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Induction of volatile organic compounds in chrysanthemum plants following infection by *Rhizoctonia solani*

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Plant volatiles comprise organic compounds derived from the breakdown of secondary metabolites that are emitted by leaves and flowers in response to stress. The composition of these volatiles varies among plant species. Green leaf volatiles (GLVs) are a crucial subgroup within the biogenic VOCs. They comprise alcohols, aldehydes, and esters with a six-carbon (C6) structure and are emitted by nearly all plant species. They can repel or attract herbivores and their natural predators induce plant defense mechanisms, prime plants for enhanced defense, activate abiotic-stress related genes and exhibit direct toxicity against bacteria and fungi.

GLVs that induce systemic resistance against pathogens could be utilized as “green vaccines” in agriculture to defend against impending pathogen attacks. However, our understanding of the mechanisms by which volatiles induce systemic resistance is still in its early stages. It remains uncertain whether a broad application of volatiles would significantly impact plant productivity. Also, the specific interactions between plants and VOCs when attacked by different fungal species have only been explored in a limited number of studies.

Chrysanthemum (*Chrysanthemum* × *morifolium* /Ramat./ Hemsl.) is known for its decorative qualities and hence is a highly popular ornamental plant worldwide, second only to roses. It can be cultivated as a potted plant or used as cut flowers. This plant belongs to the *Chrysanthemum* genus of the Asteraceae family and is native to Central-East Asia.

The major goal of this study was to investigate the induction of VOCs in *Chrysanthemum × morifolium* (Ramat.) Hemsl. plants of different cultivars following inoculation with fungal pathogen *Rhizoctonia solani*. The aims of the research presented here are: (1) to determine whether different cultivars of chrysanthemum release varying amounts and types of VOCs, resulting in a qualitatively and quantitatively diverse bouquet of odors; (2) to investigate whether the mode of inoculation (e.g., stem leaf infection versus soil inoculation) leads to distinct VOCs and temporal VOC profiles, thereby indicating different defense responses in the plants; and (3) to establish whether the VOCs emitted by chrysanthemum plants have any detrimental effect on the growth of *R. solani* mycelium, and thus potentially act as a defense mechanism against the pathogen.

Materials and methods

Following the inoculation of chrysanthemum plants with *R. solani*, VOCs emitted by the plants were sampled at specific times and analyzed using gas chromatography/mass spectrometry (GC/MS). The growth of *R. solani* mycelium was evaluated by measuring mycelial growth rates or other relevant parameters in the presence or absence of VOCs.

Plant material

Ten cultivars of chrysanthemum (*Chrysanthemum* × *morifolium* /Ramat./ Hemsl.) were used in this study, namely, ‘Ania’, ‘Beata’, ‘Brda’, ‘Kasia’, ‘Lidka’, ‘Luczniczka’, ‘Malgosia’, ‘Polka’, ‘Wda’, and ‘Zofia’, all of which were cultivated under greenhouse conditions. These plants were vegetatively propagated via shoot-tip cuttings and rooted in 64-cell propagation trays using a peat: perlite 2:1 substrate mixture for two weeks, and placed under a perforated transparent film tunnel.

***Rhizoctonia solani* infestation and volatile collection system**

Rhizoctonia solani J.G. Kühn used in the experiments was isolated from an infected chrysanthemum plant and cultured on Potato Dextrose Agar (PDA) medium (Sigma Aldrich, USA) according to the common protocol. There were two types of *R. solani* inoculation applied: leaf infestation and soil inoculation. For leaf infestation, a total of 12 plants from each cultivar were sprayed with a mycelium fragments suspension that was prepared as follows: *R. solani* was grown on PDA medium in 85 mm diameter Petri plates for 14 days.

For soil inoculation, wheat kernels overgrown with *R. solani* were used. The preparation of kernels was as follows. Triple sterilized wheat kernels of approximately 300 mL volume were placed in sterile plastic bags. These kernels were then inoculated with five 1 cm² fragments of *R. solani* mycelium. The mycelium was cultured on the kernels for five weeks in darkness at a temperature of 22 ± 1 °C.

