https://doi.org/10.26651/allelo.j/2022-55-1-1367 0971-4693/94 Euro 20.00
Allelopathy Journal 55 (1): 11-24 (January, 2022) International *Allelopathy Foundation* 2022
Table: 4, Figs : 6

Effects of invasive *Chenopodium ambrosioides* L. volatile oil on stomatal movement and signal transduction of *Vicia faba* L., *Arachis hypogaea* L. and *Pisum sativum* L.

L. Chen, H.T. Zeng[1], S. Huang and D.W. Ma[1*]
College of Life Science, Sichuan Normal University,
Chengdu 610101, China
E. Mail: danwei10ma@126.com, 549693288@qq.com

(Received in revised form: November 25, 2021)

ABSTRACT

We studied the allelopathic stress of *C. ambrosioides* volatile oil using the epidermal strip bioassay, microscopy and histochemical techniques on stomatal movement in leaves from broad beans (*Vicia faba* L.), peanuts (*Arachis hypogaea* L.) and peas (*Pisum sativum* L.). We also studied the changes in vacuoles, microfilaments and signalling molecules ROS, NO and Ca^{2+} in guard cells. The stomatal aperture of 3-test legume species decreased significantly ($P < 0.05$) in volatile oil concentration-dependent manner. Compared with control, the applied volatile oil increased the numbers of vacuoles but decreased their volume in guard cells of all 3-test crops. Aggregation of microfilament skeletons, increased the in ROS, NO and Ca^{2+} concentrations. These effects could be alleviated, when related inhibitors (Cytochalasin B, AS, $LaCl_3$ and L-NAME) were used with volatile oil. It is speculated that the dynamic changes in guard cell microfilaments caused by volatile oil of *C. ambrosioides* could lead to increased ROS and NO levels by activating the related enzymes, which increased the cytoplasmic free Ca^{2+} levels in guard cells. Thereby the ion channels in plasma membrane and vacuolar membrane were affected. The bigger vacuoles in guard cells were divided into smaller ones, turgor pressure was decreased and stomata were closed. The stomatal movement of broad bean was most sensitive to volatile oil of *C. ambrosioides*, which showed strong allelopathy.

Key words: Allelopathy, *Arachis hypogaea*, broad bean, *Chenopodium ambrosioides*, peanuts, peas, *Pisum sativum*, signal transduction, stomatal movement, *Vicia faba*, volatile oil.

Abbreviations used: AO: Acridine orange, AS: Ascorbic acid, Ca^{2+}: Calcium ion, CB: Cytochalasin B, $LaCl_3$: Lanthanum(III) chloride, DAF-FMDA: DAF-FM diacetate, DCFH-DA: 2',7'- Dichlorodihydrofluorescein diacetate, DMSO: Dimethyl sulfoxide, L-NAME: NG-Nitro-L-arginine Methyl Ester, MES: 2-(4-Morpholino) ethane sulfonic acid, NADPH: Nicotinamide adenine dinucleotide phosphate, NO: Nitric Oxide, ROS: Reactive oxygen species.

INTRODUCTION

Leguminosae is the third largest family worldwide. There are 1485 species in this family in China, from 172 genera (7). Legumes biologically fix the atmospheric nitrogen in the soil, which reduces the nitrogenous fertilizer dose of crops, hence, they are used as rotation crops to maintain the soil fertility and crop diversity (16). With the development of trade, transportation and tourism, more and more alien plants species have become invasive, competing with the indigenous crops or even inhibiting their growth of crops. *Chenopodium ambrosioides* L., an annual or perennial weed, is extremely invasive and widely distributed

*Correspondence author, [1]Co-first author: School of Biological Science and Engineering, Shaanxi University of Technology, Shaanxi Hanzhong 723001, China.

in China and has become dangerous to the farmlands (22,33). The whole plant of *C. ambrosioides* is rich in volatile oil, which causes strong allelopathic inhibition of crops when released into the environment (12,14,33). The volatile oil of *C. ambrosioides* is highly cytotoxic to legumes. The determination of the inhibitory mechanism of *C. ambrosioides* on legumes will be beneficial for its management in farmland ecosystem. Stomata plays important role in regulating water and CO_2 exchange between plants and atmosphere (20,27). Stomatal density and distribution are closely related to plant growth and productivity (3) and the regulation of stomatal movement is important for carbon assimilation and water loss. Many biotic and abiotic factors induce rapid changes in stomatal aperture (25). For example, under the allelopathic effects of *Lantana camara* (26) or *Cynodon dactylon* (4), the stomatal aperture of receptor plants changed significantly. Stomatal responses to these stimuli depend on complex signal transduction network in guard cells (25), followed by changes in turgor pressure and volume. The changes in vacuole morphology and quantity in guard cells regulates the stomatal movement (10). When stomata open, smaller vacuoles in guard cell fuse with each other to form larger ones and the number of vacuoles is decreased, that is, the fusion and division of vacuoles regulates the cell volume, thus controlling stomatal aperture (28). Microfilaments in guard cells are involved in a variety of signal transductions during stomatal movement. During the stomatal movement, the microfilament skeleton of guard cells is in dynamic change (13) and regulates the vacuolar dynamics in vacuolar ion channels (10). The network of reactive oxygen species (ROS) (1,8), Nitric Oxide (NO) (2), calcium ion (Ca^{2+}) (23,31) is not only widely involved in stomatal movement, but is also closely related to the dynamic changes in microfilaments and vacuoles in guard cells in response to various stimuli. Zhou *et al*. (33) found that ROS and NO, as signal molecules, synergistically regulated the change in Ca^{2+} level in guard cells during the death process of guard cell induced by volatile oil, α-terpinene and ρ-cymene from *C. ambrosioides*.

