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Allelopathic effects of volatile oil and its two main components from *Chenopodium ambrosioides* L. on maize (*Zea mays*) root border cells

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ABSTRACT

Chenopodium ambrosioides L. (family Chenopodiaceae) is native to Central and South America. Introduced *C. ambrosioides* poses threat to ecosystems in China. To explore allelopathy mechanism, effects of volatile allelochemicals produced by *C. ambrosioides* on maize root border cells (RBCs) were tested. *C. ambrosioides* volatile oil and its two main components (α-terpinene and cymene), inhibited the maize root growth, changed RBCs number, increased pectin methyl esterase activity and up-regulated *rcpme1* expression in the maize roots. Volatile oil decreased maize RBCs survival rate to 70.20 % when treated with 5 µl for 48 h. RBCs nuclei showed abnormalities (fragmentation, marginalization, malformation and dispersion) when treated with volatile oil, α-terpinene and cymene. Exposed to volatile oil, nuclear abnormalities rate > 70%, while other treatments were < 27%. There were more vacuoles, chromatin condensation and marginalization in RBCs treated with high doses of volatile oil. TUNEL assay showed death of RBCs was possibly due to programmed cell death or cell necrosis. Thus, the volatile oil, α-terpinene and cymene were cytotoxic and genotoxic to RBCs, caused nuclear abnormalities, decreased the RBC viability and induced programmed cell death and necrosis, thereby inhibited the growth of young maize roots. The allelopathic toxicity of oil on the RBCs was significantly greater than either α-terpinene or cymene individually.

Keywords: Allelochemical stress, allelopathic effects, *Chenopodium ambrosioides* L., cymene, maize, nuclear abnormalities, programmed cell death, RBCs, root border cells, α-terpinene, volatile oil, *Zea mays*.

INTRODUCTION

Root border cells (RBCs) are released from the root cap as individual cells or as a group of attached cells (13). Root cap pectin methyl esterase (PME) is key enzyme associated with the production and release of RBCs (24). RBCs and their associated mucilage are defensive barrier between the plant roots and the soil that in protects the plants from biotic and abiotic stresses (1,4,7,8,15,35,42). The expression of *rcpme1* influences the RBCs separation (26,41). Programmed cell death (PCD) of RBCs is way for plants to survive the environmental stress when the stress exceeds the tolerance limits of plant (9). Damages such as chromatin condensation, chromatin breakage, deformed nuclei, organellar swelling or rupture, cell shrinkage (11,14), increased mitochondrial membrane potential (3), increased caspase activity (34), or typical DNA laddering (16,17) can be observed in RBCs as a result of PCD (43).

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Allelopathic stress occurs when plant directly or indirectly impact on another plant by leaching, volatilization, root exudates, decomposing stumps or other means of chemicals release (25,31). Allelopathy is one of main mechanisms accounts for the success of invasion. *C. ambrosioides* is an annual or perennial herb, native to Central and Southern America, that is now widely distributed in tropical and temperate regions of the world. The species has become a serious environmental threat in China, it is on the list of invasive alien species in China (18,30). It can release allelopathic substances that inhibit the growth of surrounding plants through leaching 23), residue (45), emission of volatiles (21) and other ways and has become the dominant invasive species in several ecological systems. *C. ambrosioides* is rich in volatile oil composed mainly of α-terpinene and cymene (19,22,39). Its root exudates or litter decomposition can release the volatile oil into the soil, leading to the production of reactive oxygen species (ROS) in root tip cells, disturbing mitosis in the recipient plant root tip cells and inducing oxidative damage or even death in apical cells, thereby inhibiting the growth and development of plants (5,18,25,45). Plant RBCs can also help to alleviate the stress from the allelopathic effects of *C. ambrosioides*, for example, apical growth in pea radicles was significantly inhibited when the RBCs were removed (38). Extracellular DNA and proteins in RBC mucilage play key role in resisting the allelopathic stress (40). One of the successful invasion mechanisms of *C. ambrosioides* is that it releases allelochemicals to disarm the protective barrier established by RBCs, which will impact division and elongation of the recipient plant root tip cells, thereby inhibiting the plant growth. However, it is still unclear how *C. ambrosioides* volatile oil and its two main components affects the root border cells. So, RBCs of maize, which is widely grown in the region invaded by *C. ambrosioides*, were used in our experiments. The morphological characteristics and activity of RBCs were studied after exposure to the volatile oil and its two main ingredients, α- terpinene and cymene.

