https://doi.org/10.26651/allelo.j/2020-49-1-1252 0971-4693/94 Euro 20.00
Allelopathy Journal 48 (2): 35-50 (January, 2020) *International Allelopathy Foundation* 2020
Table: 3, Figs : 5

Metabolomics profiling reveals the potential metabolites and biological pathways of broad bean (*Vicia faba* L.) under allelochemical stress of *Chenopodium ambrosioides* L.

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(Received in revised form: December 10, 2019)

ABSTRACT

We studied the allelopathy of *Chenopodium ambrosioides* L. volatile oil and its two monomer components (α-terpinene and ρ-cymene) on *Vicia faba* L. using physiological measurements and metabolomics profiling. The results showed that the allelochemicals decreased leaf area, seedling height, main root length, biomass and increased stomatal density, among which, volatile oil caused the greatest changes. Variable accumulation of metabolites, in response to treatment with the volatile oil and the two monomers, were identified by GC-MS analysis. Volatile oil significantly decreased the polyols and sugars (myo-Inositol and maltose) and significantly increased the TCA cycle metabolites such as succinic acid, as well as amino acids (glutamic acid, asparagine, beta-alanine and 3-cyano-L-alanine). The two monomers (a-terpinene and ρ-cymene), significantly increased the amino acids (glutamine and 3-cyano-L-alanine and ρ-cymene) but decreased the sugar gentibiose. Due to the antagonistic effects among the components, they interfere with photosynthesis, amino acid metabolism and TCA cycle of receptor plants. The 3-cyano-L-alanine, the only differential accumulating metabolite was increased in all three treatments, suggesting that allelochemical stress induced the accumulation of cyanide and other toxic substances in receptor plants, thus inhibiting its growth.

Key words: Allelochemical stress, allelopathy, amino acids, *Vicia faba* L., *Chenopodium ambrosioides* L., GC-MS, metabolites, metabolomics, photosynthesis, seedling growth, sugar, Volatile oil.

INTRODUCTION

With global integration and climate change, China is most seriously invaded by alien plant species (33). Invasive alien plant species spread through numerous modes; have strong tolerance and adaptability to invasive sites, so they become dominant population in relatively short period of time (3). Many invasive plants exert strong allelochemical stress on their surrounding plants, which leads to a decline in biological diversity, destroys the balance in ecosystem and harms the agricultural production (23,29). *Chenopodium ambrosioides* L. (annual or perennial erect herb, origin: tropical America), is distributed in most parts of China (Figure 1). It suppresses and excludes other native plants and poses great threat to the ecosystem and biodiversity of invaded areas. Jiménez-Osornio *et al*. (16) first discovered its strong allelopathic effects on surrounding plants and that water extracts from its root, stem and leaf inhibited the seed germination and hypocotyl growth of

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Amaranthus hypochondriacus L. Previous studies by our team showed that its volatile allelochemicals had cytotoxicity effects on receptor plants. They increased the level of reactive oxygen species (ROS) in receptor plants cells (4,12) caused cell structure disorder, chromosome aberration, blockage of mitotic process (11,28), abnormality of gene expression, decreased cell activity and even apoptosis (13,18,36).

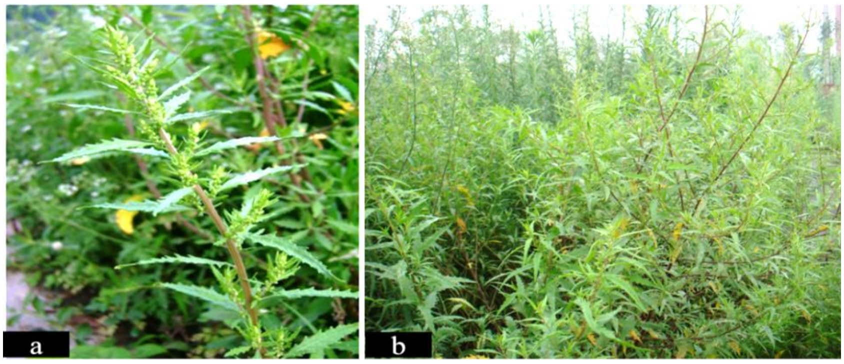

Figure 1. Full *Chenopodium ambrosioides* L. plant (a) and its population (b)

There are about 0.2-1 million endogenous metabolites in plants. Qualitative and quantitative analysis of these metabolites is important, to understand the plant life activities in the post-genomic era (6). Plant response to adversity is complex physiological process. A large number of metabolic intermediates and end products, including metabolic regulators and signal transduction factors, are produced in the whole regulatory network. Metabolomics technology is field of systems biology. It can quickly reflect the final results of all cellular events (transcription, translation and post-translation) and provides evidence of phylogenesis and biochemical phenotype (21). Plant response to allelopathy involves a series of complex metabolic regulation processes. Non-targeted metabolomics is a method that provides global metabolite detection and is powerful tool to reveal the allelopathy mechanisms (20). By GC-MS metabolomics, Jian *et al.* (15) revealed that the allelochemical 1-octene-3-alcohol accelerated the primary metabolism of *Pyropia haitanensis* L.; Araniti *et al* (1) revealed that *Zea mays* L. increased the contents of ascorbic acid and carotenoids to eliminate the free radicals induced by the allelochemical cinnamic acid. However, it is not clear how the metabolite spectrum of receptor plants changes in response to allelochemical stress, caused by volatile oil and its two main components (α-terpinene and ρ-cymene). In this study, *Vicia faba* L, a crop plant widely cultivated in the invaded area of *Chenopodium ambrosioides* L., was used as receptor plant to study the changes in the metabolic spectrum of *Vicia faba* L. leaves. We studied seedlings under the action of volatile oil and its main components, α-terpinene and ρ-cymene, using non-targeted Gas chromatography-mass spectrometry (GC-MS) metabolomics technology, to provide theoretical basis to further reveal the mechanism of allelopathy and spread of *Chenopodium ambrosioides* L.

MATERIALS AND METHODS

The mature plants of *Chenopodium ambrosioides* L. were collected in October 2018 from Baojiang Bridge Community, Chengdu City, Sichuan Province (N 30°35'34″, 104°5'57″E). The volatile oil was extracted from 1-2 cm pieces of fresh or dry plant material by steam distillation method (22). (The contents of volatile oil, α-terpinene and ρ-cymene were 843 mg/mL, 151 mg/mL and 156 mg/mL, respectively. The standard products of α-terpinene (Shanghai Yuanye Biotechnolgy, ≥ 90%) and ρ-cymene (Adamas-beta, ≥ 90%) were used as references in this study. The test variety ‘Chenghu 1#’of *Vicia faba* L. was used.