For volatile collection, the experimental chrysanthemum plants were tightly enclosed within nalophan bags, 35 cm × 60 cm, with one plant per bag. A volatile collector trap (6.35 mm outside diameter and 76 mm long glass tube (ARS, Inc., Gainesville, Florida, USA) containing 30 mg of Super-Q adsorbent (Alltech Associates, Inc., USA) provided a passive chemical filter designed for the collection of extremely low-level (ppb-ppm) VOCs from the nalophan enclosed plants. The Super-Q adsorbent was inserted into each of four Tygon tubes, which were connected between the airflow meter and the collector trap. The collection system allowed for the simultaneous collection of volatiles from four plants, with a total collection time of 2 hrs.

Analytical methods

For the volatile elutions from the Super-Q adsorbent in each volatile collection trap, 225 μL of hexane was used, supplemented with 7 ng of decane as an internal standard, where the quantity of hexane used was sufficient to extract all trapped VOCs. Individual samples (1 μL) were then injected and analyzed by GC/MS using an Auto System XL/Turbomass instrument (Perkin Elmer, USA) with a capillary column (30 m Rtx-5MS, 0.25 mm ID, 0.25 μm film thickness, Restek, USA). The identification of the volatiles was verified using authentic standards (Sigma-Aldrich, USA). The emission rate (ng h^{-1}) of each VOC was determined by comparing the VOC's peak area relative to the peak area of the internal standard.

Statistical analysis

The Shapiro-Wilk's test was used to test normality of the distribution of the fourteen VOCs, i.e., for Z-3-HAL, E-2-HAL, Z-3-HOL, E-2-HOL, β -PIN, β -MYR, Z-3-HAC, (Z)-OCI, LIN, BAC, MAT, IND, β -CAR, and β -FAR and the metabolites contents in order to be able to conduct an analysis of variance (ANOVA).

Since all VOCs and metabolites had normal distributions, multivariate analysis of variance (MANOVA) as well as two-way analysis of variance (ANOVA) were undertaken to determine the effects of cultivar, collection time and interaction between cultivar and collection time on VOC values. Arithmetic means, standard deviations and Fisher's least significant differences (LSDs) were calculated. Pearson's linear correlation coefficients were used to assess the correlation between various VOCs at each collection time. All calculations for statistical analyses were carried out using the GenStat v.23 statistical package (VSN International, England UK).