Our previous study reported, that when the guard cells of broad bean (*Vicia faba* L.), peanut (*Arachis hypogaea* L.) and pea (*Pisum sativum* L.) were exposed to the volatile oil of *C. ambrosioides*, the activities of guard cells were decreased, the nuclear morphology was caspase dependent and cell apoptosis occurred (15). However, the response of stomatal movement of 3-test legume species to allelopathic stress and the related signal transduction pathway are still unclear. In this study, the volatile oils of *C. ambrosioides* plants harvested in August-September 2017 in Chengdu and Anshun cities were used as donors, and broad bean, peanut and pea were used as receptors. The changes in microfilaments, vacuoles, ROS, NO and Ca^{2+} of stomatal guard cells and stomatal aperture of receptor plants were studied under allelopathic stress of *C. ambrosioides* by microscopy and cytochemistry. This study aimed to understand the responses of stomatal movement of 3-test legume species to allelopathic stress of *C. ambrosioides*, and the variability in the allelopathic effects of growing *C. ambrosioides* plants on crop diversity in its invaded areas.

MATERIALS AND METHODS

The shoots of *C. ambrosioides* were harvested in August-September 2017 in reproductive phase at Baojiangqiao in Chengdu city, Sichuan Province and Puding Railway Station in Anshun city, Guizhou Province. The natural environmental conditions of these 2-collection sites are shown in Table 1. The plants were dried in shade for 2 weeks at 20 °C. The volatile oils were extracted by steam distillation. The volatile oil stock solution was prepared with dimethyl sulfoxide (DMSO), to the concentration of 0.1 µL/µL. The volatile oil from Chengdu and Anshun plants were recorded and abbreviated as VC and VA, respectively. The seeds of broad beans (*Vicia faba* L., 'Cheng Hu14' species), peanuts (*Arachis hypogaea* L., 'Silihong' species) and peas (*Pisum sativum* L., 'Kewan 1' species) were purchased from Wukuaishi seed market, Chengdu city and the nutrients soil and vermiculite were purchased from Hengaoda Fertilizer Technology Co. Ltd.

Table 1. Weather conditions in study areas

Study sites	Geographical information			Annual precipitation (mm)	Temperature (℃)		
	Longitude	Latitude	Altitude (m)		Annual	Max.	Min.
Chengdu, Sichuan Province	30°35 ' 05 " N	104° 05' 25 " E	450	643.3~ 1256.2	16.5~ 17.9	36.2~ 39.2	-6.7~ -1.0
Guiyang, Guizhou Province	26°18 ' 30 " N	105°11' 51 " E	1300-1400	1290.6	15.6	32.7	-2.1

Pot culture: This study involved Pot Culture and Petri Plate Bioassay. To get the experimental Epidermis strips, 3-test legume species (Broadbean, Pea, Peanut) were grown in pots and their leaves were used to prepare the epidermis strips for the Petri plate bioassay. Healthy uniform seeds of these 3-test legumes were used. The seeds were sterilized with 0.5 % $KMnO_4$ (broad bean for 15 min, pea and peanut for 10 min), and then immersed in distilled water at 25 ºC for 24 h in dark. The seeds were sown in trays (Length 40 cm, Width 30 cm and Depth 6 cm) with moist gauze and germinated for 2-3 d at 25 ºC. The one day old germinated seeds were transplanted into pots (14 cm dia,12 cm height) containing 300 g soil each. These were cultured for 28 days at 25 ºC (14 h light/10 h dark) and their leaves were used to prepare the epidermis strips (1 cm × 0.5 cm) for the petri plate bioassay.

PETRI PLATE BIOASSAY

The Experimental treatments consisted of 3-Factors: (i). *C. ambrosoides* collection Sites: 2 (Chengdu, Anshun), (ii) Test crops: 3 (Broadbean, Pea, Peanut) and (iii). *C. ambrosoides* Extract concentrations: 7 [prepared with Dimethyl sulfoxide (DMSO), 0,2,4,6,8,10]. (Table 2). To study the effects of *C. ambrosides* volatile effects on various parameters (stomata, vacuole, microfilaments and to determine, ROS, NO and Ca ions), there were 3-main treatments (a). Pure volatile oil, (b). Volatile oil + DMSO and (c). MES

buffer. In (b) Solvent control 10 µl DMSO was mixed with 0,2,4,6,8 µl of volatile oil from Chengdu or Anshun. In (c) Negative control only 10 µl MES was applied (Table 3).

We prepared plastic tubes (volume 10 mL) and added 5 mL 2-(4-Morpholino) ethane sulfonic acid (MES) buffer (0.1 mmol/L $CaCl_2$, 50 mmol/L KCl, 0.1 mol/L Tris, 10 $mmol^{-1}$ MES, pH 7.0). The lower epidermis of second leaves of 3-test legumes (broad beans, peanuts and peas) were cut into epidermis strips (1 cm × 0.5 cm). Five epidermal strips were put into a plastic tube and completely immersed in MES buffer and thereafter used for further studies viz., stomata, vacuole, micro filaments and to determine, ROS, NO and Ca ion for 1.0 h.

Table 2. Details of Experimental treatments (Petri plate bioassay).

Treatment	Full details
T1	25 % DMSO
T2	MES buffer (Negative control)
T3	5 concentrations of volatile oil (VA, VC) were respectively denoted as T3-1, T3-2, T3-3 and T3-4 and T3-5
T4	Volatile oil and CB (VA+CB, VC+CB)
T5	Volatile oil and AS (VA+ AS, VC+ AS)
T6	Volatile oil and LaCl3 (VA+LaCl3, VC+LaCl3)
T7	Volatile oil and L- NAME (VA+ L- NAME, VC+ L- NAME)

DMSO: Dimethyl sulfoxide, MES: 2-(4-Morpholino) ethanesulfonic acid, VA: Volatile oil of Anshun *C. ambrosioides*, VC: Volatile oil of Chengdu *C. ambrosioides*, CB: Cytochalasin B, AS: Ascorbic acid, $LaCl_3$: Lanthanum (III) chloride, L-NAME: NG-Nitro-L-arginine Methyl Ester.