The uniform size seeds of *Vicia faba* L. were selected and sterilized by soaking in 0.5 % $KMnO_4$ for 10 min and then soaked in distilled water for 24 h. Then germinated in the dark at 25 ℃ for 2-3 days. The uniform mature seeds were sown 1 seed per pot (10 cm dia and 6 cm in height, filled with 300 g quartz sand) and added 50 mL Hoagland nutrient solution. The pots were Kept on Growth Chamber [14 h/10 h light-dark period (25℃ during the light period and 18 ℃ during the dark period) and relative humidity is 80%] for 12 days. Hoagland nutrient solution ($CaNO_3$•$4H_2O$ 945 mg/L, KNO_3 506 mg/L, NH_4NO_3 80 mg/L, KH_2PO_4 136 mg/L, $MgSO_4$•$7H_2O$ 493 mg/L, 2.5 mL of iron salt solution, 5 ml of trace element solution, pH 6.0) was added at 10 mL per pot every 2 days.

Allelochemical stress was applied to *Vicia faba* L. seedlings when they had true leaves at 4 days. Five treatment concentrations T1: volatile oil, α-terpinene and ρ-cymene are 1.00×10^{-3}, 1.86×10^{-4}and1.69×10^{-4}, respectively. T2: volatile oil, α-terpinene and ρ-cymene are 2.00×10^{-3}, 3.72×10^{-4} and 3.38×10^{-4}, respectively. T3: volatile oil, α-terpinene and ρ-cymene are 3.00×10^{-3}, 5.58×10^{-4} and 5.07×10^{-4}, respectively T4: volatile oil, α-terpinene and ρ-cymene are 4.00×10^{-3}, 7.44×10^{-4} and 6.76×10^{-4}, respectively, T5: volatile oil, α-terpinene and ρ-cymene are 5.00×10^{-3}, 9.30×10^{-4} and 8.45×10^{-4}, respectively. from previous studies were used (9). Samples without any treatment were used as control (Table 1). The healthy plants with same growth condition were selected and placed in Special Glass Box (25 cm × 20 cm × 40 cm) with sealed lid, each treatment has 6 plants and 3 repeats. Volatile oil, α-terpinene and ρ-cymene in different treatment concentrations were added to small petri dish, which was placed in the centre of the bottom of glass box and sealed tightly with Vaseline. Allelochemical stress was applied for 3 h (10:00-13:00) per day and then the glass boxes were uncovered and the volatile oil, α-terpinene and ρ-cymene were completely volatilized. After 7 d of continuous treatment and 1 d of resumption of culture, the required parameters were determined. The whole process was done in incubator at 25℃ and 5000 lx.

Table 1. Concentrations of *Chenopodium ambrosioides* L. volatile oil and its two main components, α-Terpinene and ρ-Cymene used in study

Treatment groups	**Treatment concentrations (μl/cμ^3)**					
	Control	T1	T2	T3	T4	T5
volatile oil	0	1.00×10^{-3}	2.00×10^{-3}	3.00×10^{-3}	4.00×10^{-3}	5.00×10^{-3}
ρ-Cymene	0	1.69×10^{-4}	3.38×10^{-4}	5.07×10^{-4}	6.76×10^{-4}	8.45×10^{-4}
α-Terpinene	0	1.86×10^{-4}	3.72×10^{-4}	5.58×10^{-4}	7.44×10^{-4}	9.30×10^{-4}

Indexes Determination

Stomatal density: The stomatal density was measured by nail polish blotting as per slightly modified method of Xie *et al* (32). The colourless nail polish was evenly smeared in the center of upper epidermis and lower epidermis of the leaves in the middle part of the *Vicia faba* L. plant. After the nail polish was dried, the imprinted specimen was collected (area: 5 mm × 12 mm) to make temporary mounting. A Leica DM3000 microscope (Leica Corp., Germany) was used to examine the specimen, photographs were taken of visual fields (0.276 mm^2) from each mounting. Then the number of stomata was counted by Adobe Photoshop CS5 and the stomatal density (No. mm^{-2}) was calculated as under:

$$SD = N / M$$

Where, SD: Stomatal density, N: Stomatal number per unit area and M: Area of visual field.

Leaf area: The area (mm^2) of leaves in the middle part of the *Vicia faba* L. plant was measured by leaf area meter (AM 300-002, ADC BioScientific Ltd, England). Two leaves were measured for each plant. A total of 12 leaves were measured for each treatment.

Seedling Growth: After measuring the seedlings stomatal density and leaf area, the quartz sand adhered on the roots was removed and the main root length (cm) and seedling height (cm) was measured by ruler. Then the aboveground parts and underground parts were separated by scissors. Thereafter, plant samples were dried in oven at 110℃ for 60 min, 80℃ for 48 h until the samples reached a constant weight and then dry weight (g) was recorded.

Metabolomics Analysis

After allelopathic stress, the samples treated with highest treatment concentration in each group were used for GS-MS analysis. Leaves hanol (pre-cooled at -20℃) was added in 1400 μL volume and the tube was vortex for 30 s. Then, 60 μL of Ribitol (0.2 mg/mL stock in methanol) was added as an internal quantitative standard and the tube was vortexed for 30 s. The tubes were placed in ultrasound machine at room temperature for 30 min. Then 750 μL chloroform (pre-cooled at -20 ℃) and 1400 μL deionized water (dH_2O) (4 ℃) was added and the tube was vortexed for 60 s. The tube was centrifuged for 10 min at 14000 rpm at 4℃ and 1 mL of the supernatant was transferred into a new centrifuge tube. Samples were blow-dried by vacuum concentration. To the dried sample, 60 μL of 15 mg/mL methoxyamine pyridine solution was added and the tube was vortexed for 30 s and allowed to react for 120 min at 37 ℃. To the mixture, 60 μL BSTFA reagent (containing 1 % TMCS) was added and allowed to react for 90 min at 37 ℃. Then the tube was centrifuged at 12000 rpm at 4 ℃ for 10 min and the supernatant was transferred to a bottle.