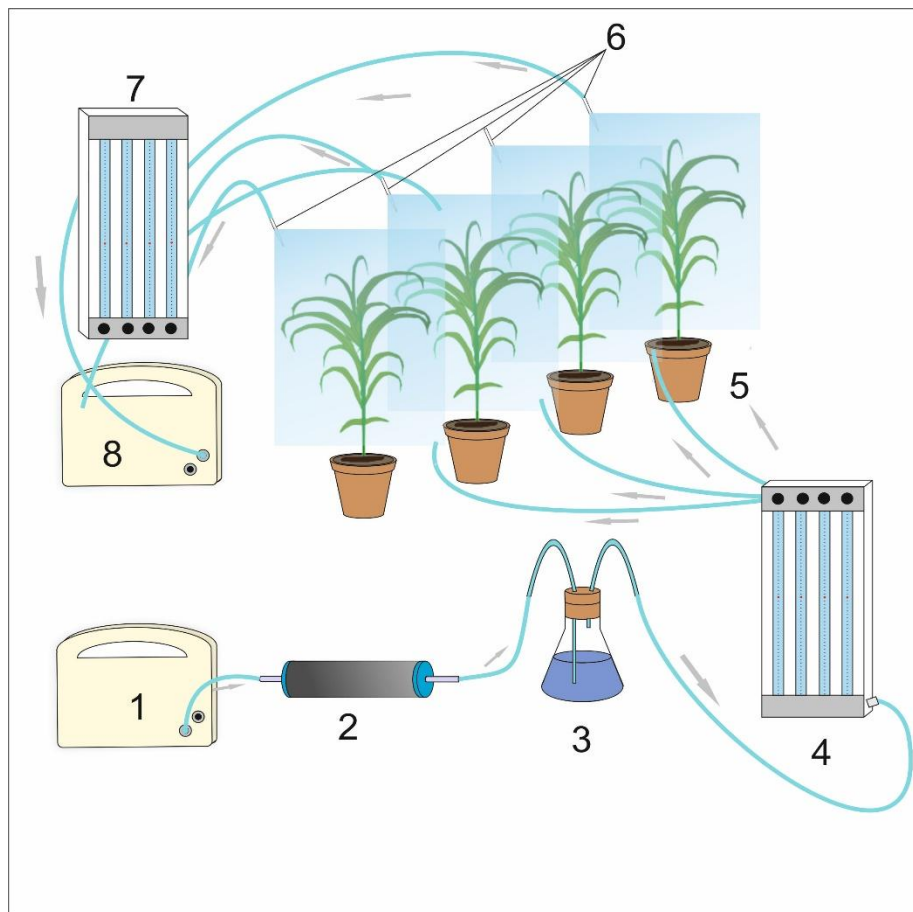
VOCs detected

Fourteen VOCs that were consistently detected to have a rate > 0.1 ng h⁻¹ are only reported in this paper, namely, (Z)-3-hexenal = (Z)-3-HAL, (E)-2-hexenal = (E)-2-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, β -pinene = β -PIN, β -myrcene = β -MYR, (Z)-ocimene = (Z)-OCI, linalool = LIN, benzyl acetate = BAC, methyl salicylate = MAT, indole = IND, β -caryophyllene = β -CAR, and (E)- β -farnesene = (E)- β -FAR.

Scheme

Installation diagram of
VOC collection
equipment:

1. Air pump
2. Carbon filter
3. Air humidifier
4. Air flow meter
5. Tested plants placed in Nalophan bags
6. Super Q-Traps and Tygon tubes
7. Air flow meter
8. Suction pump.





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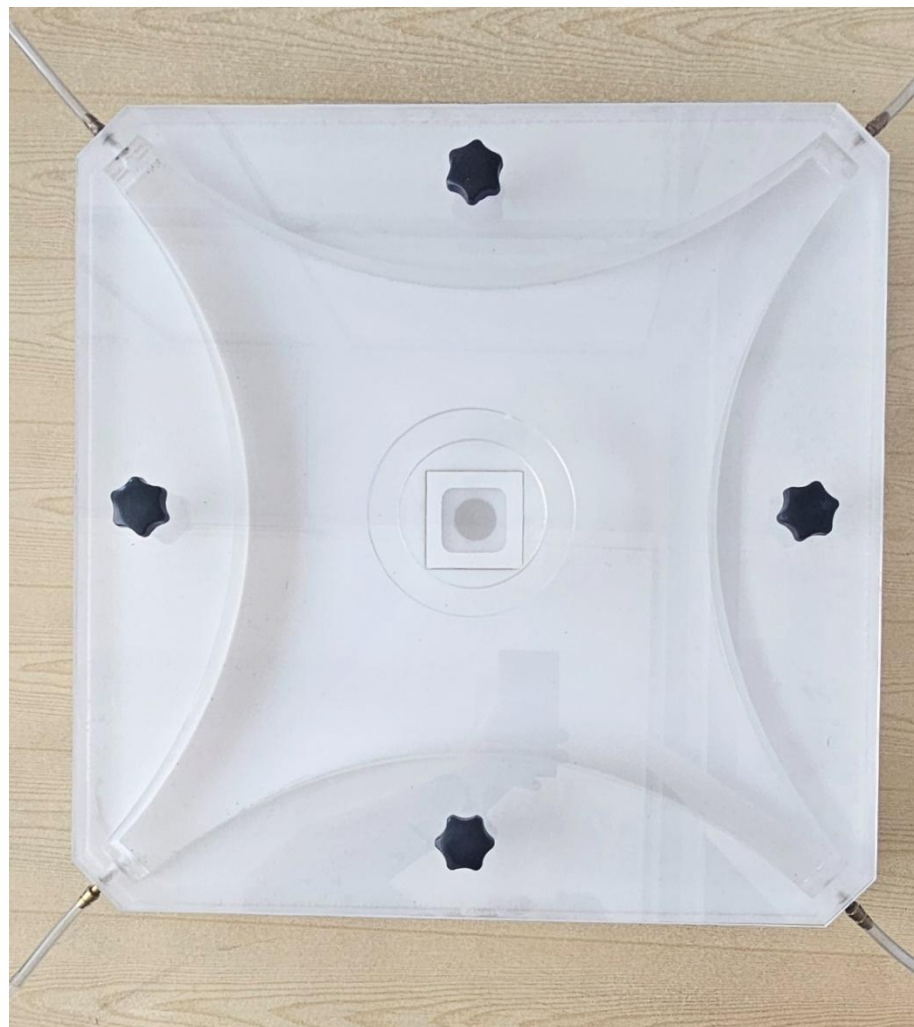


