



Methyl salicylate as an airborne signal in neighboring cowpea plants' systematic defense responses against spider mites

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With 4 figures

Abstract: Herbivore induced plant volatiles (HIPVs) play a crucial role in regulating interactions among multiple species, including triggering defense responses in neighboring plants. In this study, cowpea plants infested with *Tetranychus urticae* released substantial amounts of HIPVs, particularly methyl salicylate (MeSA). To investigate the impact of exposure to HIPVs at different distances and exposure to single MeSA on plant defense against mites, a series of experiments integrating physiology, biochemistry, behavioral, and chemical analysis were conducted. Exposure of healthy cowpea plants to either HIPVs or single MeSA enhanced their direct defense responses, with effects varying according to distance and dosage. These responses included improved antioxidant enzyme activities and increased phytohormone production. Furthermore, exposure to single MeSA increased the release of volatiles, decreased the survival rate of spider mites on leaves, and reduced leaf damage. Feeding on leaves exposed to HIPVs or single MeSA also influenced detoxification and defense-related enzymatic activities in spider mites. Additionally, plants which exposed to HIPVs or single MeSA chemical attracted predatory mites. These results emphasize the distance-dependent effect of HIPVs and highlight the role of MeSA as an airborne signal that primes direct and indirect defense responses against *T. urticae*, providing a scientific foundation for the development of eco-friendly pest management strategies based on plant defense responses to manage spider mites.

Keywords: Herbivore-induced plant volatiles (HIPVs); induced defense; plant-plant communication; salicylic acid (SA); *Tetranychus urticae*

1 Introduction

Plant volatiles serve as signaling molecules, facilitating long-distance communication among plants, other species, and the surrounding environment (Bouwmeester et al. 2019). Particularly, herbivore-induced plant volatiles (HIPVs) play a crucial role in regulating interactions among multiple species, such as protecting plants (Turlings & Erb 2018; Timilsena et al. 2020). HIPVs can safeguard plants by repelling pests directly or influencing herbivores' reproductive behavior (Loreto & D'Auria 2022). Furthermore, HIPVs act as attractants for natural enemies like parasitic and predatory species, allowing them to locate their host swiftly and accurately (Turlings & Erb 2018; Karban 2021). Additionally,

neighboring healthy plants exposed to HIPVs can trigger defense responses, mounting a prompt and robust defense response against incoming pest (Kalske et al. 2019; Pérez-Hedo et al. 2021).

Plants exposed to HIPVs can prime their defense responses through modifying their secretion of extrafloral nectar, emission of volatiles, upregulation of defense-related genes, and enhancement of defensive enzymes activities (Timilsena et al. 2020; Depalo et al. 2022). Large amounts of HIPVs capable of inducing plant defense responses have been identified, such as (*Z*)-3-hexenol, DMNT, β -ocimene, indole and methyl salicylate (MeSA) (Jing et al. 2021; Ye et al. 2021; Gong et al. 2023). Therein, MeSA, a crucial HIPVs, would be largely released into the air when plants are

infested by spider mites, attracting many predators, such as predatory mites (Wu et al. 2023). MeSA functions not only to attract natural enemies for pest population control, but also to mediate airborne defense like triggering the activation of anti-aphid immune responses in plants (Yang et al. 2022; Gong et al. 2023; Mahmood et al. 2024). The effectiveness of MeSA has been successfully demonstrated in field studies and has been developed into commercial applications for agricultural pest control (Dong & Hwang 2017).

Cowpea (*Vigna unguiculata*) is a globally important economic crop, particularly in semi-arid area regions, but susceptible to numerous pests, including spider mites (Dong & Hwang 2017; Amorim et al. 2018). The two-spotted spider mite, *Tetranychus urticae*, is a highly destructive and polyphagous pest that frequently infests cowpea plants and easily develops resistance to miticides, resulting in significant economic losses. Cowpea plants are typically planted together, with fixed row spacing and plant spacing. By this, HIPVs emitted by *T. urticae*-infested cowpea plants are likely to affect the neighboring plants, and MeSA is recognized as a significant HIPV. We therefore hypothesize that the distance of exposure to HIPVs affects induced plant defense responses, with MeSA acting as an airborne signal that primes the plant defense responses against spider mites. To investigate this hypothesis, healthy cowpea plants were exposed to varying distances from *T. urticae*-infested plants, and the differences in phytohormones and enzymes between the plants and the enzymes between spider mites residing on the plants were analyzed. Then the MeSA level was quantified using chromatography and mass spectrometry (GC-MS) analysis. Additionally, the impact of MeSA on plant defense against spider mites was investigated by considering the responses of plants, spider mites and natural enemy. Taken together, these findings emphasize the importance of exposure distances to HIPVs, and the role of MeSA as an airborne signal in priming plant defense responses against spider mites.

2 Materials and methods

2.1 Plants growth and mites rearing

Laboratory colonies of *T. urticae* were reared on cowpea plants in a greenhouse. The predatory mite *Neoseiulus barkeri* was fed with *T. urticae*. Cowpea plants were grown in plastic pots with dimensions of 9 cm in depth and 5 cm in bottom diameter, filled with a mixture of vermiculite and nutritional soil (2:1). To minimize odor interference, all experimental cowpea plants were cultivated in incubators for 7 days until they developed two tender leaves and reached a height of approximately 15 cm. The cowpea plants used to maintain the colony of spider mites were cultivated in the greenhouse. Both the greenhouse and incubator conditions were set at a temperature of $25^{\circ}\text{C} \pm 2^{\circ}\text{C}$, $75\% \pm 5\%$ relative humidity (R.H.), and a 16: 8 hours (light: dark) photoperiod.