GC-MS was performed on a HP-5MS capillary column (5 % phenyl/95 % methylpolysiloxane 30 m × 250 μm i.d., 0.25 μm film thickness, Agilent J & W Scientific, Folsom, CA, USA) to separate the derivatives at constant flow of 1 mL/min helium. The sample (1 μL volume) was injected in split mode in a 20:1 split ratio by the auto-sampler. Injection temperature was 280 °C, the interface set to 150 ℃ and the ion source adjusted to 230 ℃. The programmes of temperature-rise was followed by initial temperature of 60 ℃

for 2 min, 10 ℃/min rate up to 300 ℃ and staying at 300 ℃ for 5 min. Mass spectrometry was determined by full-scan method with range from 35 to 750 (m/z).

Statistical analysis

SPSS20.0 (IBM) was used for variance analysis and multiple comparisons. Principal component analysis (PCA) and orthogonal partial least squares-discriminant analysis (PLS-DA) were used to observe the abnormal values of sample distribution and further analyze the metabolites, which contributed greatly to the separation between groups. SIMCA 13.0 was used to analyze the differentiation of metabolites.

RESULTS AND DISCUSSION

Effects of allelochemicals stress on the seedling growth

Volatile allelochemicals released from plants into the surrounding environment enter the soil (13) through soil adsorption, root exudation and decomposition of residues, while most of them are released into the atmosphere, affecting the receptor plants. After the volatile compounds from *Chenopodium ambrosioides* are released into the environment, strong allelochemical stress inhibits the growth of its surrounding plants (26). The results of this study further confirmed this conclusion. Figure 2 showed that the length of the main root, seeding height and dry weights of *Vicia faba* seedlings treated with volatile oil, α-terpinene and ρ-cymene were significantly lower than control ($P< 0.05$) and showed a concentration-dependent effects. When the concentration reached the maximum(Volatile oil: 5.00×10^{-3}, α-terpinene : 9.30×10^{-4} and ρ-Cymene:8.45×10^{-4}), the seedling height in the volatile oil, α-terpinene and ρ-cymene groups was 58.07 %, 74.10 % and 72.43 % of the control group, respectively, the length of main root was 74.77 %, 81.51 % and 85.45 % of the control group, respectively, the aboveground dry weights were 63.26 %, 92.69 % and 80.90 % of the control, respectively. However, this inhibitory effects of α-terpinene and ρ-cymene were less than volatile oil, and there was no significant difference between them. Comparing the results of the main root length, seedling height and biomass it appears that the effect of allelochemicals on the growth of aboveground part was greater than that of the underground part in the receptor plant.

Effects of allelochemicals stress on the seedling leaf area and stomatal density

Environmental stress often affects plant growth through stomatal restriction and reduction of CO_2 intake. At the same time, a change in leaf morphology could directly affect photosynthetic efficiency (8). Exposed to volatile oil, α-terpinene and ρ-cymene, the seedlings leaves exhibited deformity, with the main veins appearing bulged and the middle veins showing increased thickness. In addition, the leaf area was significantly smaller than that of control leaves ($P < 0.05$) (Figure 3). The decrease in leaf area due to treatment with volatile oil and α-terpinene was concentration-dependent, but there was no significant difference in leaf area between different concentrations of ρ-cymene ($P > 0.05$). At the maximum treatment concentration (Volatile oil: 5.00×10^{-3}, α-terpinene: 9.30×10^{-4} and ρ-Cymene: 8.45×10^{-4}), the leaf area in the volatile oil, α-terpinene and ρ-cymene treatment groups was 33.08 %, 84.56 % and 83.5 0% of control, respectively.

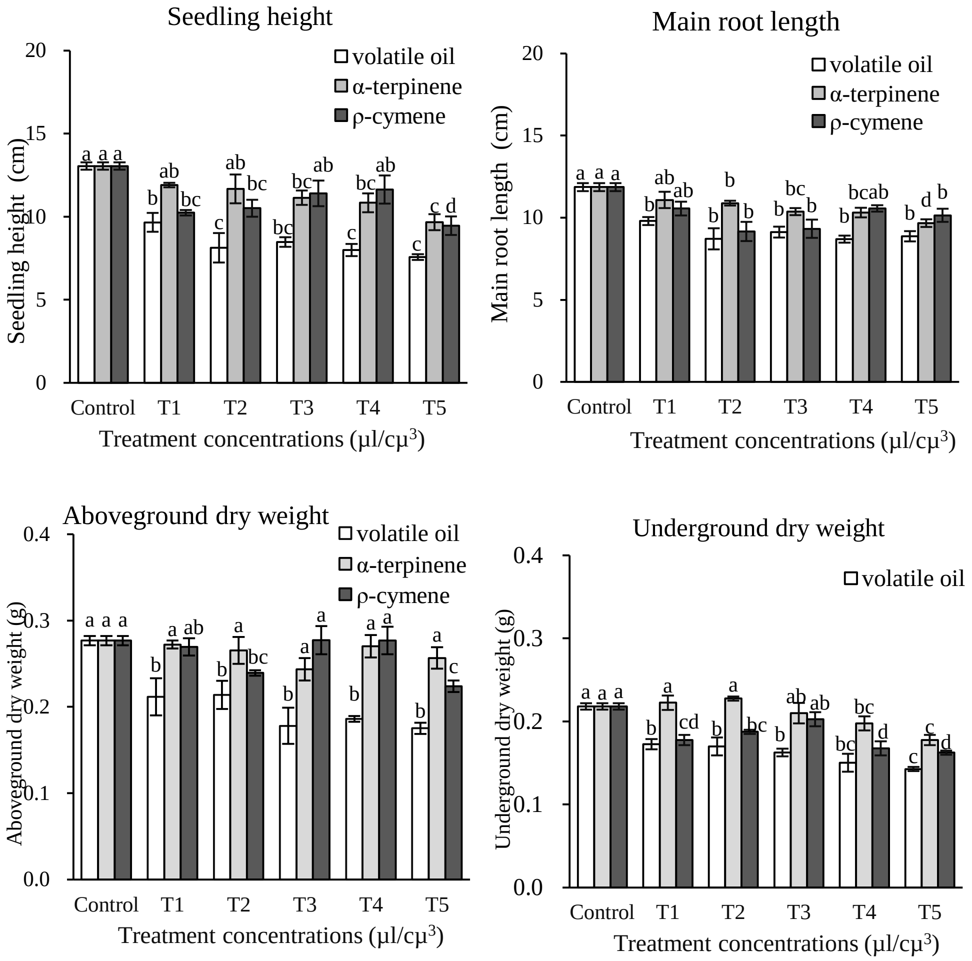

Figure 2. Effects of volatile oil, α-terpinene and ρ-cymene on seedlings growth of *Vicia faba* L. Different letters means there are significant differences between the treatments and control groups ($P < 0.05$).