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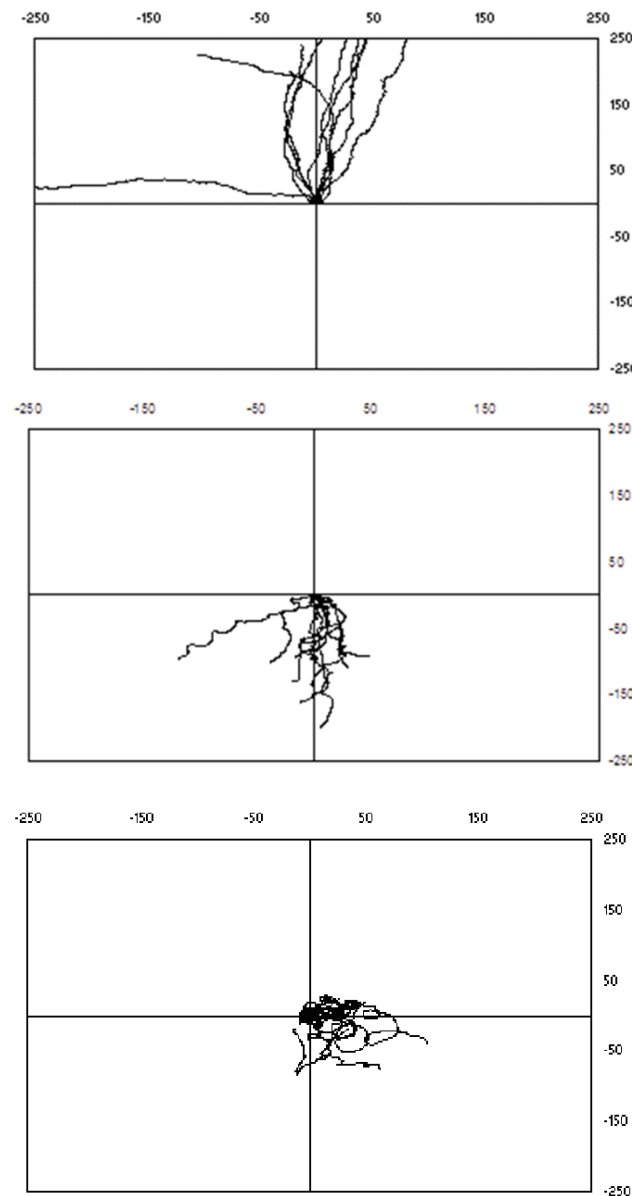


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Results

Table 1. Influence of cultivars, days past inoculation (dpi) and inoculation treatment (leaf (L) or soil (S)) with *Rhizoctonia solani* on disease symptoms incidence and re-isolation. Fisher's least significant differences (LSDs) and values of *F*-statistics were used in the comparison of mean values for studied cultivars.

Cultivar	Visual assessment (% of leaf area with disease symptoms)			Rhizoctonia solani re- isolation on PDA medium
	3 dpi L	6 dpi L	42 dpi S	
Ania	0.0±0.0	0.18±0.13	0.0±0.0	+
Beata	0.0±0.0	0.32±0.11	0.01±0.01	+
Brda	0.0±0.0	0.11±0.09	0.0±0.0	+
Kasia	0.0±0.0	0.27±0.14	0.02±0.01	+
Lidka	0.0±0.0	0.36±0.17	0.0±0.0	+
Luczniczka	0.0±0.0	0.15±0.08	0.0±0.0	+
Malgosia	0.0±0.0	0.21±0.10	0.0±0.0	+
Polka	0.14±0.13	1.24±0.46	0.03±0.02	+
Wda	0.76±0.35	4.78±1.66	0.22±0.12	+
Zofia	0.0±0.0	0.03±0.02	0.0±0.0	+
LSD _{0.05}	0.137	0.643	0.045	
F-statistic	19.21***	41.05***	18.60***	

Table 2. Mean values of the VOC emission rates (ng hr^{-1}) and standard deviations for ten cultivars for all of the fourteen VOCs. Fisher's least significant differences (LSDs) and values of F -statistics were used in the comparison of mean values for studied cultivars (*means of cultivars; statistically significant difference).

