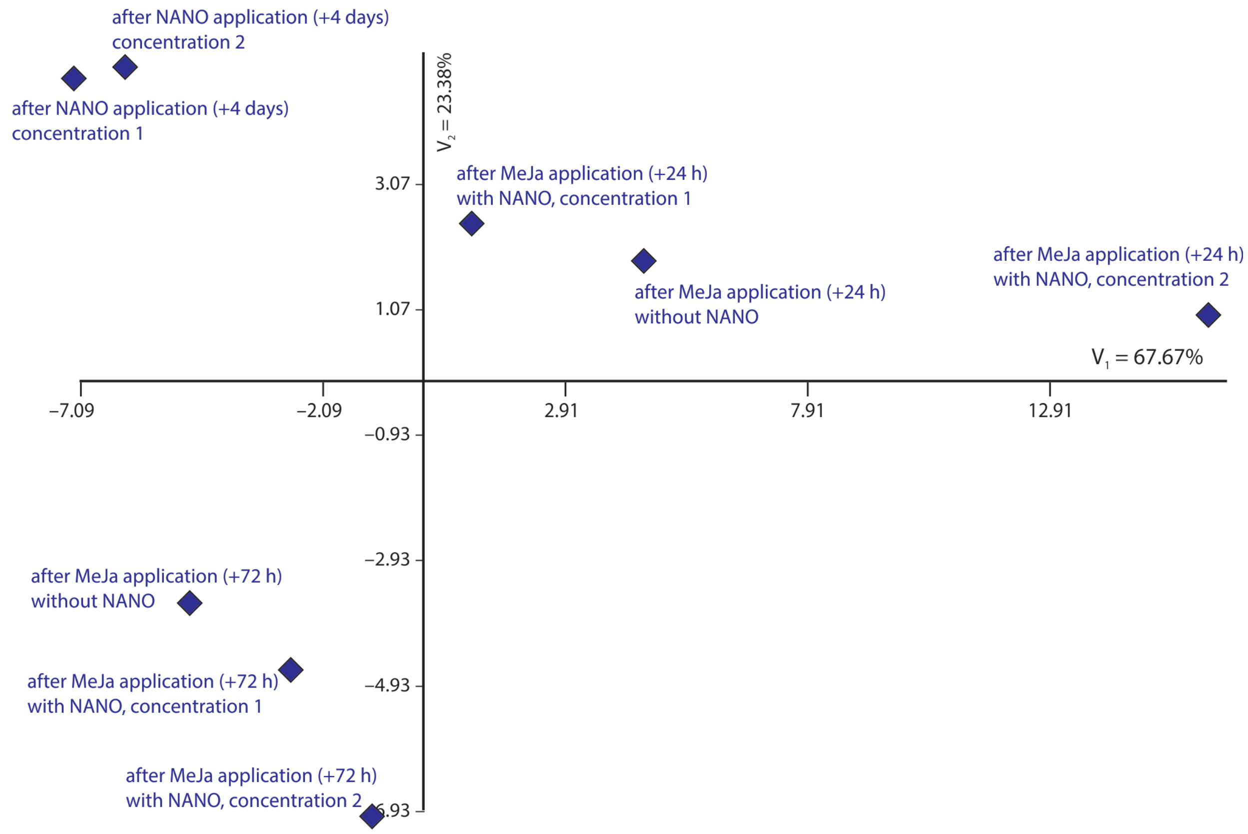

Fig. 2. Distribution of eight applications in the space of the first two canonical variates for MeJa. In the diagram, the coordinates of a given application are values of the first (V_1) and second (V_2) canonical variate, respectively

similarity was found between both applications of iron-NPs (+4 days), concentration 1 and iron-NPs (+4 days), concentration 2 (0.518) (see Table S4, supplementary material).

All VOCs were found to have a normal distribution. The results of MANOVA indicated that applications (Wilks' λ = 0.00040; $F_{77;283}$ = 9.88) were statistically significantly different (p < 0.0001) for all 11 VOCs jointly. Analysis of variance indicated that the main effects of application were significant for all the VOCs of study (Table 3). Mean values and standard deviations for observed VOCs for studied applications for MeSa are presented in Table 3.

Statistically significant positive correlations for MeSa were observed between all pairs of VOCs, except *Z*-3-HAL and *E*-β-FAR, *E*-2-HAL and *E*-β-FAR, *Z*-3-HOL and *E*-β-FAR, *Z*-3-HAC and *E*-β-FAR, as well as *E*-2-HAL and LIN (see Table S1, supplementary material, and Fig. 3).