2.2 Collection and analysis of plant volatiles

Plant volatiles were analyzed qualitatively and quantitatively using solid-phase micro-extraction (SPME) (50/30 μm DVB/CAR/PDMS, Supelco, USA) fiber and GC-MS (7090B-5977B, Agilent, USA) (Tholl et al. 2006). Cowpea plants were infested with two-spotted spider mites for 3 days to collect the volatiles. After removing the spider mites from detached leaves, the leaves (6 leaflets) were placed in a 50 ml conical flask and sealed with parafilm. The flask was then heated in a water bath at 60°C for 20 min. Subsequently, the SPME fiber was introduced into the flask to absorb the volatiles for 30 min (Fig. S1) (Jing et al. 2019). Following the collection, the SPME fiber was inserted into a GC injector with a GC column (HP-5, 30 m $\times$ 0.25 mm, film thickness 0.25 μm) operating in splitless mode for 5 min. Helium gas was employed as the carrier gas. The initial GC temperature was set at 45°C for 3 min, followed by a temperature increase of $10^{\circ}\text{C min}^{-1}$ to 130°C . Subsequently, the temperature was increased at a rate of $5^{\circ}\text{C min}^{-1}$ to 180°C , and then raised to 250°C at a rate of $20^{\circ}\text{C min}^{-1}$. It was held at 250°C for 1 min, followed by a temperature reduction to 200°C at a rate of $10^{\circ}\text{C min}^{-1}$ and held for 3 min. The detector temperature was set to 225°C . The volatile compounds were identified by matching their mass spectra with the NIST 14.0 GC-MS library. For accurate qualitative and quantitative analysis of MeSA, the external standard method was utilized with the MeSA (purity $\geq 99.5\%$ GC, Aladdin, China) standard.

2.3 Effect of distances of exposure to HIPVs on plant defense responses

To examine the impact of distances exposure to HIPVs on plant defense responses, healthy cowpea plants were exposed to volatiles emitted by *T. urticae*-infested cowpea plants at varying distances. Given the standard row spacing of approximately 25 cm and plant spacing of about 50 cm for cowpea plants, 7-day-old uniform-height cowpea plants were placed at distances of 0 cm, 25 cm (equivalent to row spacing), 50 cm (equivalent to plant spacing), and 100 cm (twice the plant spacing) within a controlled environment measuring $180 \times 100 \times 105$ cm at room temperature (Fig. S2). Subsequently, over 500 spider mites (averaging 30 mites per leaf) were introduced to the plants at 0 cm, while the intact plants at other distances were exposed to the volatiles emitted by the *T. urticae*-infested plants. To prevent the movement of spider mites between the groups, a water barrier was placed between each group of plants. After 3 and 7 days of exposure, a leaf sample was collected from each potted plant. To evaluate the influence of distance, indicators such as the activities of defense enzymes in the exposed plants at both 3 days and 7 days, the levels of hormones in the exposed plants at both 3 days and 7 days, the enzyme activities of *T. urticae* reared on 3-day-exposed leaves for 3 days, and the behavior choices of *N. barkeri* were analyzed.

2.3.1 Activities of plant defense enzyme analysis

To analyze the plant defense enzyme activities, 0.1 g of cowpea leaves were ground to a fine power in liquid nitrogen. The protein concentration was determined using the Bicinchoninic Acid Assay (BCA, Solarbio Life Sciences, Shanghai, China). Subsequently, the reaction mixture was prepared and processed as per the instructions provided with the enzyme activity assay kits (Solarbio Life Sciences, Shanghai, China). The activities of phenylalanine ammonia-lyase (PAL), peroxidase (POD), dismutase (SOD), and catalase (CAT) were evaluated by measuring the changes in absorbance at 290 nm, 470 nm, 560 nm and 240 nm, respectively.

2.3.2 Quantification of SA and JA

Considering that the SA and JA pathways are two principal signaling pathways related to plant resistance, we quantitatively analyzed the contents of SA and JA to assess the plant defense responses. To quantify SA and JA, 0.1 g of cowpea leaves were ground to a fine power in liquid nitrogen and extracted with 1 ml of ethyl acetate. The JA-SA extract was vortexed at 4°C for 30 min and then centrifuged at 12,000 rpm at 4°C for 10 min. The resulting supernatant was transferred to a new tube, dried under a nitrogen flow, and then concentrated by adding 250 µl of methanol. After centrifuging at 12,000 rpm at 4°C for 20 min, the supernatant was filtered through a 0.22 µm organic filter and analyzed using liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS, Shimadzu). The retention times of JA and SA were determined based on the mass spectrum library. Dihydrojasmonic acid (H₂JA) was utilized as an internal standard for phytohormone quantification. The reversed-phase B consisted of acetonitrile, while phase A was composed of 20 mmol l⁻¹ ammonium acetate. All chemicals were of high-performance liquid chromatography (HPLC) grade (Pan et al. 2010).

2.3.3 Enzyme activities analysis of spider mites

Cowpea leaves exposed to HIPVs for 3 days were collected to create leaf discs. The leaf discs were prepared by placing a slightly larger absorbent sponge underneath the leaves, covering it with filter paper, arranging the leaves on top, and then inoculating female spider mites. These leaf discs with spider mites were placed in an incubator set at a temperature of 25°C ± 1°C, 75% ± 5% relative humidity (R.H.), and a 16: 8 hours (light: dark) photoperiod for 3 days. Approximately 300 spider mites were then collected into a centrifuge tube and ground to a fine power in liquid nitrogen. The activities of acetylcholinesterase (AChE) and glutathione S-transferase (GST) were assessed by measuring the changes in absorbance at 412 nm and 340 nm, respectively. The methods used for testing the activities of the other enzymes (POD, SOD and CAT) were consistent with those described above.

2.3.4 Behavior choices of *N. barkeri* to plants exposed to HIPVs

To investigate the impact of HIPVs-exposed plants on the olfactory behavior of *N. barkeri*, Y-tube olfactometer was used to assess the preference of predatory mites on plants positioned at different distances (0 cm, 25 cm, and 50 cm) in comparison to plants located at 100 cm away (Wu et al. 2023). The plants, exposed to HIPVs for 3 days, were placed in individual 4-L glass containers. The Y-tube consisted of two 5 cm arms with a 5 cm handle length and an inner diameter of 0.8 cm. Prior to the experiment, female predatory mites were starved for 24 hours. A choice was considered valid when a single mite selected one of the two odor sources and reached at least 2/3 of one of the arms within 3 minutes. After testing five mites, the direction of the Y-tube was altered, and after examining ten mites, the tube was substituted with a new one. Each trial involved testing 100 female predatory mites.