Trait	Ania	Beata	'Brda'	Kasia	Lidka	Luczniczka	Malgosia	Polka	Wda	Zofia	LSD _{0.05}	F-statistic
Z-3-HAL	4.9±1.3	30.6±7.5	3.7±1.2	3.2±1.3	7.4±1.7	12.5±2.4	6.7±5.2	7.6±2.2	11.9±2.0	4.3±3.9	4.02	33.28***
E-2-HAL	2.9±0.8	9.6±3.9	4.1±2.4	1.8±1.0	4.2±1.1	5.3±1.7	4.8±5.0	5.3±2.0	5.9±2.3	1.8±0.9	2.88	5.09***
Z-3-HOL	1.2±0.8		3.8±1.8	1.7±1.3	3.5±2.0	2.7±1.0	4.4±2.9	4.5±1.5	4.3±2.3	2.8±0.3	1.99	2.87*
E-2-HOL	3.5±2.8		2.7±0.7	2.4±0.5	2.8±0.9	3.3±0.6	5.4±4.0	2.4±0.8	3.2±1.9	2.8±1.1	2.16	1.47
β-PIN	3.3±1.1	8.1±3.9		2.7±0.5		4.8±2.2	5.2±3.7		5.0±1.8		2.97	3.37*
β-MYR	3.9±1.8	5.1±1.6		0.9±0.5		2.5±0.5	4.8±1.9		1.9±0.8		1.56	9.74***
Z-3-HAC	4.4±1.0	12.8±7.4	2.6±2.1	3.5±3.2	5.2±2.4	6.7±1.1	4.8±2.4	9.6±2.3	15.2±6.6	2.0±0.7	4.22	8.97***
(Z)-OCI	2.4±0.6	18.8±4.9	3.2±0.9	1.0±0.6	6.3±2.7	6.3±0.9	4.7±1.1	6.2±1.2	12.4±4.2	3.2±1.6	2.77	30.02***
LIN	3.2±0.3	15.0±4.0	2.2±0.5	1.5±1.0	5.3±2.0	6.8±1.7	4.1±1.1	4.4±1.3	9.7±5.5	2.8±1.7	2.86	16.76***
BAC	2.3±0.8			2.8±0.6	3.6±3.3	3.5±0.5	5.4±3.8	3.0±1.1	4.4±1.5		2.43	1.57
MAT	2.1±0.6	4.5±1.6		3.2±0.7		4.3±1.8		2.6±1.1	3.7±0.6		1.37	3.86**
IND	2.8±0.7	6.6±1.2		1.9±0.9		2.0±2.0		1.7±0.8	1.7±0.9		1.37	16.46***
β-CAR	3.6±2.7	10.0±4.0	1.6±0.7	1.7±0.7	4.7±2.6	4.8±1.0	4.0±1.7	3.9±1.4	4.6±2.2	2.7±0.6	2.38	7.84***
β-FAR	1.7±1.0	11.1±4.7	2.7±0.8	3.2±1.1	2.9±1.1	5.6±1.6	3.6±1.7	4.5±1.9	4.7±1.4	1.9±1.2	2.29	11.5***