T1: volatile oil, α-terpinene and ρ-cymene are 1.00×10^{-3}, 1.86×10^{-4}and1.69×10^{-4}, respectively.
T2: volatile oil, α-terpinene and ρ-cymene are 2.00×10^{-3}, 3.72×10^{-4} and 3.38×10^{-4}, respectively.
T3: volatile oil, α-terpinene and ρ-cymene are 3.00×10^{-3}, 5.58×10^{-4} and 5.07×10^{-4}, respectively
T4: volatile oil, α-terpinene and ρ-cymene are 4.00×10^{-3}, 7.44×10^{-4} and 6.76×10^{-4}, respectively,
T5: volatile oil, α-terpinene and ρ-cymene are 5.00×10^{-3}, 9.30×10^{-4} and 8.45×10^{-4}.
Volatile oil from *Chenopodium ambrosioides* L., α-terpinene and ρ-cymene were pure.

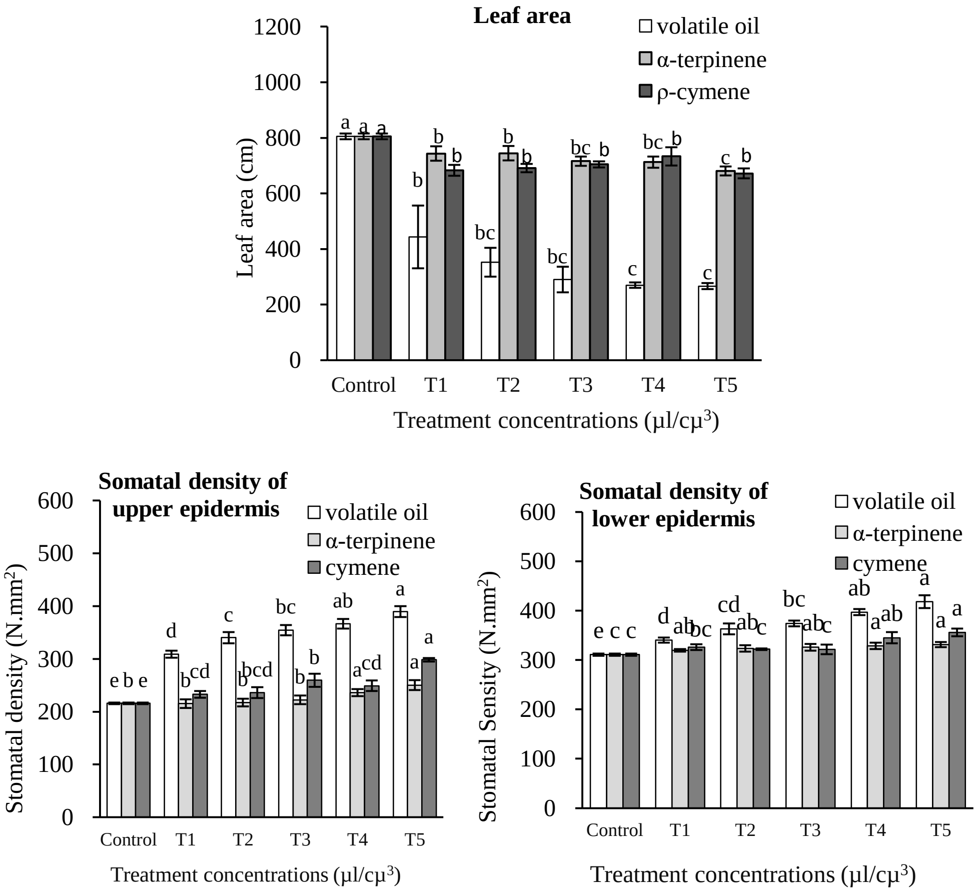

Figure 3. Effects of applied volatile oil, α-terpinene and ρ-cymene on leaf area and stomatal density of *Vicia faba* L.
Different letters means show significant differences between the treatments and control ($P < 0.05$).

The increase in stomatal density and the decrease in leaf area under stress may be an adaptive mechanism of soybean (*Glycine Max* L.) (8). Similar results were also found in this study, the stomatal density of the upper and lower epidermis of *V. faba* were increased with the increase in volatile oil, α-terpinene and ρ-cymene concentrations. At the highest concentration, the stomatal density of the upper epidermis in seedlings treated with volatile oil, α-terpinene and ρ-cymene was 1.80, 1.16 and 1.38 times higher than that of control seedlings respectively and the stomatal density of the lower epidermis was 1.35, 1.07 and 1.14 times higher than that of the control respectively. Therefore, when cytotoxicity of these allelochemicals exceeds the tolerance range of *V. faba*, it could lead to guard cell aberration and even apoptosis (14, 36), which could damage the photosynthesis process of receptor plants (9).

Effects of allelochemicals stress on metabolites in *Vicia faba* L. leaves

The metabolites of *Vicia faba* L. leaves after allelochemical stress were studied by using GC-MS non-targeted metabolomics. 218 peaks were detected, 82 metabolites were annotated. According to chemical structure, they were divided into nine categories: amino acids (37.00%), carbohydrates (13.00%), nucleotides (1.00%), organic acids (20.00%), fatty acids (3.00%), phosphoric acid (7.00%), amines (4.00%), polyols (7.00%) and others (8.00%). Principal component analysis (Figure 4) was carried out to study the differential distribution of metabolites in seedlings treated with the allelochemicals (volatile oil, α-terpinene and ρ-cymene) and the control seedlings. All samples were in 95% confidence interval. According to PCA score plot, abnormal samples were eliminated. In order to eliminate the influence of background noise and intra-group differences and highlight inter-group differences, a two-component PLS-DA model with cross-validation was established (Figure 4). The results showed that volatile oil, α-terpinene and ρ-cymene treatment had obvious separation effect, but the dispersion in these three groups was large, indicating that the metabolic process of *V. faba* leaves was in a changing stage during allelochemical stress exerted by *Chenopodium ambrosioides* L. While the effects of α-terpinene and ρ-cymene on secondary metabolites in *Vicia faba* L. leaves were similar, there were significant differences between the control group and the volatile oil treatment group.