2.4 Effect of MeSA on induced plant defense responses

To assess the impact of MeSA on induced plant defense responses, three pots of 7-day-old intact cowpea plants were placed in a 4-L glass container. Inside the container, a 1.5 ml sterilized centrifuge tube was hung, filled with 0.05 g sterilized cotton, adsorbed 1 ml of different chemicals. The air-flow was split into two streams using a purification system (empty-activated charcoal-activate charcoal). Each stream was then directed into individual glass containers at a constant flow rate of 100 ml min⁻¹. One container received the MeSA, while the other served as a control with hexane (purity ≥ 98%, Sigma-Aldrich) (Fig. S3). After 3 days of exposure, leaf samples were collected from the plants for analysis. Plant enzyme activities, plant hormone levels, emission of plant volatiles, and plant leaf areas were used to assess the MeSA-induced plant defense responses. Moreover, enzyme activities and survival rates of *T. urticae* fed on MeSA-exposed leaves, as well as leaf damages resulting from spider mites and the behavioral choices of *N. barkeri* to MeSA were also evaluated.

2.4.1 Determination of plant leaf area

The transparent grid paper method was utilized to analyze the leaf areas of cowpea plants exposed to MeSA for 3 days. The grid paper comprised large squares measuring 1 cm on each side and small squares measuring 1 mm on each side. Subsequently, the leaves were positioned on the grid paper, and their outlines were traced. The leaf area was quantified by counting the number of small squares encompassed by the traced outline. In cases where the outline did not completely fill a small square, the calculation with 1/2 small square was the boundary. If the filled area was greater than or equal to 1/2, it was recorded as 1, whereas if it was less than 1/2, it was recorded as 0. Each small square represented an area of 1 mm². Each treatment was replicated 9–12 times.

2.4.2 Behavior choices of *N. barkeri* to MeSA

To evaluate the behavioral response of *N. barkeri* to MeSA, a Y-tube olfactometer was used, following the same procedure as described previously, with the only variation being the odor sources. In this experiment, 20 μ L each of MeSA and hexane were introduced into two separated 50 mL glass tubes as the odor sources.

2.4.3 Development and survival rate of *T. urticae* feeding on MeSA-exposed leaves

To assess the impact of MeSA-exposed cowpea leaves on the developmental stage and survival rate of spider mites, adult female mites were transferred to healthy leaf discs to collect eggs of the same age. The eggs were subsequently transferred to new leaf discs made from cowpea leaves exposed to MeSA and hexane for 3 days the following morning. These new leaf discs, measuring 1.5 cm in diameter, were placed on sponges with a diameter of 2.5 cm, each disc containing a single egg. The developmental stage and survival rate of the mites were recorded twice daily. The survival rate of *T. urticae* was calculated by dividing the number of surviving mites by the total number (initial number-number dead). There were 50 replicates for each treatment.

2.4.4 Leaf damage on MeSA-exposed leaves caused by *T. urticae*

To investigate the leaf damage on cowpea leaves exposed to MeSA caused by *T. urticae*, about 30 female adult spider mites were introduced on leaf discs obtained from cowpea leaves exposed to 5 mM MeSA for 3 days. After a 36-hour infestation, the mites were meticulously removed, and the damaged area was subsequently analyzed using ImageJ (National Institutes of Health, Bethesda, Maryland, USA). The percentage of leaf damage was calculated by dividing the damaged area by the total leaf area.

2.5 Statistical analysis

Statistical analysis of the data was performed using R 4.3 and SPSS 27.0 (IBM, Chicago, USA). Graphs were generated with GraphPad Prism 9.5, SigmaPlot 15.0, Origin 9.0 (Origin Lab) and Adobe Illustrator 27.0. Normality testing of the data was done using the Kolmogorov-Smirnov test or Shapiro-Wilk test (all $P > 0.05$). Variations in peak area of plant volatiles between two groups were evaluated through a Mann-Whitney U test. The data on plant defense enzyme activities, plant hormone levels, and enzyme activities of spider mites were compared among different exposure distances to HIPVs by the general linear model (GLM) at $P = 0.05$. Predatory mites' choice was analyzed using the Chi-square test. The data on plant defense enzyme activities, plant hormone levels, percentage of leaf damage, and enzyme activities of spider mites in MeSA exposed treatment were compared with the control group using an independent t test. Leaf area differences among treatment groups were assessed using One-way ANOVA. Survival curves were evaluated by

the Kaplan-Meier method, and differences between groups were determined by the log-rank test.

3 Results

3.1 Analysis of plant volatiles

Uninfested leaves emitted low levels of volatile compounds, whereas infested leaves, particularly those with severely infested, released a higher quantity and variety of compounds (Fig. S4). A total of eleven major volatiles were identified in the NIST 14.0 GC-MS library. Severely infested leaves exhibited a notable release of lipoxygenase-derived volatiles, including (*E*)-3-hexen-1-ol ($P = 0.004$), 1-octen-3-ol ($P = 0.004$), (*Z*)-3-hexen-1-ol, acetate ($P = 0.028$), and 2-ethyl-1-hexanol ($P = 0.004$). Additionally, terpenoids like linalool ($P = 0.006$) and (*E*)-4,8-dimethyl-1,3,7-nonatriene ((*E*)-DMNT) ($P = 0.004$), as well as aromatic compound MeSA ($P = 0.006$), showed a significant increase in severely infested cowpea leaves (Fig. S4). To determine the emission of MeSA accurately, a standard curve ($y = 4.61e + 8x - 3.34e + 7$, $R^2 = 0.99$, Fig. S5) was employed based on different concentrations of MeSA. It was revealed that severely infested leaves released approximately 0.22 mM of MeSA.