Stomatal aperture measurement: DMSO (Dimethyl sulfoxide) solvent was used as solvent control (namely T1), and MES buffer was used as negative control (namely T2). A stock solution with 0.1 µL/µL concentration of volatile oil was prepared by mixing the 100 µL volatile oil of *C. ambrosoides* collected from Chengdu (VC) or Anshun (VA) with 900 µL DMSO. Then 2, 4, 6, 8 and 10 µL of the stock solution (T3) was mixed with 5 mL T2 (MES buffer) to get the final concentration of 2, 4, 6, 8, 10 µL/5mL (namely T3-1, T3-2, T3-3, T3-4 and T3-5). Ten µL of T1, T2 or T3 (T3-1……..T3-5) was applied to epidermis strips as per treatments (Table 3). Each experiment was repeated thrice and cultured under light at 25 °C for 30 min. Thirty guard cells were randomly selected for each treatment. The epidermis strips were washed thrice with MES buffer for 5 min each. Stomata morphology was observed and stomatal aperture was measured by LEICA DFC450C light microscope.

Table 3. Quantity and Chemical Composition of *C. ambrosioides* volatile oil solutions conc. used (Petri plate bioassay).

Compound (µL)	T1 (DMSO)	T2 (MES buffer)	T3 （VC, µL/5 mL)					T3（VA µL/5 mL)				
			T3-1	T3-2	T3-3	T3-4	T3-5	T3-1	T3-2	T3-3	T3-4	T3-5
Volatile oil	0	0	2	4	6	8	10	2	4	6	8	10
DMSO	10	0	8	6	4	2	0	8	6	4	2	0
MES buffer	0	10	0	0	0	0	0	0	0	0	0	0

Note: VC: Volatile oil of Chengdu *C. ambrosioides*; VA: Volatile oil of Anshun *C. ambrosioides* (V2).

Fluorescent labelling of vacuoles: Epidermal strips treated with 10 μL/5mL of VC and VA stock solutions (T3-T5, Table 2) were washed thrice with MES buffer for 5 min, dried with filter paper, as per Gao *et al*. (11). 10 μmol/L acridine orange (AO) was used to stain the epidermal strips at 25 ºC and kept in dark for 15 min. Microscopy was used to observe the changes in vacuoles.

Microfilament inhibition assay: We dissolved 23.980 μg cytochalasin B (CB) and 47.961 μg CB respectively in 5 mL EMS buffer containing 10 μL VC or VA stock solutions (T3-5, Table 2), this gave final concentrations of 10 and 20 μmol/L (T4, Table 2). The epidermal strips of 3-test legumes were treated with T4 and 10 μL VC or VA stock solution (T3-5), respectively (Table 2). MES buffer (T2) was used as the control. and incubated under light at 25 ºC for 30 min. Experiments were repeated thrice. Afterwards, the epidermal strips were rinsed twice with MES buffer and examined by LEICA DFC450C microscope. 30 stomata were randomly selected to determine this parameter (6).

Measurement of ROS, Nitric Oxide and Calcium ion contents: Ascorbic acid (AS, final conc 0.1 $mmol \cdot L^{-1}$), Lanthanum (III) chloride. ($LaCl_3$, final concentration 0.1 $mmol \cdot L^{-1}$), and NG-Nitro-L-arginine Methyl Ester (L-NAME, final concentration 0.05 $mmol \cdot L^{-1}$) were mixed with 10 μL VA or VC stock solutions (T3-5), respectively. These solutions (Table 2: T5, T6 and T7) were applied to the epidermal strips of 3-test legumes (Broadbean, Pea, Peanut). Ten μL VA or VC stock solutions (Table 2: T3-T5) and 10 μL MES buffer (Table 2: T2) were used as control. Experiments were repeated thrice (Table 2: T2, T3-5, T5, T6 and T7) and incubated under light at 25 ºC for 30 min. After that, the epidermal strips were rinsed with MES buffer twice and dried with filter paper and further treated as under:

(i). AS treated epidermal strips (Table 2: T5) were co-incubated with 2',7'-Dichlorodihydrofluorescein diacetate (DCFH-DA) (20 $\mu mol \cdot L^{-1}$) at 25 ºC and kept in dark for 1 h;

(ii). $LaCl_3$ treated epidermal strips (Table 2: T6) were co-incubated with Calcium Indicator (fluo-3AM) (10 $\mu mol \cdot L^{-1}$) at 25 °C and kept in dark for 2 h;

(iii). L-NAME treated epidermal strips (Table 2: T7) were co-incubated with DAF-FM diacetate (DAF-FMDA, 10 $\mu mol \cdot L^{-1}$) at 25 ºC and kept in dark for 1.5 h.

The morphology of guard cells was observed and photographed with LEICA DM 3000 fluorescence microscope (40×) under blue excitation light. Image Pro Plus 6.0 software was used to measure the fluorescence value of guard cells. 300 cells were randomly measured for each treatment, and the fluorescence value of the control group was set as 1. The ratio of the fluorescence value of each treatment group to the control group were the relative fluorescence value of different signal molecules, which represented their content (29,32).

Statistical analysis

The allelopathic sensitivity index was calculated as under (30):

$RI = 1 - C/T$, $(T \geq C)$

$RI = T/C - 1$, $(T < C)$

Where, *C*: Control value and *T*: Treatment value. The comprehensive allelopathic effects were expressed by the arithmetic mean of allelopathic index.

SPSS 17.00 was used for one-way Analysis of Variance (ANOVA) and Least-Significant Difference (LSD), and Microsoft Excel 2019 was used for mapping.

RESULTS AND DISCUSSION

Stomatal aperture

Stomata regulates the gas and water exchange between the internal and external environment of leaves. Thus, changes in stomatal aperture induced by external environmental factors and internal signals regulates the transpiration (17,21). The allelopathic effects significantly changes the stomatal movement of receptor plants (4,27). There was no significant difference in the stomatal aperture between the solvent control (Table 3: T1) and negative control (Table 3: T2), indicating that DMSO had little influence on the stomatal aperture (Table 4). Compared to negative control (Table 3: T2), the stomatal aperture of 3-legume species decreased with the increase in volatile oil concentrations ($P < 0.05$). The highest volatile oil concentration (10 μL/5 mL) treatment decreased the stomatal aperture of broad bean, peanut and pea by 68.67 %, 59.77 % and 25.05 % in VC (Chengdu treatment) and by 75.75 %, 75.52 % and 52.16 % in VA (Anshun treatment). According to the comprehensive allelopathic index, the allelopathic intensity of *C. ambrosioides* growing in Anshun (0.65) was higher than that in Chengdu (0.47). The sensitivity of three receptors to volatile oil of *C. ambrosioides* followed the order: broad bean > peanut > pea. These results showed that the volatile oil of *C. ambrosioides* could regulate the stomatal movement of receptor plants in a concentration dependent manner.