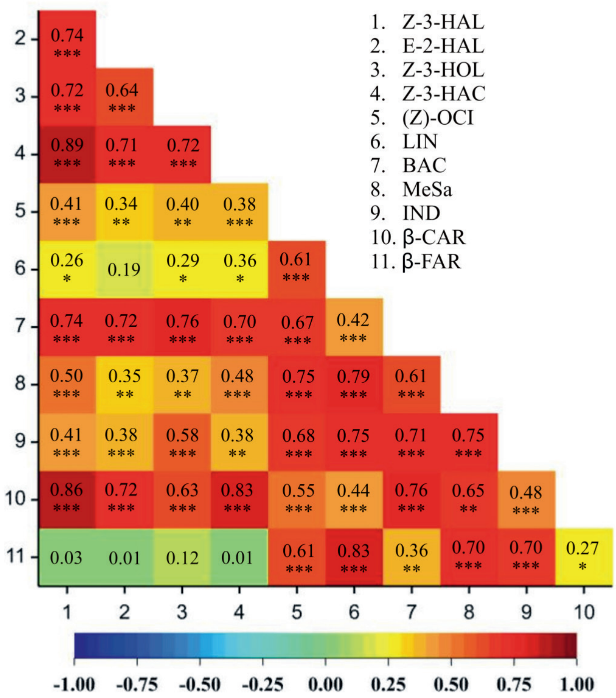

Fig. 3. Heatmaps for linear Pearson's correlation coefficients between the observed VOCs for MeSa ($r_{0.05}$ = 0.25, $r_{0.01}$ = 0.32, $r_{0.001}$ = 0.40) ; * p < 0.05; ** p < 0.01; *** p <0.001; *Z*-3-hexenal=*Z*-3-HAL, *E*-2-hexenal=*E*-2-HAL, *Z*-3-hexenol=*Z*-3-HOL, *Z*-3-hexenyl acetate=*Z*-3-HAC, *Z*-β-ocimene=*Z*-OCI, linalool=LIN, benzyl acetate=BAC, methyl salicylate=MeSa, indole=IND, β-caryophyllene=β-CAR, and *E*-β-farnesene=*E*-β-FAR

The first two canonical variates explained 92.90% of the total variation between the applications (see Table S2, supplementary material, and Fig. 4) for MeSa. A significantly positive linear relationship with the first canonical variate was found for *Z*-3-HAL, *E*-2-HAL, *Z*-3-HOL, *Z*-3-HAC, *Z*-OCI, BAC and β-CAR (see Table S2, supplementary material). The second canonical variate was significantly negatively correlated with LIN, MeSa and *E*-β-FAR (see Table S2 supplementary material). For MeSa the greatest variation in terms of all 11 VOCs jointly measured with Mahalanobis distances was found after iron-NPs application (+4 days), concentration 1 and after MeSa application, (+24 h), with iron-NPs, concentration 2 (distance between them amounted to 16.495). The greatest similarity was found between after iron-NPs application (+4 days), concentration 1 and after iron-NPs application (+4 days), concentration 2 (0.518) (see Table S4, supplementary material).

Results of contrast analyses between MeJa and MeSa for particular VOCs are presented in Table S5, supplementary material. Contrasts were significant for *Z*-OCI, LIN, BAC, MeSa, and β-CAR for the following: after MeJa application (+24 h), without iron-NPs versus after MeSa application (+24 h), without iron-NPs, after MeJa application (+72 h), without iron-NPs vs. after MeSa application (+72 h), without iron-NPs, after MeJa application, (+24 h), with iron-NPs, concentration 1 vs. after MeSa application, (+24 h), with iron-NPs, concentration 1, after MeJa application, (+72 h), with iron-NPs, concentration 1 vs. after MeSa application, (+72 h), with iron-NPs, concentration 1, after MeJa application, (+24 h), with iron-NPs, concentration 2 vs. after MeSa application, (+24 h), with iron-NPs, concentration 2, after MeJa application, (+72 h), with iron-NPs, concentration 2 vs. after MeSa application, (+72 h), with iron-NPs, concentration 2. Additionally, contrasts were significant for *Z*-3-HAL, *Z*-3-HAC and *Z*-β-FAR for the following: after MeJa application (+24 h), without iron-NPs vs. after MeSa application (+24 h), without iron-NPs, after MeJa application, (+72 h), with NANO, concentration 1 vs. after MeSa application, (+72 h), with iron-NPs, concentration 1, after MeJa application, (+24 h), with iron-NPs, concentration 2 vs. after MeSa application, (+24 h), with iron-NPs, concentration 2, after MeJa application, (+72 h), with iron-NPs, concentration 2 vs. after MeSa application, (+72 h), with iron-NPs, concentration 2. Comparison was significant for *Z*-3-HAL and *Z*-3-HAC for the following: between post MeJa application (+72 h), without iron-NPs and post MeSa application (+72 h), without iron-NPs was statistically significant for *Z*-β-FAR. Comparison between post MeJa application, (+24 h), with iron-NPs, concentration 1 and post MeSa application, (+24 h), with iron-NPs, concentration 1. However, comparison between post MeJa application, (+24 h), with iron-NPs, concentration 2 and post MeSa application, (+24 h), with iron-NPs, concentration 2 was significant for *E*-2-HAL (Table S5, supplementary material).