3.2 Exposure distances affect neighboring plants defense responses

The results of plant defense enzyme activities indicated that infested plants (0 cm) exhibited higher PAL and POD activities compared to healthy plants (25, 50 and 100 cm), regardless of being exposed to HIPVs for 3 days or 7 days (All $P < 0.001$, more details in supplementary material Table S1) (Fig. 1A). Furthermore, when exposed to HIPVs for 3 days, healthy plants at a proximity of 25 cm exhibited higher activities of PAL ($P < 0.001$), POD ($P < 0.001$) and SOD ($P < 0.001$) than those at a distance of 100 cm, while the enzyme activities of 50 cm were intermediate between the two distances (Fig. 1A, Table S1). After a 7-day exposure, the changes in enzyme activities followed a similar pattern to the 3-day exposure (Fig. 1A). Additionally, CAT activity was significantly lower in plants exposed at a 25 cm distance compared to those at 100 cm after exposure for both 3 ($P = 0.005$) and 7 days ($P < 0.001$) (Fig. 1A, Table S1). The proximity of neighboring plants to the source of HIPVs influenced the defense enzyme activities, with closer proximity resulting in enzyme activities more similar to those of infested plants.

After being infested with *T. urticae* for 3 days, the levels of both SA and JA in the infested plants (0 cm) were significantly higher than those in healthy plants (25, 50 and 100 cm), while after 7 days of infestation, the SA level in infested plants remained higher than SA level in healthy plants (All $P < 0.001$, more details in supplementary material Table S1) (Fig. 1B). Furthermore, the JA level in plants

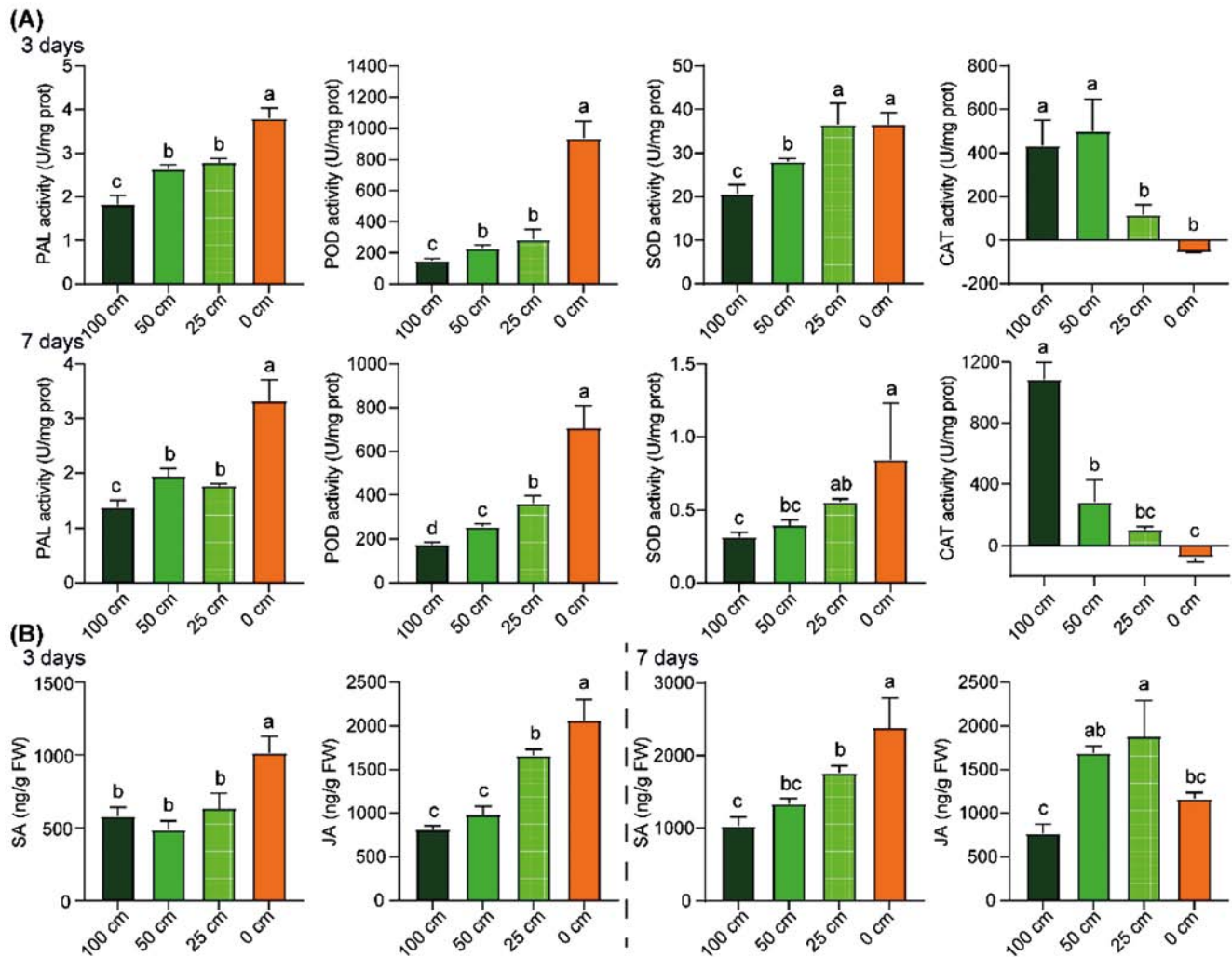


Fig. 1. The distances of exposure to HIPVs affect (A) plant defense enzyme activities and (B) plant hormone levels (mean $\pm$ SE). Different letters indicate significant differences (GLM followed by LSD post-hoc test).

exposed to HIPVs at distance of 25 cm for both 3 days ($P < 0.001$) and 7 days ($P < 0.001$) were higher than those at a distance of 100 cm, with the level at 50 cm being intermediate between the two distances (Fig. 1B, Table S1). Similarly, the SA level in plants after 7 days of exposure showed the same trend (Fig. 1B, Table S1). It is worth noting that *T. urticae*-infested plants exhibited a notable increase in SA levels ($t = -4.066$, $df = 6$, $P = 0.007$) and a decrease in JA levels ($t = 3.659$, $df = 6$, $P = 0.011$) after being infested for 7 days compared to plants infested for 3 days (Fig. S6).