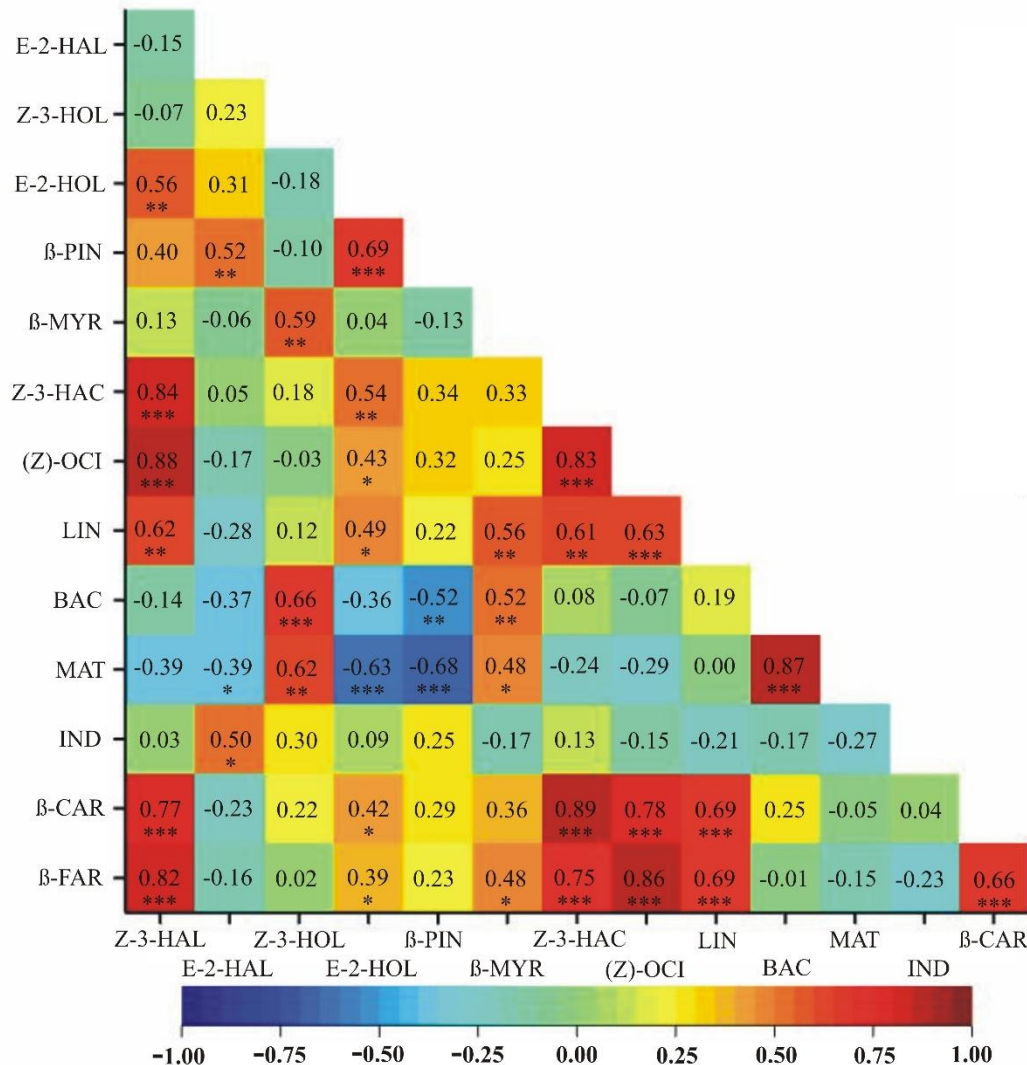


Fig 2. A heatmap showing correlation coefficients between VOC emissions assessed on day 3 post leaf-inoculation. Dark green depicts the highest and red the lowest mean emission rates. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