Table 4. Stomatal aperture of three legumes exposed to volatile oil of *C. ambrosioides* L. (μm) (Petri plate bioassay).

Receptor	Sites	T1 (DMSO)	T2 (MES buffer)	T3 (*C. ambrosioides* oil μL/5 mL)				
				T3-1	T3-2	T3-3	T3-4	T3-5
Vicia	Chengdu	10.94±0.18a	11.30±0.02a	4.05±0.08b	4.00±0.11b	3.71±0.12b	3.66±0.07b	3.54±0.28b
faba	Anshun	10.94±0.18a	11.30±0.02a	3.03±0.14b	2.98±0.11b	2.92±0.17b	2.76±0.05b	2.74±0.04b
Arachis	Chengdu	5.17±0.06a	5.27±0.06a	2.98±0.07b	2.86±0.03b	2.56±0.07bc	2.43±0.08bc	2.12±0.12c
hypogaea	Anshun	5.17±0.06a	5.27±0.06a	1.54±0.13b	1.42±0.14b	1.32±0.06b	1.29±0.10b	1.29±0.02b
Pisum	Chengdu	4.77±0.16a	4.87±0.01a	3.82±0.00b	3.79±0.22b	3.70±0.17b	3.70±0.28b	3.65±0.18b
sativum	Anshun	4.77±0.16a	4.87±0.01a	2.76±0.00b	2.62±0.05bc	2.57±0.09bc	2.41±0.01bc	2.33±0.06c

Note：V: Volatile oil of *C. ambrosioides*; Different letters in the same line represent the significant difference (P <0.05).

Microfilaments in guard cells

The microfilament skeleton is the key node of stomatal movement regulatory network in dicotyledons, which plays important roles in integrating multiple exogenous stimuli and regulating stomatal aperture (3). When the guard cells are stimulated by the external environment, the microfilaments in guard cells are subjected to dynamic changes between polymerization and depolymerization (19). Our previous results showed that the volatile oil had significant effects on stomatal movement. After the volatile oil was added

with different concentrations of CB co-treatment (Table 2: T4), the effect of volatile oil on stomatal movement gradually decreased with the increase in CB concentration. At 20 μmol/L CB concentration, the volatile oil did not affect the stomatal aperture of pea and peanut compared with control ($P > 0.05$). The results showed that the volatile oil of *C. ambrosioides* caused microfilament polymerization and stomatal closure. Among the three plants, the microfilament skeleton in guard cells of broad bean was most sensitive to the volatile oil of *C. ambrosioides*. The effects of volatile oil from *C. ambrosioides* on microfilaments were VA > VC.

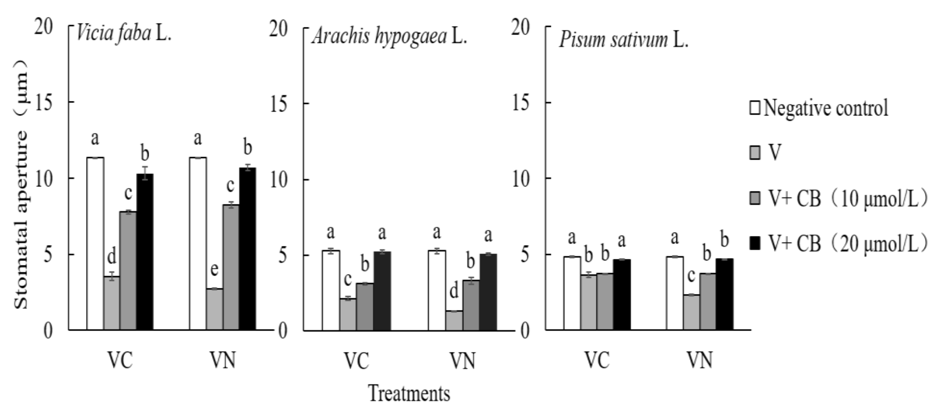

Figure 1. Effects of applied *C. ambrosioides* volatile oil solutions on stomatal apertures in leaves of 3-test legume species in petri plate bioassay. Treated with negative control: 2-(4-Morpholino) ethane sulfonic acid (MES) buffer, V: volatile oil of *C. ambrosioides*, V+ CB (10 μmol/L): Volatile oil of *C. ambrosioides* +10 μmol/L cytochalasin B, V+ CB (20 μmol/L): Volatile oil of *C. ambrosioides*+ 20 μmol/L cytochalasin B. VC: Volatile oil of Chengdu *C. ambrosioides*, VA: Volatile oil of Anshun *C. ambrosioides*. Different letters indicate the significant differences between various treatments in the same group（$P < 0.05$).

Vacuoles in Guard Cells

Guard cells sense and respond to the environmental changes, which influences the stomatal aperture through the changes in turgor pressure and volume, that are mediated by complex intracellular signals (10). The morphological and quantitative changes in vacuoles of guard cells are closely related to these processes. The results of labelling vacuole assay by acridine orange (AO) were shown in Fig. 2. The applied volatile oil reduced the stomatal aperture of epidermal strips and decreased the volume of vacuoles in guard cells compared with control. These results indicated that the stomatal closure caused by volatile oils were related to the decrease in vacuole volume and increase in vacuole number in guard cells. In comparison to VA treatments, the volume and number of vacuoles in guard cells increased under VC treatments. The vacuole volume of broad bean was larger than that of peanut and pea, which was consistent with the dynamic changes in microfilament. Therefore, the results showed that allelopathic stress changed the shape and number of vacuoles in guard cells, leading to decreased turgor pressure and eventually causing stomatal closure.