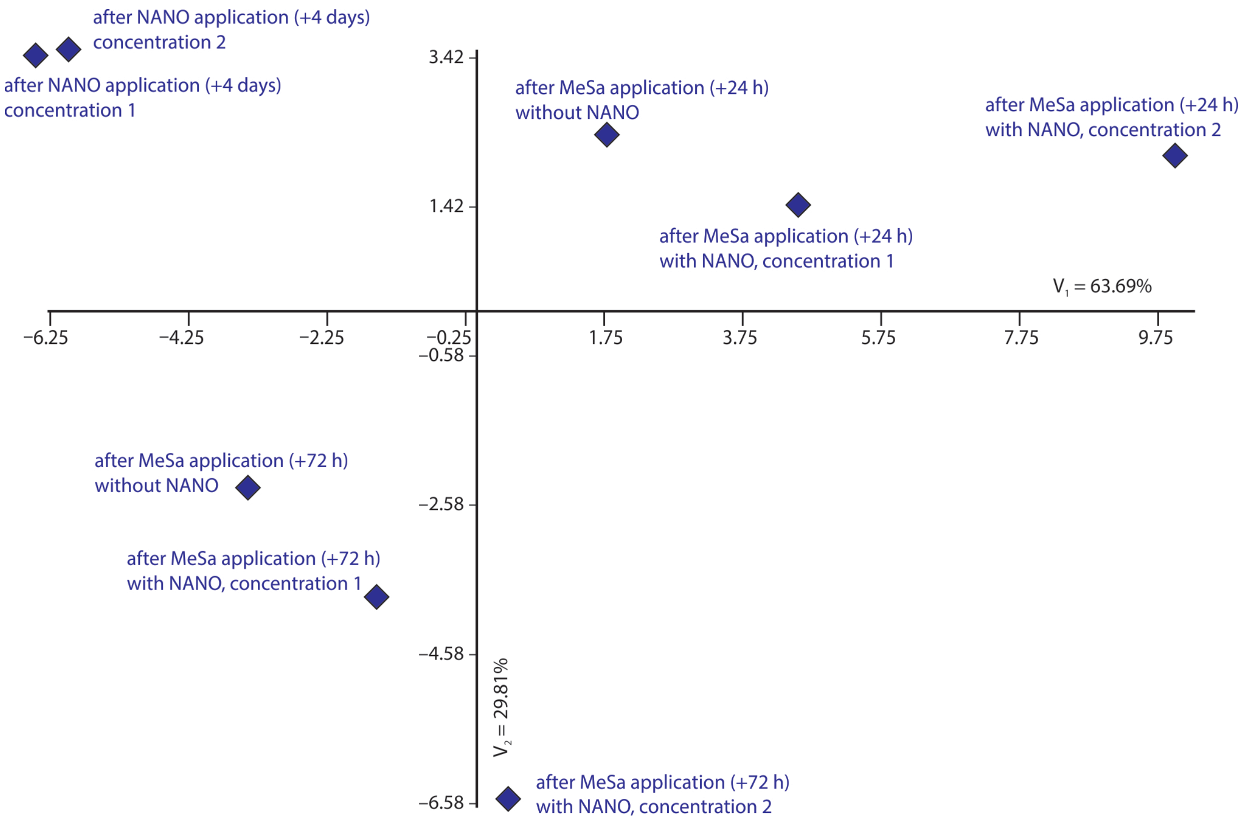

Fig. 4. Distribution of eight applications in the space of the first two canonical variates for MeSa. In the diagrams, the coordinates of a given application are values of the first (V_1) and second (V_2) canonical variate, respectively

Discussion

In our study we identified a number of volatiles which are classified as green leaf volatiles, namely *Z*-3-HAL, *E*-2-HAL, *Z*-3-HOL, *Z*-3-HAC (Engelberth and Engelberth 2020) or as stress induced plant volatiles, namely *Z*-OCI, LIN, MeSa, β-CAR and E-β-FAR (Cascone *et al.* 2015; Scandiffio *et al.* 2020). Surprisingly, one of the VOCs was indole, which is characterized by an unpleasant and irritating fragrance. Its presence is suspected to be related with indole acetic acid, an auxin which has a significant role in a plant's defense mechanism, which was discussed by Lecube *et al.* (2014).