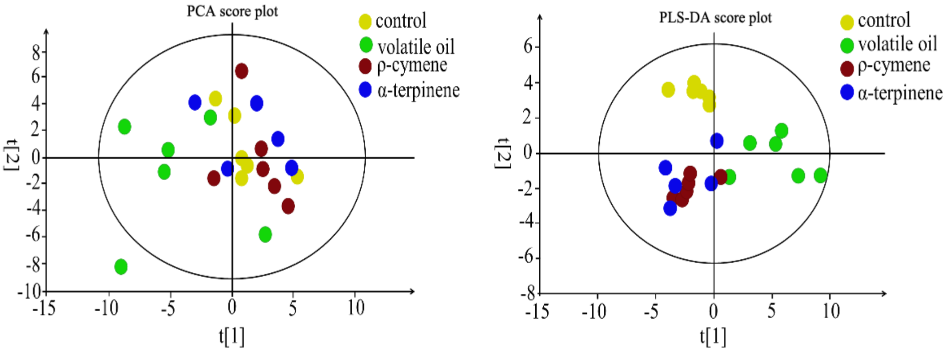

Figure 4. Principal component analysis (PCA) and partial least squares-discriminant analysis of metabolites in *V. faba* leaves as affected by volatile oil, α-terpinene and ρ-cymene

Effects on differential accumulation of metabolites in *Vicia faba* L. leaves

Because the plant cannot escape the unexpected stress, a complex metabolic regulation mechanism has been formed in the long-term evolution process (10). In this study, exposed to volatile oil and its two main components, the metabolite spectrum of *Vicia faba* L. leaves changed significantly, but the effects of different treatments were obviously different. The results showed that there were significant changes in the levels of 27 metabolites, including 8 amino acids, 6 organic acids, 2 fatty acids, 4 sugars, 2 amines, 1 nucleotide, 2 phosphoric acids and 2 other metabolites (Table 2). Compared with control,

Table 2. Effects of volatile oil, α-Terpinene and ρ-Cymene on various metabolites in *Vicia faba* L. seedlings

Metabolites	**VIP**	***P***	**FC**
Volatile oil			
3-cyano-L-Alanine	1.9192	0.00824	2.53825
Succinic acid	1.42668	0.03064	2.01354
beta-Alanine	1.88708	0.01307	1.92660
Suberyl glycine	1.60407	0.01307	1.87890
4-hydroxyButyric acid	1.59863	0.03064	1.87832
2-amino-Butyric acid	1.30846	0.04533	1.82978
Malonic acid	1.33032	0.03064	1.73742
Monomethylphosphate	1.65150	0.04533	1.67974
Adenosine	1.59604	0.01307	1.66017
Glutamic acid	1.64911	0.01307	1.57379
Gulonic acid	1.67302	0.03064	1.52546
Asparagine	1.38541	0.03064	1.34130
Ethanolamine	1.59821	0.03064	1.28570
Glycine	1.43163	0.04533	0.77635
myo-Inositol	1.45854	0.01307	0.64818
Maltose	1.76781	0.01307	0.61549
α-Terpinene			
3-cyano-L-Alanine	2.19848	0.01371	3.20086
Glutamine	1.96962	0.02248	2.93451
Threonic acid-1,4 -lactone	1.60077	0.03576	1.29850
Threitol	2.23094	0.03576	1.29850
Octadecanoic acid	1.73851	0.03576	0.83949
Hexadecanoic acid	1.70705	0.03576	0.81435
ρ-Cymene			
3-cyano-L-Alanine	2.42022	0.005075	4.60965
Glutamine	2.16289	0.01307	2.24400
Parabanic acid	2.01907	0.02024	2.12906
Threonic acid-1,4- lactone	2.30037	0.00824	1.40096
Hydroxylamine	2.3442	0.00824	1.25344
Glycolic acid	1.45044	0.04533	1.24204
Threitol	2.11857	0.00824	1.23900
Xylose	1.08367	0.04533	1.14040
2-Ketoglutaric acid	1.47279	0.04533	0.84810
gentibiose	1.83	0.013065	0.35997

Note: The screening was based on the VIP (variable importance in the projection) (threshold value ≥ 1) of the first principle component of the OPLS-DA model and the *p* value (threshold value ≤ 0.05) from the Student's *t*-test; FC: fold change.

the volatile oil treatment group had 16 differentially accumulating metabolites, of which 3 (myo-inositol, maltose and glycine) were significantly decreased and 13 were significantly increased. The change in the levels of 3-cyano-L-alanine was the largest, followed by succinic acid and beta-alanine. In the α-terpinene treatment group, 6 differentially accumulating metabolites were identified, of which 2 (hexadecanoic acid and octadecanoic acid) were significantly decreased. Interestingly, these two metabolites were the only fatty acids in the 27 differentially accumulating metabolites detected in this study. The change in the levels of 3-cyano-L-alanine was the greatest, followed by glutamine. In the ρ-cymene treatment group, there were 10 differentially accumulating metabolites, of which 8 increased and 2 (gentibiose and 2-ketoglutaric acid) decreased compared to the control group. The change in the levels of 3-cyano-L-alanine was the greatest, followed by glutamine and parabanic acid.

Amino acid metabolism played an important role in the response of seedlings to volatile oil from *Chenopodium ambrosioides*. 4-aminobutyric acid (GABA) is an important product of transamination metabolism and plays an important role in the regulation of osmotic potential, pH and TCA cycle (27, 31). In this study, volatile oil from *Chenopodium ambrosioides* L. stimulated the accumulation of glutamic acid and GABA in *V. faba* leaves. GABA as a supplementary loop of TCA cycle promotes the accumulation of succinic acid and maintains the TCA cycle. In addition, volatile oil stimulated the metabolic pathways related to aspartic acid and β-alanine metabolism. β-alanine is a non-protein amino acid and its content in plants is positively correlated with the degree of stress. Inositol, an important precursor and raw material in plants (24), has the function of protecting chloroplast membrane structure and antioxidant system, increasing photosynthetic rate and biomass (34). At night, chloroplast starch decomposes to form maltose, which is transported to the cytoplasm to form sucrose, providing energy metabolism for plants (30). Inositol and maltose in *Vicia faba* L. leaves were significantly decreased in seedlings exposed to volatile oil, indicating that volatile oil reduced photosynthetic rate and carbohydrate synthesis of seedlings, which was consistent with the changes of biomass, root growth, seedling height and leaf area in this study. In conclusion, the volatile oil had a great influence on amino acid metabolism and photosynthesis, which confirmed our previous research (9).