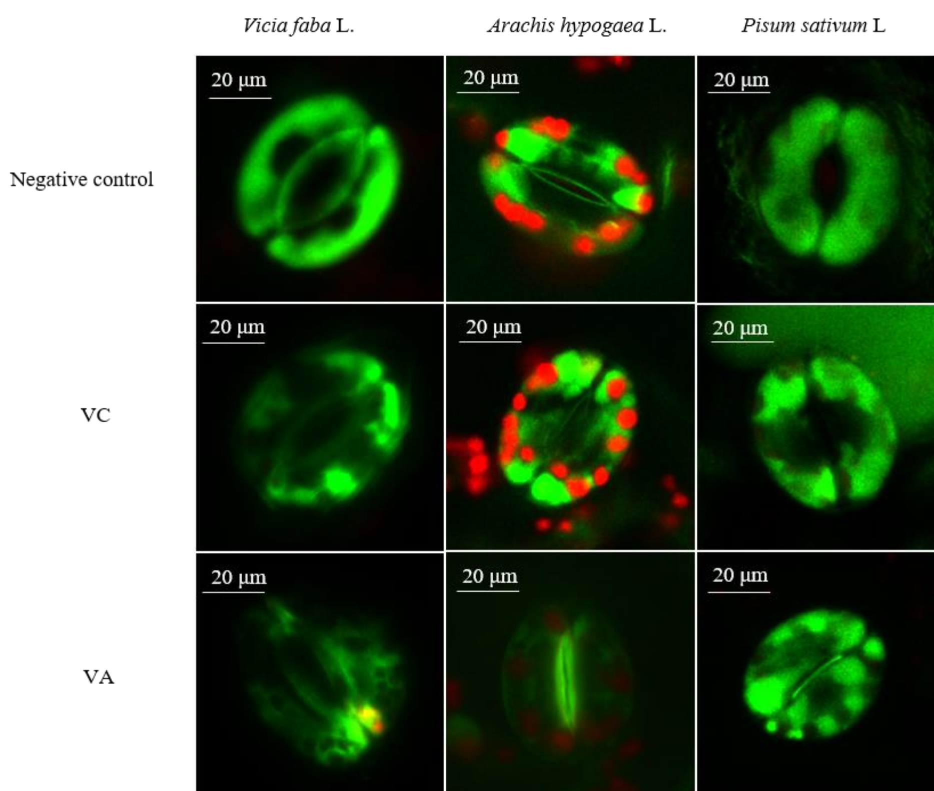

Figure 2. Effects of applied *C. ambrosioides* volatile oil solutions from Chengdu and Anshun and DMSO, on fluorescence localization images of vacuoles in leaves of 3-test legume species in petri plate bioassay. Negative control: MES buffer, VC: Volatile oil of Chengdu *C. ambrosioides*, VA: Volatile oil of Anshun *C. ambrosioides*.

Effects on ROS, Nitric Oxide and Calcium ion contents

Reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, which can catalyze the ROS production in plant cells, is located in the plasma membrane under stress (24). The localization and activity of NADPH oxidase in plant cells may be influenced by the dynamic changes in microfilament (13). Our previous studies showed that allelopathic stress increased the ROS level in cells and oxidative burst (5,18,33). Free Ca^{2+} in guard cells could affect the stomatal aperture as the second messenger of stimuli. Cytoplasmic free Ca^{2+} mainly comes from the release of Ca^{2+} from intracellular calcium pool and the inflow of Ca^{2+} through plasma membrane channels. ROS is the upstream regulator of Calcium (18). ROS could affect K^+ channels on the plasma membrane and tonoplast of guard cells by increasing the Ca^{2+} concentration and activating the Ca^{2+} signalling transduction pathway (10). Nitric Oxide usually promotes the release of Ca^{2+} from intracellular calcium pool and increase the content of cytoplasmic free Ca^{2+}, and thus activating K^+ channel (9). As shown

in Fig. 3-A, the stomatal apertures of broad bean, peanut and pea were increased by 26.11 %, 17.84 % and 12.73 % in VC and AS co-treatment group, 30.00 %, 32.07 % and 38.17 % in VA and AS co-treatment group, respectively, when compared to *C. ambrosioides* volatile oil treatments. The results also showed that ROS scavenges the AS effectively, alleviating the inhibitory effects of volatile oil on stomatal aperture (P <0.05). When labelling ROS with fluorescent probe DCFH-DA (Fig. 3-B), the fluorescence intensity of the volatile oil and AS co-treatment group was significantly decreased than group treated with volatile oil (P < 0.05), but was not significantly different from negative control (P >0.05).