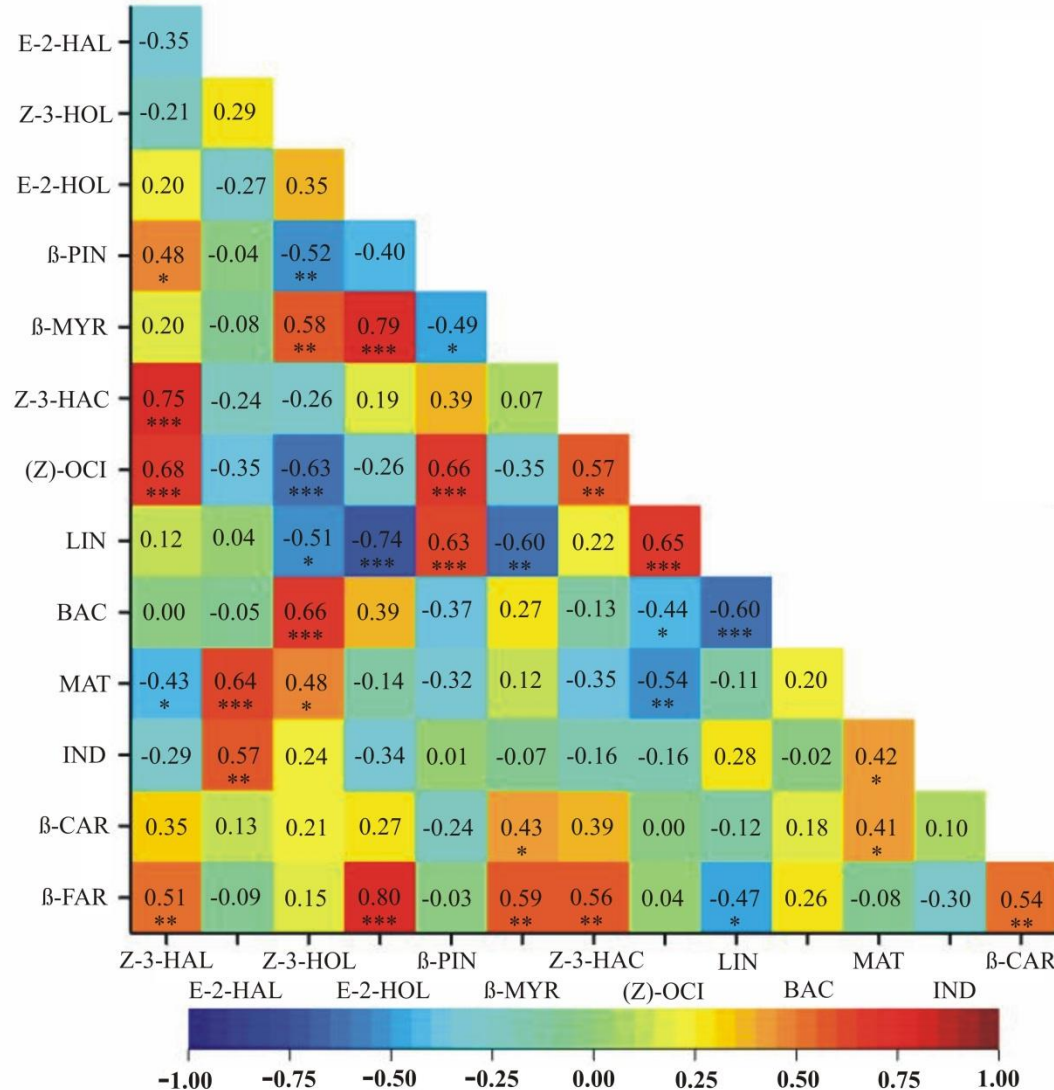


Fig 3. A heatmap showing correlation coefficients between VOC emissions assessed on day 6 post leaf-inoculation. Dark green depicts the highest and red the lowest mean emission rates. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