This study aimed to determine the allelopathic effects of volatile oil and its two main components from C. *ambrosioides* on maize root border cells and to find mechanism of invasion of C. *ambrosioides.*

MATERIALS AND METHODS

Materials

C. ambrosioides plants were collected at Baojiangqiao in Chengdu, Sichuan Province (N 30° 35′ 17, E 104° 05′ 18) and their aboveground shoots were cut into small pieces after drying. The essential oil from *C. ambrosioides* was extracted by steam distillation (33) with a yield of ~0.8 %. The oil was then dried with anhydrous sodium and finally stored at 4^{o}C. The essential oil of *C. ambrosioides* consists of 156 mg/mL cymene and 151 mg/ml α-terpinene as analyzed by GC-MS. Known standards for cymene (mass fraction 99.5%) and α-terpinene (mass fraction 90 %) were purchased from Ehrenstoefer Quality Company, Augsburg, Germany and Sinopharm Chemical Reagent Co., Ltd., Shanghai, China, respectively. Maize seed (Ya-yu 26#) was purchased from Southwest Kelian Seed Co., Ltd., Sichuan.

Maize culture and allelopathic stress treatments

Maize seeds were surface sterilized with 0.5% (v/v) $KMnO_4$ for 20 min, washed 6-times with sterile water, soaked in distilled water for 12 h and then germinated between the folds of wet gauze at 25 ^oC for 24 h on white porcelain plates. After the radicles had appeared, seeds were transferred to 50 ml sterile agar medium in covered glass bottles (volume 200 mL, 9 cm dia, height 8.5 cm), 10 seeds/bottle, with the radicle facing outwards until the seeds had fully germinated. The bottles were then capped, inverted and cultured at 25 ^oC in the dark.

Allelopathic stress treatment: Through pre-experiment we found that the root length of the group treated with 5 μL oil was about half of the control group, there were distinct rot in root tip when volatile oil was higher than 5 μL. Experimental treatments consisted of *C. ambrosioides* volatile oil, cymene, α-terpinene, cymene+α-terpinene and five concentrations of each as shown in Table 1, add ddH_2O to final volume of 2 μL. The concentration of volatile oil was 843 g/L. The concentrations of cymene and α-terpinene were determined based on their contents in the volatile oil. Different volumes of volatile oil, cymene and α-terpinene were added to the inside of the bottle covers. The seeds were cultured for 12 h, 24 h and 48 h prior to analysis.

Table 1. Doses of *C. ambrosioides* volatile oil and its two components added to maize seedings

Treatments	Treatments dose (μL)					
	Control	1	2	3	4	5
V (Volatile oil)	0	1	2	3	4	5
C (Cymene)	0	0.186	0.372	0.558	0.744	0.930
AT (α-terpinene)	0	0.169	0.338	0.507	0.676	0.845
M (Cymene+α-terpinene)	0	0.186+0.169	0.372+0.338	0.558+0.507	0.744+0.676	0.930+0.845

Note: Add double distilled water to each treatment for total 2μL.

Root lengths were measured at 12, 24 and 48 h after treatment. Ten roots were measured in each treatment, with three replicates/treatment.

Preparing and counting RBCs

After treating the roots with volatile oil from *C. ambrosioides* and its two main components for 12, 24 and 48 h, five apical root sections (5 mm) were excised and placed in 0.5 mL eppendorf tube with 100 μl dd H_2O and vortexed for 30 s to make a suspension of RBCs. The cells were stained by adding of 50 μL 0.4% trypan blue and counted using hemocytometer under a bright-field microscope. Each treatment was repeated five times.