After feeding on cowpea leaves that were exposed to HIPVs at various distances, the enzyme activities of *T. urticae* were analyzed. The results indicated that the activities of SOD ($P < 0.001$), CAT ($P < 0.001$), POD ($P < 0.001$), AchE ($P = 0.009$) and GST ($P < 0.001$) in *T. urticae* reared on cowpea plants exposed to HIPVs at a distance of 25 cm were significantly lower than those in spider mites reared on plants exposed to HIPVs at a distance of 100 cm (Fig. 2A,

Table S2). The activities of CAT, POD, AchE and GST in *T. urticae* reared on cowpea plants exposed to HIPVs at a distance of 50 cm were found to be intermediate between the activities observed in spider mites reared on plants at two distances (Fig. 2A, Table S2). Moreover, when compared to plants exposed to HIPVs at a distance of 100 cm, predatory mites displayed a preference for plants exposed to HIPVs at a distance of 25 cm ($\chi^2 = 4.840$, $df = 1$, $P = 0.028$) and infested plants ($\chi^2 = 19.360$, $df = 1$, $P < 0.001$) (Fig. 2B). Additionally, there was a difference in the selection of infested plants by predatory mites compared to their choice of plants exposed to HIPVs at a distance of 50 cm ($\chi^2 = 3.668$, $df = 1$, $P = 0.055$) (Fig. 2B).

3.3 MeSA mediates plant defense responses against *T. urticae*

To investigate the impact of MeSA on plants, cowpea plants was exposed to MeSA concentration approximately 2 and

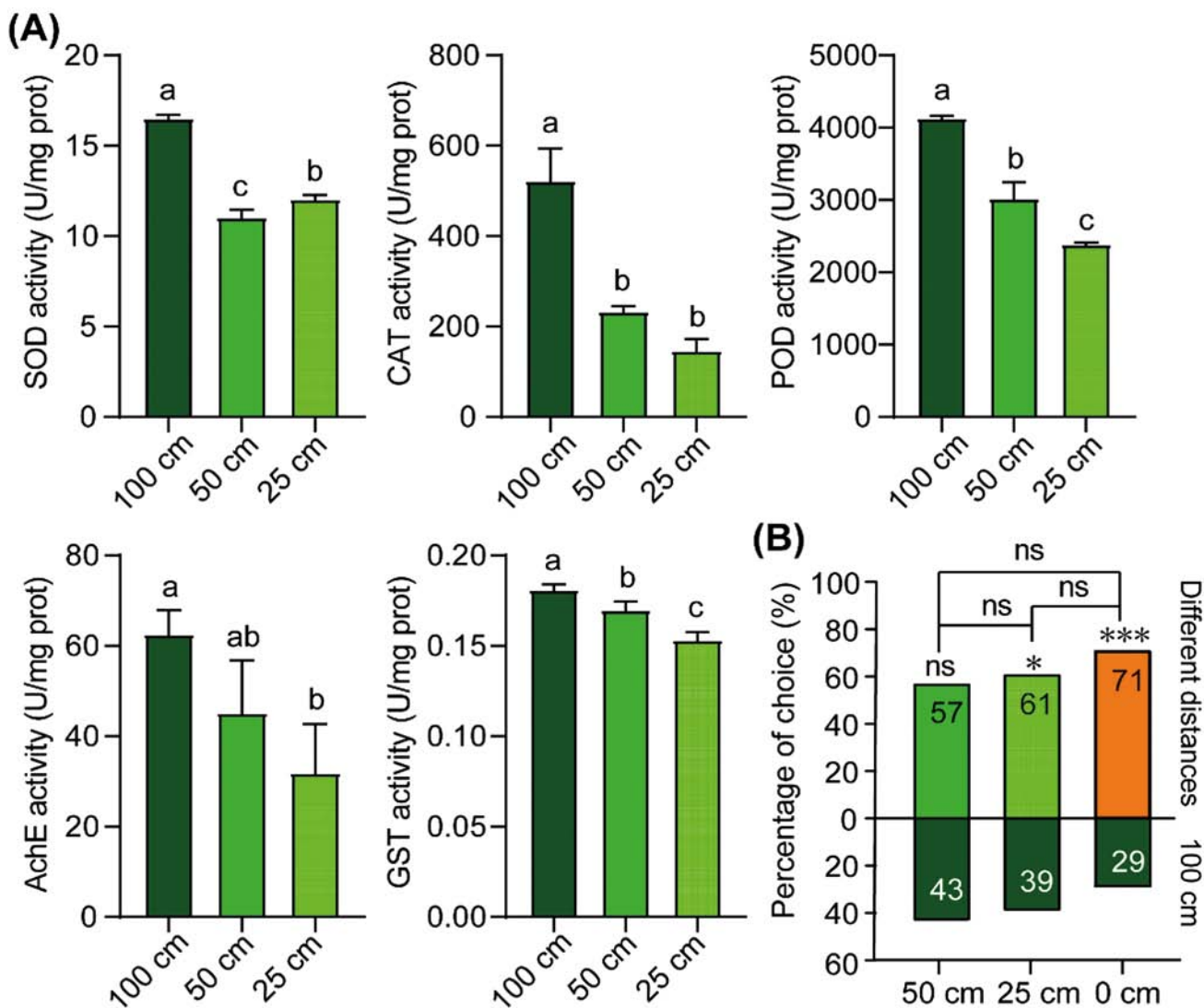


Fig. 2. (A) The enzyme activities of spider mites reared on leaves at different distances of exposure to HIPVs (mean ± SE). Different letters indicate significant differences (GLM followed by LSD post-hoc test); (B) Olfactory behavior of *N. barkeri* to different odors (Chi-square test). *, $P < 0.05$; ***, $P < 0.001$.