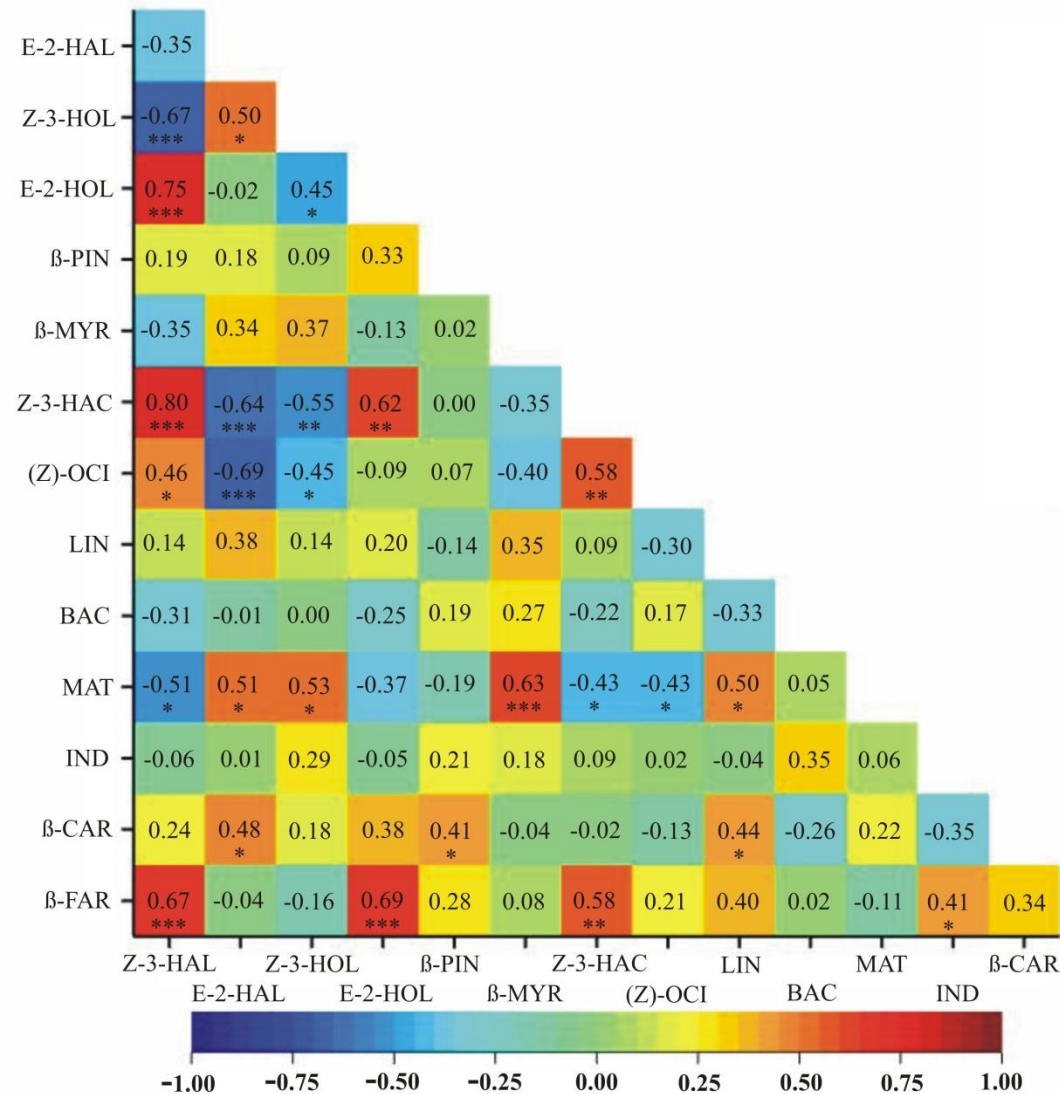


Fig 4. A heatmap showing correlation coefficients between VOC emissions assessed on day 42 after soil inoculation. Dark green depicts the highest and red the lowest mean emission rates. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

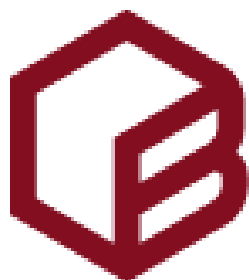
Conclusions

Our study investigated the effects of *Rhizoctonia solani* infection on the emission of volatile organic compounds (VOCs) and selected primary and secondary metabolites present in the leaves of ten chrysanthemum cultivars grown in a greenhouse. Our findings suggest that chrysanthemum cultivars exhibit distinct responses to *Rhizoctonia solani* infection, with some cultivars emitting higher quantities of certain VOCs compared to others. Furthermore, the composition of VOCs emitted by infected plants varied over time, indicating dynamic changes in plant-pathogen interactions and metabolic responses.

These results represent a step forward in understanding the mechanisms of plant defense and susceptibility to fungal pathogens in chrysanthemum cultivars. By elucidating the specific VOCs involved in plant-pathogen interactions, future research can be focused on investigating the role of individual compounds in mediating plant defense mechanisms and pathogen virulence. Further studies could involve transcriptomic, proteomic, and metabolomic analyses to identify key genes, proteins, and metabolic pathways involved in VOC biosynthesis and regulation. The future prospects incorporate research that investigates the potential use of VOC profiles as diagnostic markers for early detection and monitoring of *Rhizoctonia solani* infection in chrysanthemum crops, which could help improve environmental-friendly disease management strategies and minimize crop losses.

Thank you for attention

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