Compared with allelopathic effects of volatile oil from *Chenopodium ambrosioides*, the amount of differential metabolites and metabolic pathways were less affected by its two monomer components, α-terpinene and ρ-cymene in *Vicia faba* L. seedlings. Glutamine, the precursor of glutathione in leaves, showed a marked upward trend under the action of both monomer components, but its downstream metabolites proline, putrescine and spermidine did not change significantly, indicating that α-terpinene- and ρ-cymene- treated may promote the synthesis of glutamine and glutathione, regulate nitrogen metabolism and enhance antioxidant capacity in *V. faba* seedlings (35). In addition, threonine-1, 4-lactone and threitol related to ascorbic acid metabolism were also significantly increased by α-terpinene and ρ-cymene. Our previous studies showed that ROS was increased in the receptor plants cells (4,36) exposed to α-terpinene and ρ-cymene, which indicated that *Vicia faba* L. seedlings could eliminate ROS induced by allelochemical stress and alleviate oxidative damage by up-regulating the levels of glutathione and ascorbic acid. Plants regulate the metabolism of saturated and unsaturated fatty acids and maintain the relative fluidity of the cell membrane through desaturation during stress (7). In this study, only α-terpinene treatment showed significant decreases in octadecanoic acid and hexadecanoic acid. In addition, the significant increase in glutamine suggests that allelochemical stress by α-terpinene promotes fatty acid catabolism and maintains the metabolic balance of glycolysis and Citrate cycle.

3-cyano-L-alanine was the only metabolite that was significantly increased in all treatments and its levels in volatile oil, α-terpinene and ρ-cymene treatment group were 2.53, 3.2 and 4.6 times higher compared to the control group, respectively. In response to stress, cyanide is often released with the formation of ethylene. On the one hand, cyanide, as a signaling molecule, plays an important role in seed germination and root hair development (2). On the other hand, cyanide can inhibit plant growth, reduce chlorophyll content and induce nuclear degradation (5). Cyanide metabolism of plants is closely related to the growth and development. 3-cyano-L-alanine is the product of cyanide detoxification in plants. It is synthesized with cysteine and cyanide by the enzyme 3-cyano-L-alanine synthetase. It could further lead to the synthesis of aspartic acid or asparagine, or produce alanine (19,25) through the enzyme nitrile lyase. 3-cyano-L-alanine was the only co-expressed and significantly increased metabolite in all groups. Combined with the significant increase in asparagine and the significant change of β-alanine metabolic pathway, it could be speculated that allelochemical stress of *Chenopodium ambrosioides* L. activates shikimic acid metabolic pathway, accelerates the production of secondary metabolites such as phenylalanine, tryptophan and tyrosine and promotes the accumulation of cyanide in large quantities.

KEGG pathways associated with accumulating metabolites in *Vicia faba* L. leaves

The differentially accumulating metabolites were assigned to specific metabolic pathways based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (https://www.genome.jp/kegg). For the volatile oil treatment group, the largest number of differentially accumulating metabolites were observed in the ‘alanine, aspartate and glutamate metabolism’, ‘cyanoamino acid metabolism’ and ‘glutathione metabolism’ pathways. Differential metabolite accumulation was also observed in the ‘alanine, aspartate and glutamate metabolism’ and ‘cyanoamino acid metabolism’ pathways for both the ρ-cymene and α-terpinene treatment groups. Additionally, one differentially accumulating metabolite in the ρ-cymene treatment group was associated with the citrate (TCA) cycle pathway (Table 3).

To analyze the overall changes in metabolic pathways of *Vicia faba* L. seedlings in response to volatile substances from *Chenopodium ambrosioides* L., differential accumulating metabolites were located on a metabolic network map (Figure 5). Figure 5 showed that antioxidant, membrane phospholipid, fatty acid, glutamic acid and cyanoamino acid metabolism were affected to varying degrees. Interestingly, the glycolysis pathway was not affected, but the disaccharides, maltose and xylose associated with glycolysis were significantly reduced. One explanation for this could be that the components of volatile allelochemicals are complex and there are some synergistic or antagonistic effects among them.