20 times higher than the baseline level determined through quantitative analysis. Following MeSA exposure, there was a notable increase in the activities of defense enzymes such as POD (0.5 mM, $t = -4.674$, $df = 5$, $P = 0.005$; 5 mM, $t = -6.440$, $df = 4$, $P = 0.003$), SOD (0.5 mM, $t = -8.062$, $df = 5$, $P < 0.001$; 5 mM, $t = -3.427$, $df = 3.188$, $P = 0.038$) and CAT (0.5 mM, $t = -11.642$, $df = 4$, $P < 0.001$; 5 mM, $t = -2.603$, $df = 5$, $P = 0.048$) (Fig. 3A). The SA content in plants exposed to 0.5 mM ($t = -3.366$, $df = 5$, $P = 0.020$) and 5 mM ($t = -6.302$, $df = 6$, $P < 0.001$) MeSA was 1.95 times and 1.32 times higher, respectively, than in the control plants, while the JA content showed no significant change (Fig. 3B). Additionally, plants exposed to MeSA, particularly those exposed to 5 mM MeSA, emitted a wider range and higher quantities of volatiles compared to those exposed

to hexane. Plants exposed to 5 mM MeSA released significant amounts of lipoxygenase-derived volatiles, including 1-octen-3-ol ($P = 0.009$), (Z)-3-hexen-1-ol, acetate ($P = 0.016$), and (E)-3-hexen-1-ol, acetate ($P = 0.007$), which were also prominently increased in infested plants (Fig. 3C). Furthermore, the MeSA ($P = 0.005$) concentration significantly increased, reaching 0.17 mM in plants exposed to 5 mM MeSA (Fig. 3C).

After being exposed to MeSA for 3 days, cowpea plants showed a significant increase in leaf area ($F_{(3,37)} = 30.379$, $P < 0.001$) (Fig. 4A). In comparison to feeding cowpea leaves exposed to hexane, the activities of POD (0.5 mM, $t = -7.667$, $df = 4.205$, $P = 0.001$; 5 mM, $t = -6.255$, $df = 5$, $P = 0.002$) and AchE (0.5 mM, $t = -4.789$, $df = 6$, $P = 0.003$; 5 mM, $t = -6.144$, $df = 3$, $P = 0.009$) in *T. urticae* significantly

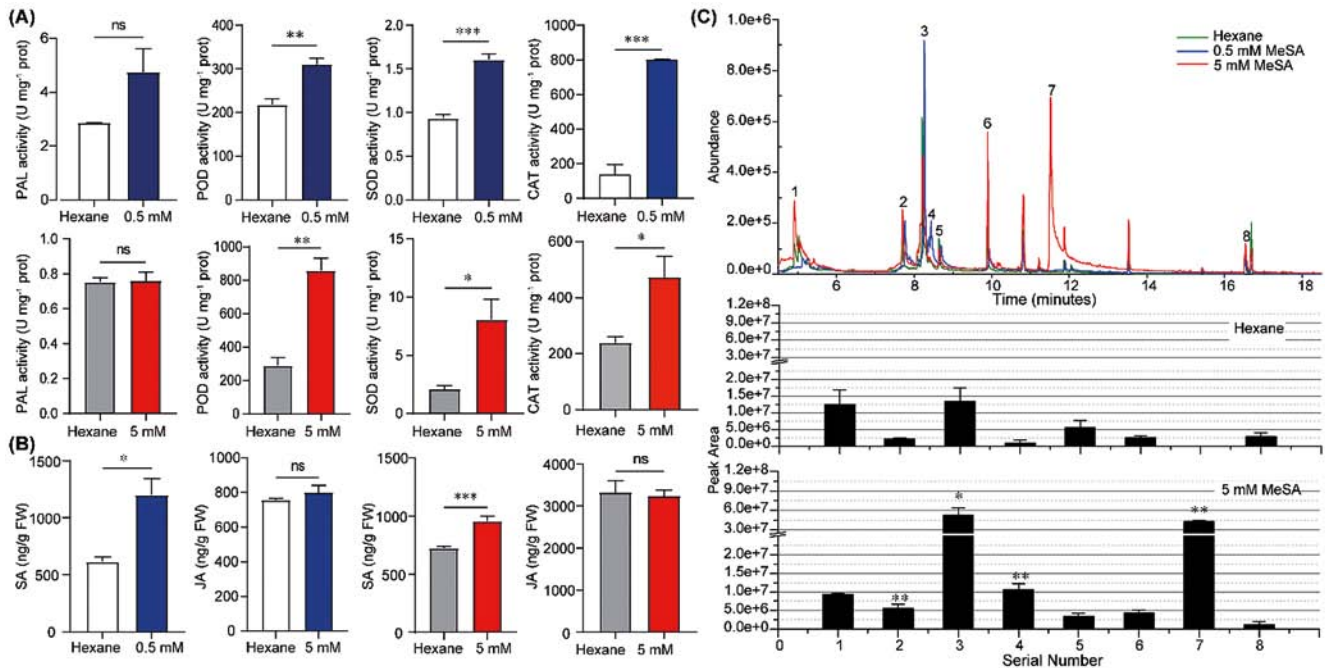


Fig. 3. MeSA primed defense responses of healthy cowpea plants. (A) Defense enzyme activities and (B) levels of SA and JA in healthy cowpea plants after exposure to MeSA for 3 days (Independent *t* test). (C) Volatiles emitted by MeSA-exposure cowpea leaves (mean $\pm$ SE) (Mann-Whitney *U* test). Eight HIPVs were represented, including (1) (*E*)-3-hexen-1-ol; (2) 1-octen-3-ol; (3) (*Z*)-3-hexen-1-ol, acetate; (4) (*E*)-3-hexen-1-ol, acetate; (5) 2-ethyl-1-hexanol; (6) linalool; (7) MeSA; (8) trans- β -ionone (3-buten-2-one, 4-(2,6,6-trimethyl-1-cyclohexen-1-yl)-). *, $P < 0.05$; **, $P < 0.01$.