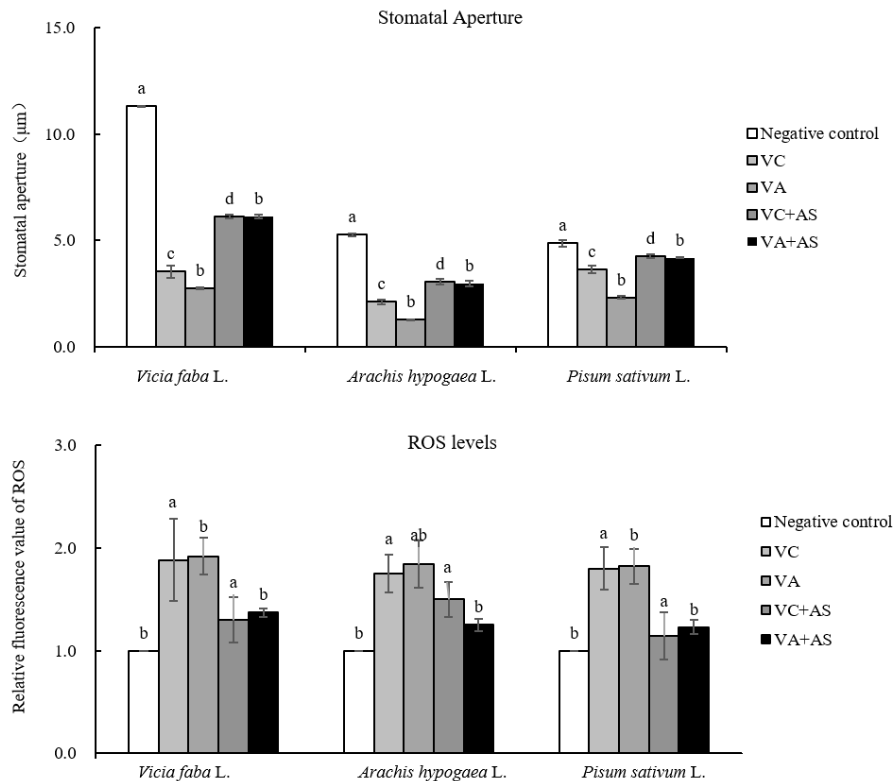

Figure 3. Effects of applied *C. ambrosioides* volatile oil and ascorbic acid (T5, Table 2) solutions on stomatal apertures and ROS in leaves of 3-test legume species in petri plate bioassay. Treated with negative control: MES buffer, VC: Volatile oil of Chengdu *C. ambrosioides*, VA: Volatile oil of Anshun *C. ambrosioides*, VC+AS: Volatile oil of Chengdu *C. ambrosioides* + ascorbic acid, VA+ AS: Volatile oil of Anshun *C. ambrosioides* + ascorbic acid. Different letters indicate the significant differences between various treatments in the same group（P <0.05).

Fig. 4-A showed that the reduction in stomatal aperture caused by volatile oil of *C. ambrosioides* could be significantly alleviated by L-NAME, an inhibitor of NO synthase (P < 0.05). The stomatal apertures of broad bean, peanut and pea were increased by 4.60 %, 11.57 % and 11.91 % in the VC group, and increased by 28.58 %, 30.17 % and 36.96 % in VA group, respectively. The relative content of NO in guard cells of leaves of three legumes co-treated with L-NAME and volatile oil decreased in comparison to the group treated with the volatile oil (P >0.05).

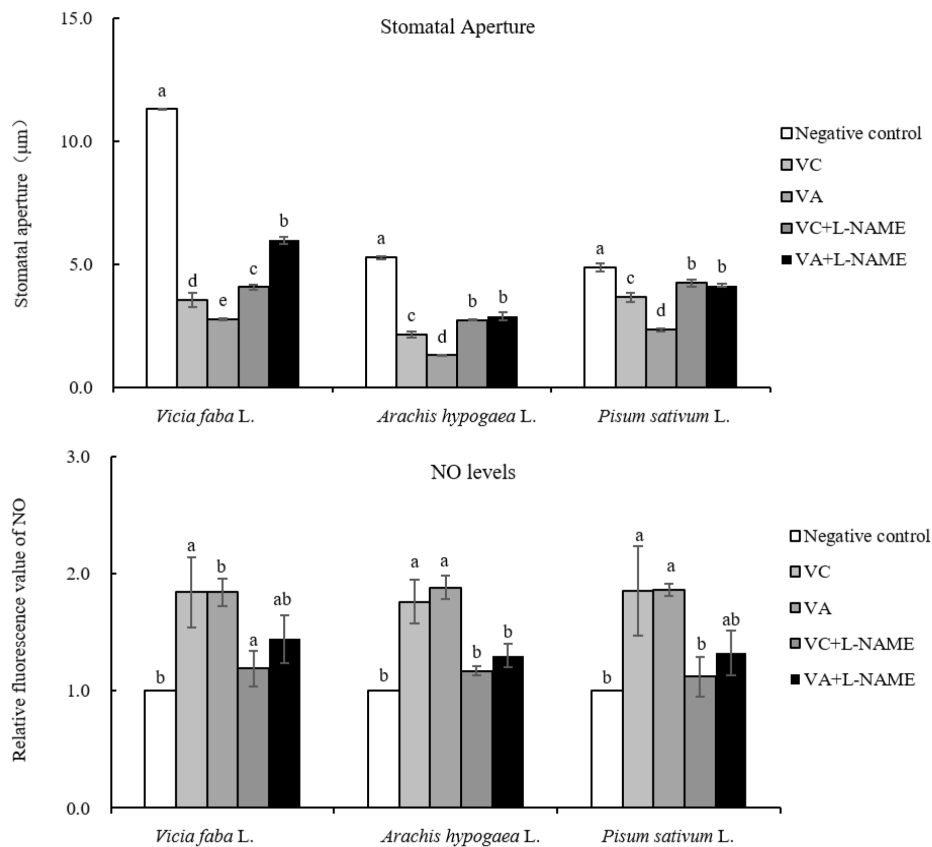

Figure 4. Effects of applied *C. ambrosioides* volatile oil L-NAME (T7, Table 2) solutions on stomatal apertures and NO levels in leaves of 3-test legume species in petri plate bioassay. Treated with negative control: MES buffer, VC: Volatile oil of Chengdu *C. ambrosioides*, VA: Volatile oil of Anshun *C. ambrosioides*, VC+ L- NAME: Volatile oil of Chengdu *C. ambrosioides* + L-NAME. VA+ L- NAME: Volatile oil of Anshun *C. ambrosioides* + L-NAME. L-NAME: NG- Nitro-L-arginine Methyl Ester. Different letters indicate the significant differences between various treatments in the same group (*P* <0.05).

Figure 5-A showed that $LaCl_3$, a Ca^{2+} channel inhibitor, significantly alleviated the inhibitory effects of *C. ambrosioides* volatile oil on the stomatal aperture (P <0.05). The stomatal apertures of broad bean, peanut and pea were increased by 12.39 %, 12.14 % and 11.29 % in VC group and increased by 26.64 %, 33.59 % and 33.68 % in VA group, respectively, when co-treated with $LaCl_3$ and the *C. ambrosioides* volatile oil. After Fluo-3/AM fluorescence labeling (Fig. 5-B), it was found that intracellular Ca^{2+} level of *C. ambrosioides* volatile oil treatment group was increased compared to negative control group and the $LaCl_3$ and volatile oil co-treated group. The results indicated that *C. ambrosioides* volatile oil could trigger the Ca^{2+} signal pathway in guard cells and cause the stomatal closure. In conclusion, allelopathic stress from *C. ambrosioides* activated ROS, NO and Ca^{2+} signalling system of three receptors, and caused stomatal closure, wherein, the effect of VA was higher than VC.