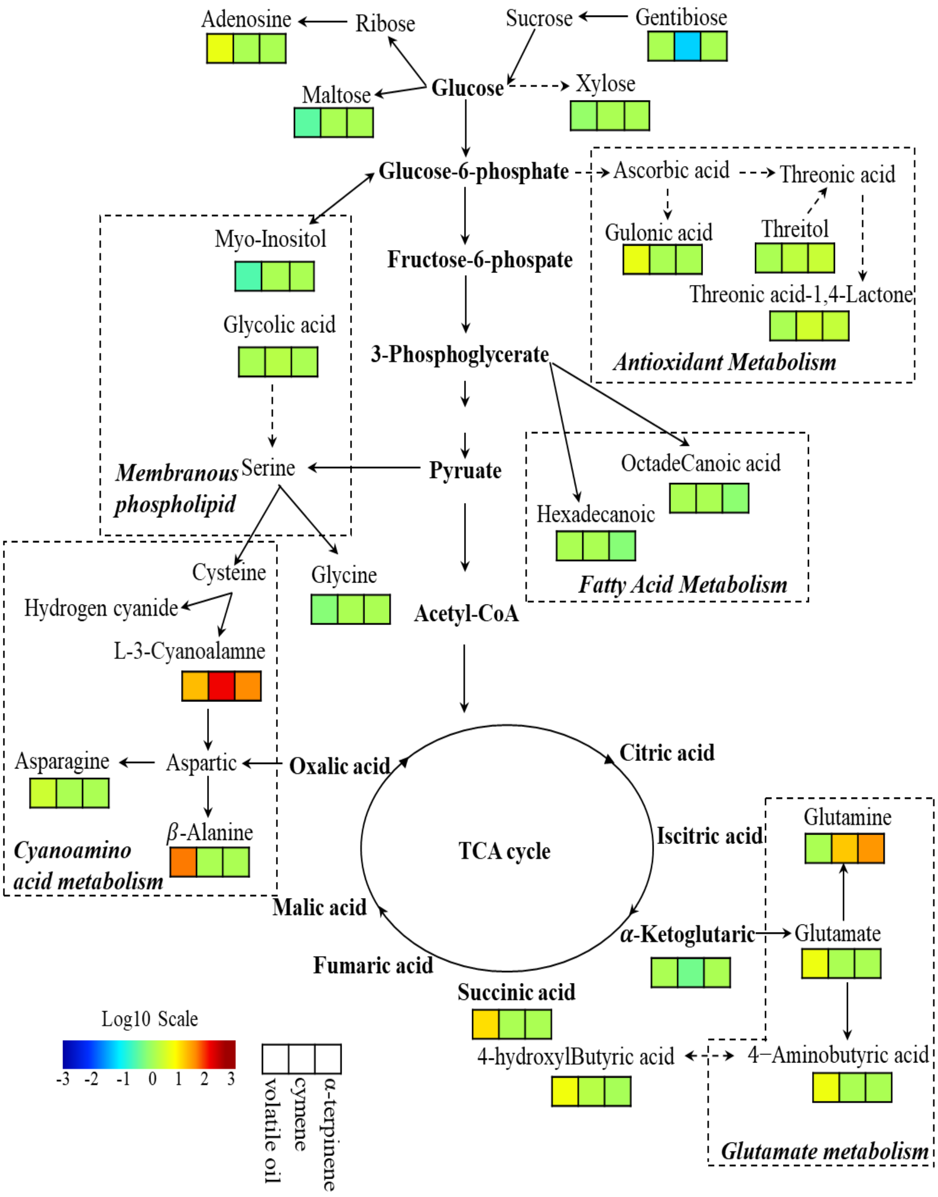

Figure 5. Effects of volatile oil, α-terpinene and ρ-cymene from *Chenopodium ambrosioides* L. on metabolic pathways of *Vicia faba* leaves.

Table 3. KEGG pathway analysis of different metabolites in leaves of *Vicia faba* L. seedlings

Treatment groups	**Metabolic pathway**	**Total**	**Hits**
Volatile oil	Alanine, aspartate and glutamate metabolism	22	3
	Cyanoamino acid metabolism	11	2
	Glutathione metabolism	26	2
	beta-Alanine metabolism	12	1
	Pantothenate and CoA biosynthesis	14	1
	Citrate cycle (TCA cycle)	20	1
	Inositol phosphate metabolism	24	1
	Starch and sucrose metabolism	30	1
	Glycine, serine and threonine metabolism	30	1
	Arginine and proline metabolism	38	1
	Purine metabolism	61	1
ρ-Cymene	Alanine, aspartate and glutamate metabolism	22	2
	Cyanoamino acid metabolism	11	1
	Citrate cycle (TCA cycle)	20	1
α-terpinene	Cyanoamino acid metabolism	11	1
	Alanine, aspartate and glutamate metabolism	22	1

Note: Total: Total metabolites in target metabolic pathway; Hits: Differential metabolites in target metabolic pathway.

CONCLUSIONS

The volatile oil from *Chenopodium ambrosioides* L. and its two main components (α-terpinene and ρ-cymene) inhibited the growth of *Vicia faba* L. seedlings, changed the shape of leaves, increased the stomatal density and altered the metabolic pathways related to amino acids, photosynthesis, fatty acids and antioxidants. In addition, the allelochemicals from *Chenopodium ambrosioides* induced cyanide accumulation, causing self-poisoning and Ultimately inhibited the growth of *Vicia faba*. seedlings.

ACKNOWLEDGEMENTS

This project was financially supported by Key Program for Basic Research of Sichuan Province (Project No. 2017JY0017) and National Natural Science Foundation of China (NO. 31971555).

REFERENCES

1. Araniti, F., Lupini, A., Mauceri, A., Zumbo, A., Sunseri, F. and Abenavoli, M.R. (2018). The allelochemical trans-cinnamic acid stimulates salicylic acid production and galactose pathway in maize leaves: A potential mechanism of stress tolerance. *Plant Physiol Biochem* **128**: 32-40.
2. Arenas-Alfonseca, L., Gotor, C., Romero, L.C. and Garcia, I. (2018). β-Cyanoalanine synthase action in root hair elongation is exerted at early steps of the root hair elongation pathway and is independent on direct cyanide inactivation of NADPH oxidase. *Plant and Cell Physiology* **59**: 1072-1083.
3. Bertelsmeier, C. and Keller, L. (2018). Bridgehead effects and role of adaptive evolution in invasive populations. *Trends in Ecol Evol* **33**: 527-534.