In agreement with other plant studies (Song and Ryu 2018; Jiang *et al.* 2022) we demonstrated that MeJa and MeSa applications had a positive influence on plant vigor. Jiang *et al.* (2022) showed that the *Cymbopogon flexuosus* treated with MeJa resulted in increased GLV emissions. Song and Ryu (2018) showed that MeSa upregulated the expression of genes related to the defense mechanism to produce volatile emissions. On the other hand, with regards to nanoparticle application, Khalid *et al.* (2022) demonstrated that Zn, Fe and Mg-based nanoparticles improved *Caesalpinia bonducell* height, mass and nutrient contents. Our results relating to the emission of VOCs by *B. napus* are reflected in other investigated strategies. Brosset *et al.* (2021) used a related plant, *B. nigra*, to verify the possibility of using the natural elicitor ((*Z*)-11-hexadecenal) to reduce damage to *P. xylostella*. Wenda-Piesik *et al.* (2016) reported that it is important to verify how the mixture of elicitors works on its own. In their research, insect behavior depended on the concentration of elicitors – with low concentrations attracting herbivores and higher concentrations repelling the insects. However, the mixture of elicitors that was similar to the VOCs emitted by *B. napus* did not attract insects at all. These results provide a positive perspective for the approach investigated in our study.

The approach of using combined natural chemical elicitors (MeJa and MeSa) with NPs is an interesting technique because of its efficiency and was crucial for this study. The use of natural compounds as inducers does not introduce compounds into the environment that can interfere with the well-being of other nontarget organisms, which is in contrast to artificial pesticides (Jamiołkowska 2020). On the other hand, NPs are still developing a strategy, which needs further investigation. For instance, phosphorus or nitrogen NPs were found to be efficient, because of the mechanism of action, which is based on the prolonged delivery of safe nutrients. However, not all NPs have been investigated in-depth, which may raise concerns about their toxicity and overall safety (Abdel-Azizi *et al.* 2018). Nonetheless, even if NPs show some level of toxicity, it is possible to reduce the potential threat by slight modifications or using potentially toxic NPs along with non-toxic ones to lower the toxicity. Farghaly *et al.* (2023) investigated the possibility of using thiol compounds as detoxifiers during ZnO-NP application. In their model study, the pomegranate calli were exposed to various doses of ZnO-NPs that negatively influenced callus growth. However, thiol compounds seemed to reduce the oxidative stress caused by ZnO. In light of multiple benefits that are related to NP application for agronomy, and which are summarized in a comprehensive review by Pramanik *et al.* (2023), further investigations on NP use and modes of actions are needed. Our current research is still in the early stages, but it provides the perspective for further investigation and legal proceedings, which will regulate the future use of NPs in agriculture.

Conclusions

In this study we have demonstrated for the first time the synergistic effect of applying volatile elicitors simultaneously, namely methyl jasmonate and methyl salicylate and iron nanoparticles. In general, methyl jasmonate induced larger emissions of volatile organic compounds than methyl salicylate. Also, the concentration of iron nanoparticles was found to have a significant influence on the plants' reaction; $10\ \mu g \cdot ml^{-1}$ of nanoparticles induced a larger reaction of the tested plants. Nevertheless, the combination of elicitors and nanoparticles induced the highest emission of the plants' volatile organic compounds. A difference was observed with particular green leaf volatiles emission times. For example, benzyl acetate, and β-caryophyllene were released in larger amounts after 24 h following nanoparticle and elicitor application, while Z-β-ocimene, linalool, methyl salicylate and E-β-farnesene were released in larger amounts after 72 h following the application of iron nanoparticles and elicitors. In the next step of research it would be worth investigating how the optimal combinations of nanoparticles and elicitors influence plant defense capability in light of actual herbivore attacks. For such a test a combination of methyl jasmonate with $10\ \mu g \cdot ml^{-1}$ of nanoparticles will be used.

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