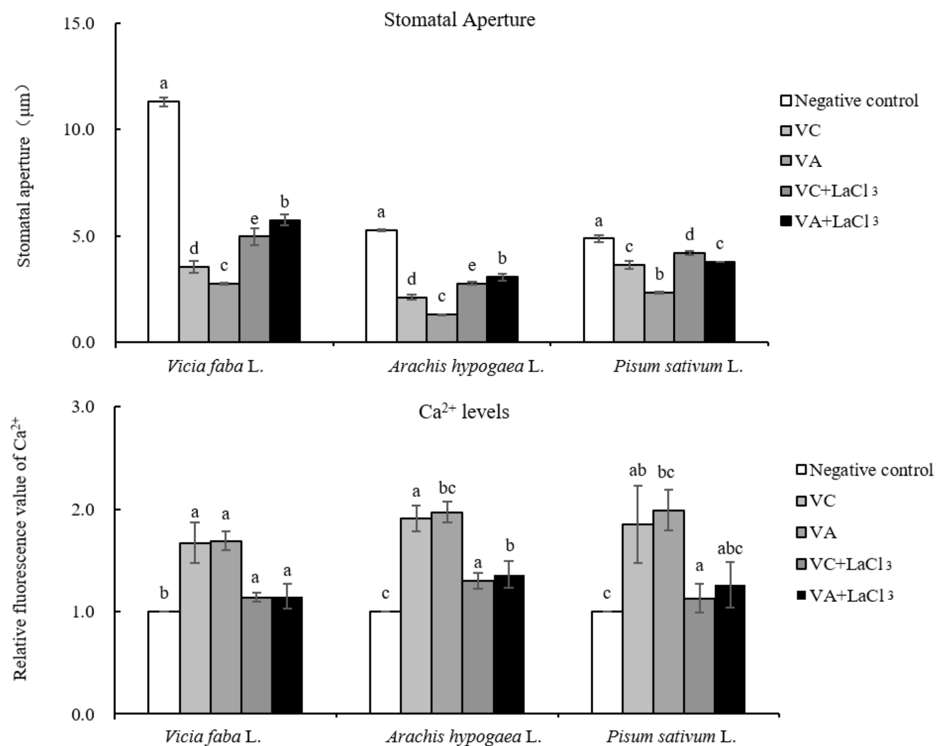

Figure 5. Effects of applied *C. ambrosioides* volatile oil $LaCl_3$ (T6, Table 2) solutions on stomatal apertures and Ca^{2+} levels in leaves of 3-test legume species in petri plate bioassay. Treated with negative control: MES buffer, VC: Volatile oil of Chengdu *C. ambrosioides*, VA: Volatile oil of Anshun *C. ambrosioides*, VC+ $LaCl_3$: Volatile oil of Chengdu *C.* Different letters indicate the significant differences between various treatments in the same group（P <0.05).

CONCLUSIONS

The effects of volatile oil on stomatal movement in 3-legume species (broad bean, peanut, pea) were greater from Anshun population (Guizhou Province) than volatile oil from Chengdu population (Sichuan Province). This indicated that allelopathy of *C. ambrosioides* population increased, when they grow in poor environment. In addition, stomatal movement of 3-legume species showed significant differences in response to allelopathy and followed the order: broad bean > peanut > pea. The *C. ambrosioides* was more harmful to broad bean than to peanut and pea. Therefore, the crops that were less sensitive to *C. ambrosioides* should be sown in fields facing invasion of *C. ambrosioides*.

ACKNOWLEDGEMENTS

The project was financially supported by National Natural Science Foundation of China (NO. 31971555) and the Fourth National Survey of Traditional Chinese Medicine Resources (NO. GZY-KJS-2018-004).

CONFLICT OF INTEREST

The authors announce that they have no conflict of interest.

REFERENCES

1. Adamipour, N., Khosh-Khui, M., Salehi, H., Razi H., Karami, A. and Moghadam, A. (2020). Role of genes and metabolites involved in polyamines synthesis pathways and nitric oxide synthase in stomatal closure on *Rosa damascena* Mill. under drought stress. *Plant Physiology and Biochemistry* **148**: 53-61.
2. Agurla, S., Gayatri, G. and Raghavendra, A. S. (2018). Polyamines increase nitric oxide and reactive oxygen species in guard cells of *Arabidopsis thaliana* during stomatal closure. *Protoplasma* **255**: 1-10.
3. Bertolino, L.T., Caine, R.S. and Gray, J.E. (2019). Impact of stomatal density and morphology on water-use efficiency in a changing world. *Frontiers in Plant Science* **10**: DOI: 10.3389/fpls.2019.00225
4. Bouchagier, P. and Efhimiadis, P. (2010). Allelopathic susceptibility of cotton to bermudagrass. *Journal of Agronomy* **9**: 23-28.
5. 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* L.) radicles. *Allelopathy Journal* **38**: 171-182.
6. Chen, H. and Han, R. (2016). Characterization of actin filament dynamics during mitosis in wheat protoplasts under UV-B Radiation. *Scientific Reports* **6**: 1-8.
7. Chinese Flora Commission, Chinese Academy of Sciences, (1988). *Flora of China* (39 volumes). Beijing: Science Press: 1-233. (Chinese)
8. Devieddy, A.R., Liscum, E. and Mittler, R. (2020). Phytochrome B is required for systemic stomatal responses and ROS signaling during light stress. *Plant Physiology* **184**: 1563-1572.
9. Duque, P., Sánchez, J.P. and Chua, N.H. (2018). Signaling and the cytoskeleton in guard cells. *Annual Plant Reviews* **10**： 290-317.
10. Gao, X.Q., Wang, X.L., Ren, F., Chen, J. and Wang, X.C. (2009). Dynamics of vacuoles and actin filaments in guard cells and their roles in stomatal movement. *Plant Cell and Environment* **32**: 1108-1116.
11. Gao, X.Q., Ren, Q.P. and Wang, Z.B. (2008). Methods for vital fluorescent labeling of guard cells vacuole. *Chinese Journal of Cell Biology* **30**: 411-414. (Chinese)