increased after feeding cowpea leaves exposure to 0.5 mM and 5 mM MeSA, while the activities of CAT ($t = -3.324$, $df = 5$, $P = 0.021$) and GST ($t = -3.556$, $df = 5$, $P = 0.016$) in the spider mites were significantly higher after feeding cowpea leaves exposure to 5 mM MeSA (Fig. 4B). Survival rates of spider mites did not significantly differ between the groups exposed to 0.5 mM and hexane ($\chi^2 = 1.952$, $df = 1$, $P = 0.162$), however, the survival rate of *T. urticae* in the 5 mM MeSA-exposed group was significantly lower ($\chi^2 = 7.431$, $df = 1$, $P = 0.006$) (Fig. 4C). No significant variances were observed in the developmental time of spider mites at each stage between the groups exposed to 5 mM MeSA and hexane (Table S3). Additionally, the proportion of damaged area caused by spider mites on 5 mM MeSA-exposed leaves was lower than that on hexane-exposed leaves ($t = 3.357$, $df = 26$, $P = 0.002$) (Fig. 4D). Of course, both 0.5 mM ($\chi^2 = 14.440$, $df = 1$, $P < 0.001$) and 5 mM ($\chi^2 = 4.000$, $df = 1$, $P = 0.046$) of MeSA significantly attracted predatory mites (Fig. 4E).

4 Discussion

Plant volatiles, particularly HIPVs, are essential for mediating communication between plants and can be harnessed as a sustainable pest management tool in agriculture (Kessler

et al. 2023). This study demonstrated that the distances of exposure to HIPVs influences the defense responses of neighboring cowpea plants when subsequently attacked by spider mites. The defense responses include direct responses such as increased activities of plant defense enzymes and elevated levels of SA and JA, as well as the indirect attraction of predatory mites. Moreover, the enzyme activities of spider mites reared on the exposed leaves were reduced. Additionally, MeSA can serve as an airborne signal mediating plant defense responses against spider mites. MeSA exposure impacted leaf area, volatiles emissions, spider mite survival rates, and leaf damage. These results elucidate the importance of HIPVs, particularly MeSA, as airborne signaling molecules that regulate plant defense against *T. urticae*, thereby enhancing the understanding of tritrophic interactions involving plants, spider mites and predatory mites (Fig. S7). This research holds promise for the development of innovative and eco-friendly pest control strategies by utilizing HIPVs to trigger efficient direct and indirect defense responses in plants (Kansman et al. 2023).

The role of HIPV in triggering plant defense responses has been widely documented in previous reports (Timilsena et al. 2020; Kessler et al. 2023). The strength of induced plant defense is known to vary based on the proximity to the source of HIPVs (Heil & Adame-Alvarez 2010). This study confirms the distance-dependent nature of induced defense