4. Chen, B., Zhou, J., Gou, X., Ma, D.W., Wang, Y.N., Hu, Z.L. and He, Y.Q. (2016). Volatiles from *Chenopodium ambrosioides* L. induce the oxidative damage in maize (*Zea mays*) radicles. *Allelopathy Journal* **38**: 171-182.
5. Chen, Y.F., Etheridge, N. and Schaller, G.E. (2005). Ethylene signal transduction. *Annals of Botany* **95**: 901-915.
6. Dixon, R.A. and Strack, D. (2003). Phytochemistry meets genome analysis and beyond. *Phytochemistry* **62**: 815-816.
7. Escandon, M., Meijon, M., Valledor, L., Pascual, J., Pinto, G. and Canal, M.J. (2018). Metabolome integrated analysis of high-temperature response in *Pinus radiata*. *Frontiers in Plant Science*. **9**: 1-15.
8. Fullana-Pericàs, M., Conesa, M.A., Soler, S., Ribas-Carbo, M., Granell, A. and Galmés, J. (2016). Variations of leaf morphology, photosynthetic traits and water-use efficiency in Western-Mediterranean tomato land races. *Photosynthetica* **55**: 121-133.
9. Gu, C.J., Zhou J., Ma D.W., Hang, S. and Meng, Q.Q. (2019). Response of photosynthetic characteristics of *Vicia faba* L. seedlings to volatile allelochemical stress from *Chenopodium ambrosioides* L. *Guihaia* **39**:1317-1326. (Chinese)
10. Guo, R., Zhou, J., Yang, F., Li, F., Li, H.R., Xia, X. and Liu, Q. (2016). Growth metabolism of wheat under drought stress at the jointing-booting stage. *Chinese Journal of Plant Ecology* **40**: 1319-1327. (Chinese)
11. Hu, W.J., Ma, D.W., Wang, Y.L., Zhang, H. and Li, Q. (2011). Allelopathic potential of volatile oil from *Chenopodium ambrosioides* L. on root tip cells of *Vicia faba* L. *Acta Ecologica Sinica* **31**: 3684-3690. (Chinese)
12. Hu, W.J., Ma, D.W., Wang, Y.N. and Zhang, H. (2012). Oxidative damage of volatile oil from *Chenopodium ambrosioides* L. on *Vicia faba* L. root tip cells. *Chinese Journal of Applied Ecology* **23**: 1077-1082. (Chinese).
13. Hu, Z.L., Wang, Y.N., Ma, D.W., Chen, B., He, Y.Q. and Zhou, J. (2015). The Alleviate effects of extracellular DNA and protein in maize root border cells on the allelochemical stress from *Chenopodium ambrosioides* L. *Scientia Agricultura Sinica* **48**: 1962-1970. (Chinese)
14. Huang, S., Ma, D.W., Lu, X. and Zhang, H. (2019). Morphological characteristics of leaf guard cells in six crops in response to the volatile stress of *Chenopodium ambrosioides* L. from different invaded habitats. *Acta Ecologica Sinica* **39**: 1517-1527. (Chinese)
15. Jian, Q., Zhu, X., Chen, J., Zhu, Z.J., Yang, R., Luo, Q.J., Chen, H.M. and Yan, X.J. (2017). Analysis of global metabolome by gas chromatography-mass spectrometry of *Pyropia haitanensis* stimulated with 1-octen-3-ol. *Journal of Applied Phycology* **29**: 2049-2059.
16. Jiménez-Osornio, F.M.V.Z.J., Kumamoto, J. and Wasser, C. (1996). Allelopathic activity of *Chenopodium ambrosioides* L. *Biochemical Systematics & Ecology* **24**: 195-205.
17. Jumrani, K., Bhatia, V.S. and Pandey, G.P. (2017). Impact of elevated temperatures on specific leaf weight, stomatal density, photosynthesis and chlorophyll fluorescence in soybean. *Photosynth Research* **131**: 333-350.
18. Li J., He, Y.Q., Ma, D.W., He, B., Wang, Y.N. and Chen, B. (2018). Volatile allelochemicals of *Chenopodium ambrosioides* L. induced mitochondrion-mediated Ca^{2+}-dependent and Caspase-dependent apoptosis signaling pathways in receptor plant cells. *Plant Soil* **425**: 297-308.
19. Piotrowski, M., Schönfelder, S. and Weiler, E.W. (2001). The *Arabidopsis thaliana* isogene NIT4 and its orthologs in tobacco encode beta-cyano-L-alanine hydratase/nitrilase. *Journal of Biological Chemistry* **276**: 2616-2621.
20. Scognamiglio, M., D'Abrosca, B., Esposito, A. and Fiorentino, A. (2015). Metabolomics: an unexplored tool for allelopathy studies. *Journal of Allelochemical Interactions* **1**: 9-23.
21. Shulaev, V., Cortes, D., Miller, G. and Mittler, R. (2010). Metabolomics for plant stress response. *Physiologia Plantarum* **132**: 199-208.
22. Singh, H.P., Kaur, S., Mittal, S., Batish, D.R. and Kohli, R.K. (2009). Essential oil of *Artemisia scoparia* inhibits plant growth by generating reactive oxygen species and causing oxidative damage. *Journal of Chemical Ecology* **35**: 154-162.
23. Stefanowicz, A.M., Majewska, M.L., Stanek, M. and Nobis, M. (2018). Differential influence of four invasive plant species on soil physicochemical properties in a pot experiment. *Journal of Soils and Sediments* **18**: 1409-1423.

24. Stevenson, J.M., Perera, I.Y., Heilmann, I., Persson, S. and Boss, W.F. (2000). Inositol signaling and plant growth. *Trends in Plant Science* **5**: 252-258.
25. Vahala, J., Ruonala, R., Keinanen, M., Tuominen, H. and Kangasjarvi, J. (2003). Ethylene insensitivity modulates ozone-induced cell death in birch. *Plant Physiology* **132**: 185-195.
26. Wang, J.R., Ma, D.W. and Tang, L. (2009). Allelopathy of volatile oil from *Chenopodium ambrosioides* L. on receptors. *Southwest China Journal of Agriculture* **22**: 777-780. (Chinese)
27. Wang, Y., Gu, W., Meng, Y., Xie, T., Li, L., Li, J. and Wei, S. (2017). γ-Aminobutyric acid imparts partial protection from salt stress injury to maize seedlings by improving photosynthesis and upregulating osmoprotectants and antioxidants. *Scientific Reports* **7**: 43609.
28. Wang, Y.N., Hu, Z.L., Hao, J.M., Qian, R.H., Ren, Q.R. and Ma, D.W. (2019). Allelopathic effects of volatile oil and its two main components from *Chenopodium ambrosioides* L. on maize (*Zea mays* L.) root border cells. *Allelopathy Journal* **46**: 215-228.
29. Warren, R.J., Labatore, A. and Candeias, M. (2017). Allelopathic invasive tree (*Rhamnus cathartica*) alters native plant communities, *Plant Ecology* **218**: 1233-1241.
30. Weise, S.E. and Sharkey, W.T.D. (2004). Maltose is the major form of carbon exported from the chloroplast at night. *Planta* **218**: 474-482.
31. Xie, M., Chen, H.H., Nie, S.P., Yin, J.Y. and Xie, M.Y. (2017). Gamma-aminobutyric acid increases the production of short-chain fatty acids and decreases ph values in mouse colon. *Molecules* **22**: 653-664.
32. Xie, Z.S., Du, H.R., Li, J.B. and Bhaskar, B. (2018). Morphological and structural changes of stomata and leaf veins during growth of grape leaves using tissue clearing technique. *Plant Physiology Journal* **54**: 237–246. (Chinese)
33. Xu, H.G. and Qiang, S. (2004). *Inventory of Invasive Alien Species in China*. China Environmental Science Press, Beijing. (Chinese)
34. Yildizli, A., Cevik, S. and Vnyayar, S. (2018). Effects of exogenous myo-inositol on leaf water status and oxidative stress of *Capsicum annuum* under drought stress. *Acta Physiologiae Plantarum* **40**: 122-131.
35. Zhao, L., Ortiz, C., Adeleye, A.S., Hu, Q., Zhou, H.J., Huang, Y.X. and Keller, A.A. (2016). Metabolomics to detect Response of Lettuce (*Lactuca sativa*) to $Cu(OH)_2$ Nano-pesticides: Oxidative stress response and detoxification mechanisms. *Environmental Science & Technology* **50**: 9697-9707.
36. Zhou, J., Wang, Y.N., Ma, D.W., Huang, S., Xin, W.Y. and Zhang, H. (2017). Volatile allelochemicals from *Chenopodium ambrosioides* L. induced guard cells apoptosis and its signal regulation in *Vicia faba* L. *Acta Ecologica Sinica* **37**: 5713-5721. (Chinese).