12. Gu, C.J., Ade, L.J., Ma, D.W., Wang, Y.N. and Zhang, H. (2019). Metabolomics profiling reveals the potential metabolites and biological pathways of broad bean (*Vicia faba* L.) under allelochemical stress of *Chenopodium ambrosioides* L. *Allelopathy Journal* **49**: 35-50.
13. Hu, Z.Y., Ren, J.J., Yu, Q., Kong, L. J. and Wang, X. L. (2017). The Role mechanism of acitn filament and ROS in regulating stomatal movement. *Chinese Journal of Cell Biology* **39**: 510-514. (Chinese)
14. Hu, Z.L., Wang, Y.N., Ma, D.W., Chen, B., He, Y.Q. and Zhou, J. (2015). The alleviate effect 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)
15. 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)
16. Jouan, J., Ridier, A. and Carof, M. (2019). Economic drivers of legume production: approached via opportunity costs and transaction costs. *Sustainability* **11**:705; DOI:10.3390/su11030705.
17. Lawson, T. and Vialet-Chabrand, S. (2019). Speedy stomata, photosynthesis and plant water use efficiency. *The New Phytologist* **221**: 93-98.
18. Li, J., He, Y.Q., Ma, D.W., He, B., Wang, Y.N. and Zhang, H. (2018). Volatile allelochemicals of *Chenopodium ambrosioides* L. induced mitochondrion-mediated Ca^{2+}-dependent and Caspase-dependent apoptosis signaling pathways in receptor plant cells. *Plant and Soil* **425**: 297-308.
19. Li, X., Diao, M., Zhang, Y.N., Chen, G.L., Huang, S.J. and Chen, N.Z. (2019). Guard cell microfilament analyzer facilitates the analysis of the organization and dynamics of actin filaments in Arabidopsis guard cells. *International Journal of Molecular Sciences* **20**: 2753.
20. Liu, J., Sun, X., Xu, F., Zhang, Y., Zhang, Q., Miao, R., Zhang, J., Liang, J. and Xu, W. (2018). Suppression of OsMDHAR4 enhances heat tolerance by mediating H_2O_2 -induced stomatal closure in rice plants. *Rice* **11**: 38. DOI: 10.1186/s12284-018-0230-5.
21. Majeed, S., Nawaz, F., Naeem, M. and Ashraf, M.Y. (2020). Nitric oxide regulates water status and associated enzymatic pathways to inhibit nutrients imbalance in maize (*Zea mays* L.) under drought stress. *Plant Physiology and Biochemistry* **155**: 147-160.
22. Monzote, L., Geroldinger, G., Tonner, M., Scull, R., Sarkar, S.D., Bergmann, S., Bacher, M., Staniek, K., Chatterjee, M., Rosnau, T. and Gille, L. (2018). Interaction of ascaridole, carvacrol, and caryophyllene oxide from essential oil of *Chenopodium ambrosioides* L. with mitochondria in *Leishmania* and other eukaryotes. *Phytotherapy Research* **32**:1729-1740.
23. Rahman, M.S., Nakamura, T., Nakamura, Y., MunemAS, S. and Murata, Y. (2020). The myrosinases TGG1 and TGG2 function redundantly in reactive carbonyl species signaling in Arabidopsis guard cells. *Plant & Cell Physiology* **61**:967-977.
24. Sagi, M. and Fluhr, R. (2001). Superoxide production by plant homologues of the gp91(phox) NADPH oxidase. Modulation of activity by calcium and by tobacco mosaic virus infection. *Plant Physiology* **126**: 1281-90.
25. Samakovli, D., Tichá, T. and Šamaj, J. (2020). HSP90 chaperones regulate stomatal differentiation under normal and heat stress conditions. *Plant Signaling & Behavior* **15**: DOI: 10.1080/15592324.2020.1789817.
26. Singh, R., Chaurasia, S., Gupta, A.D., Mishra, A. and Soni, P. (2014). Comparative study of transpiration rate in *Mangifera indica* and *Psidium guajawa* affect by *Lantana camara* aqueous extract. *Journal of Environmental Science* **3**: 1228-1234.
27. Urban, J., Ingwers, M.W. and McGuire, M. A. (2017). Teskey RO. Increase in leaf temperature opens stomata and decouples net photosynthesis from stomatal conductance in *Pinus taeda* and *Populus deltoides* x *nigra*. *Journal of Experimental Botany* **68**:1757-1767.
28. Voss, L. J., Hehrich, R. and Roelfaema, M.R.G. (2016). Current Injection Provokes Rapid Expansion of the Guard Cell Cytosolic Volume and Triggers Ca^{2+} Signals. *Molecular Plant* **9**: 741-480.
29. Wei, A.L., Han, E.Q., Zhang, X.B., Wu, F.J., Xin, X.J., Wang, T., Zhang, S.S., Wang, Y.S. and Cao, D.M. (2016). SO_2-induced stomatal movement and its signal regulation in guard cells of *Hemerocallis fulva*. *Acta Scientiae Circumstantiae* **36**: 740-746. (Chinese)
30. Williamson, G.B. and Richardson, D. (1988). Bioassays for allelopathy: Measuring treatment responses with independent controls. *Journal of Chemical Ecology* **14**:181- 187.
31. Wu, Z. G., Wu, S. J., Wang, Y. C. and Zheng, L. L. (2018). Advances in studies of calcium-dependent protein kinase (CDPK) in plants. *Acta Prataculturae Sinica* **27**: 204-214. (Chinese)

32. Yi, H.L., Yin, J.J., Liu, X., Jing, X.Q., Fan, S.H. and Zhang, H. (2012). Sulfur dioxide induced programmed cell death in *Vicia* guard cells. *Ecotoxicology and Environmental Safety* **78**: 281-286.
33. Zhou, J., Wang, Y.N., Ma, D.W., Hang, S., Xin, W.Y. and Zhang, H. (2017). Volatile allelochemicals from *Chenopodium ambrosioides* L. induced guard cells apoplosis and its signal regulation in *Vicia faba* L. *Acta Ecologica Sinica* **37**: 5713-5721. (Chinese).

PUBLISHER NOTE

Allelopathy Journal remains neutral with regard to jurisdictional claims in published Maps and Institutional Affiliations.