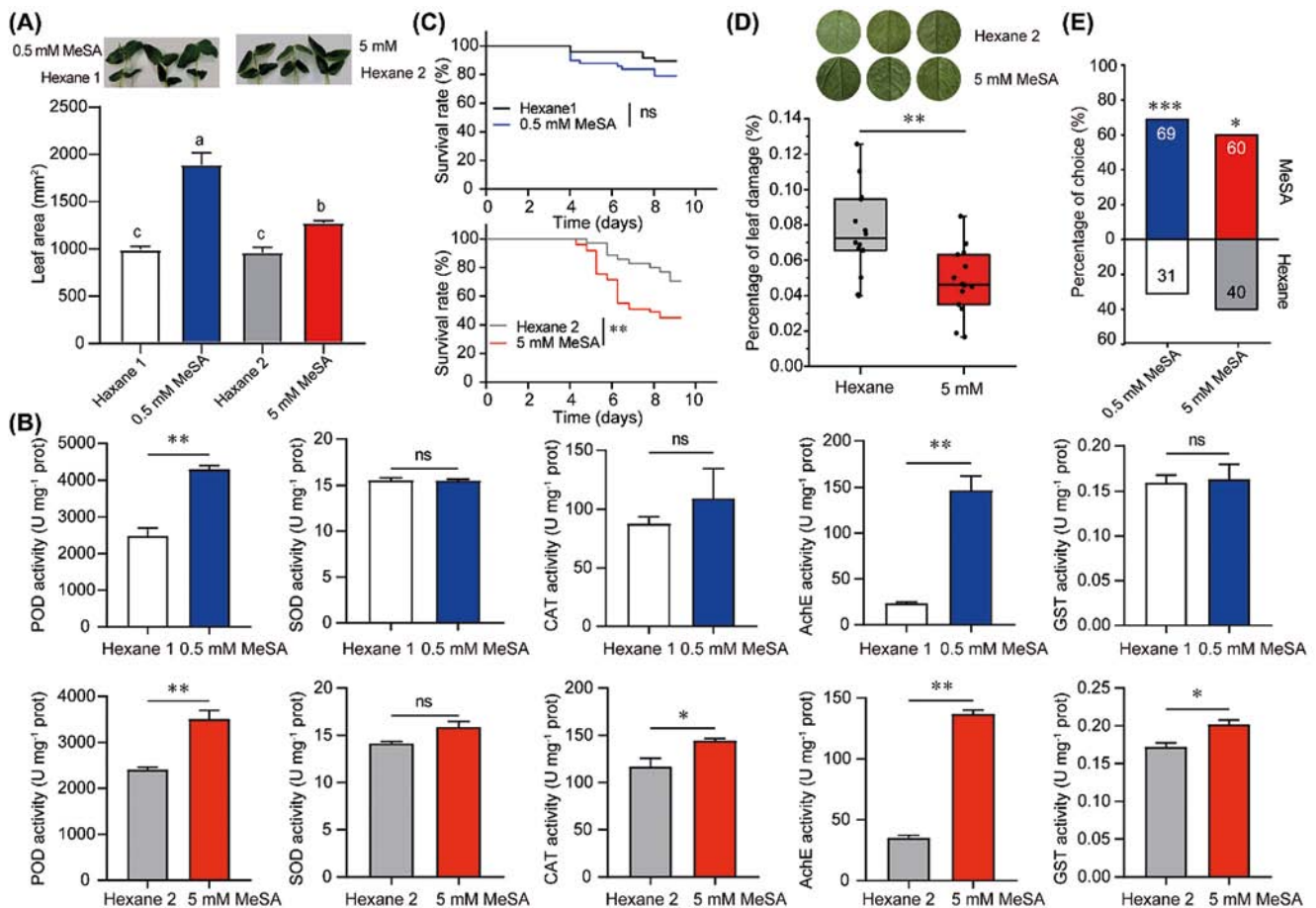


Fig. 4. MeSA primed direct and indirect defense response of plants against spider mites (mean $\pm$ SE). (A) Leaf areas of cowpea leaves exposed to MeSA (ANOVA followed by Dunnett's T3 post-hoc test). (B) Enzyme activities (Independent *t* test) and (C) survival rates of *T. urticae* after feeding on MeSA-exposed leaves (Kaplan-Meier method). (D) Percentage of leaf damage in *T. urticae*-infested MeSA-exposed cowpea leaves (Independent *t* test). (E) Olfactory behavior of *N. barkeri* to MeSA (Chi-square test). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

responses, as evidenced by changes in enzymes activities, phytohormone levels in plants, enzyme activities in spider mites, and the behavior choices of predatory mites. Unlike attacks from chewing herbivores, cell-content feeders such as aphids and spider mites trigger the SA response and promote the release of MeSA in various plant species, including tomato and lima bean (Blande et al. 2010). These findings demonstrate that severely infested plants emit higher level of MeSA, while uninfested plants emit negligible amounts. MeSA induces defense responses in healthy plants similar to HIPVs. Exposure to MeSA not only alters plant enzyme activities, plant hormone levels, and plant volatile emissions, but also affects leaf areas, leaf damages in plants, the survival rates and enzyme activities in spider mites. This suggests that MeSA may acts as an airborne signal that mediates plant defense responses against spider mites.

The activities of defense enzymes are typically used as an intuitive indicator of plant defense. PAL plays a crucial role in both the phenylpropane metabolic pathway and

SA biosynthesis pathway, while POD, SOD and CAT act as essential scavengers of reactive oxygen species (ROS) (O'Brien et al. 2012). In our study, plants exposed to HIPVs at a distance of 25 cm for 3 and 7 days exhibited increased PAL, POD, and SOD activities, along with elevated SA levels after 7 days, indicating the activation of the SA pathway and modulation of the plants' antioxidant defense systems. Conversely, the reduction in CAT activity may be attributed to the inhibitory effect of SA on CAT activity (Bittner et al. 2017). Moreover, the levels of SA and JA in plants infested with *T. urticae* for 3 days significantly increased, suggesting the activation of both SA- and JA-dependent pathways. However, the increase in SA levels after 7 days exposure and decrease in JA levels suggested that the escalation of SA-dependent responses partially suppressed JA signaling, a phenomenon also observed in plants infested by whiteflies and aphids as well (Zhang et al. 2019; Aerts et al. 2021). Furthermore, when spider mites were fed with leaves exposed to HIPVs at a 25 cm distance, their activities

of SOD, CAT, POD, AchE and GST were lower compared to fed with leaves exposure at a 100 cm distance, indicating that exposure to HIPVs may inhibit the defense mechanisms in spider mites against plant defenses, hence explaining why mites perform better on previously attacked plants compared to undamaged ones (Sarmiento et al. 2011). The results also show that predatory mites have a preference for proximity to infested plants, aiding them in locating prey, which indicates the reinforcement of indirect defense.

Plants emit a complex array of volatile compounds that serve as chemical cues to facilitate interactions between plants and other organisms, particularly in response to herbivore attacks (Hao et al. 2023). This study identified that plants subjected to spider mite attacks released significant quantities of volatile compounds, such as 3-hexen-1-ol, (Z)-3-hexenyl acetate, MeSA, DMNT, and linalool, which have been found to be associated with plant defense responses (Yang et al. 2020). Additionally, plants exposed to MeSA exhibited an increased release of 3-hexen-1-ol, (Z)-3-hexenyl acetate, and MeSA, with the extent of release being influenced by the MeSA dosage. This phenomenon has also been proved in *Betula pendula*, and figured that varying MeSA doses trigger distinct defense mechanisms (Liu et al. 2018). The roles of MeSA in enhancing plant pathogen resistance and attraction of natural enemies have been extensively studied, with MeSA functioning as a long-distance signaling molecule that mediates airborne defense against insects and viruses gaining significant attention in recent years, particularly in aphid-virus systems (Jaiswal et al. 2024; Mahmood et al. 2024). Here, we found that exposing healthy plants to MeSA led to enhanced plant defense against spider mites, as evidenced by increased activities of POD, SOD, CAT, elevated SA level, and enhanced volatile emissions. Additionally, the leaf area of cowpea plants increased when exposed to MeSA, indicating that MeSA induces growth changes in plants, consistent with the growth-promoting property of SA (Kováčik et al. 2009). Moreover, *T. urticae* fed with MeSA-exposed cowpea leaves exhibited reduced survival rates and caused less damage to the leaves, and the attractiveness of MeSA to *N. barkeri*, thus highlighting the potential application of MeSA for plant defense against spider mites (Wu et al. 2023). Regarding the increased activities of POD and AchE in spider mites reared on MeSA-exposed cowpea leaves, this may be because that *T. urticae* activate their own defense responses to counteract oxidative damage (Xin et al. 2017).

In conclusion, this study provides insights into the distance of exposure to HIPVs affects the induced defensive responses in neighboring cowpea plants against spider mites, as evidenced by changes in physiology and biochemistry analysis of plants and spider mites, as well as the behavior of predatory mites. The results also demonstrated that MeSA serves as the primary airborne signal in mediating these induced defense responses. This knowledge is of significant scientific importance in understanding the role of HIPVs and MeSA in intra- and inter-species communication among

plants. Moreover, this knowledge has practical implication for developing environmentally-friendly mite-resistant substances and advancing biological control efforts (Dong & Hwang 2017; Tang et al. 2024).

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Figure S1–S7, Table S1–S3