Extraction of pectin methyl esterase and determination of enzymatic activity

After treatment with volatile oil from *C. ambrosioides* and its two main components for 12, 24 and 48 h, pectin methyl esterase (PME) activity was determined as described by Richard *et al.* (32). Decrease of H^+ was used to measure PME activity. For the standard

curve, aliquots of 0.01 mol/L HCl (0, 30, 60, 90, 120, 150, 180, 210 and 240 μL) were mixed with 4 mL PME substrate solution containing 0.5% (w/v) citrus pectin, 0.2 mol/L NaCl and 0.15% (w/v) methyl red, pH 6.8. After incubation for 2 h at 37°C, the colour change was measured by the absorbance at 525 nm using a SpectraMax M2 multifunction plate reader.

PME (pectin methyl esterase) was extracted from 10 maize root tips (3 mm) after treatment for 12, 24 and 48 h. The root tips were placed in glass grinder with 200 μL PME extraction solution (0.2 mol/L Na_2HPO_4, 0.1 mol/L citric acid and 1 mol/L NaCl, pH 5.8). The mixture was transferred to 1.5 mL eppendorf tube after grinding completely at 4°C and kept on ice for 1 h with shaking every 20 min. The mixture was centrifuged at 15,000 g for 10 min at 4 °C and 10 μL samples of the supernatants were then mixed with 4 mL of PME substrate solution. The absorbance of supernatant was measured at 525 nm after incubation for 2 h at 37 °C. The PME activity (μmol H^+ ·root cap^{-1}·h^{-1}) was calculated based on the standard curve. Each treatment was repeated five times.

Extraction of root tip RNA and expression analysis of *rcpme1*

After treated with volatile oil from *C. ambrosioides* and its two main components for 24h, total RNA was extracted from all samples using the RNA prep pure Plant Kit (Tiangen BIOTEC, Beijing, China) according to the manufacturer's instructions.

The integrity of the RNA samples was checked by agarose gel electrophoresis. Quantity and purity of total RNA were determined by measuring the absorbance at 260 and 280 nm using an Ultrospec 2100 proUV/Visible Spectrophotometer (GE Healthcare).
Primers for amplification of the *rcpme1* gene were designed for qRT-PCR using Primer Express software (V 5.0) and synthesized by Chengdu Qinke Zixi Biotechnology Co. Ltd. (F: 5'-CCAATGGCACCGAGAACC-3', R: 5'-GGACCGACTGAACAGCACCT -3'). Real-time qRT-PCR was performed on a MiniOpticon™ Real-Time PCR Detection System using the HSYBR One Step RT-qPCR Kit (Zomanbio, Beijing, China). The qRT-PCR amplification program was 5 min at 45°C and 1 min at 95°C followed by 45 cycles of 94°C for 10 s and at 57°C for 40 s. The qRT-PCR assays were performed in a total volume of 20 μL in an optical 96-well PCR plate. Each reaction contained 10 μL of HSYBR One Step RT-qPCR Buffer (Zomanbio), 0.4 μL of each forward and reverse primer (1 pM), 0.4 μL QK Enzyme Mix (Zomanbio), 2 μL of RNA (1 μg) and 6.8 μL of RNase-free water. All real-time RT-PCR experiments were carried out in triplicate together with controls lacking template to detect potential genomic DNA contamination. Bio-Rad CFX Manager software (V 3.1) was used to analyze the RT-PCR results and the *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase) gene was used as the internal reference gene to analyze the relative expression of *rcpme1*, this software directly output relative gene expression results base on formula of $2^{-\Delta\Delta Ct}$。

RBC (root border cells) activity

After treated with volatile oil from *C. ambrosioides* and its two main components for 12, 24 and 48 h, RBC suspensions were made as described above ("Preparing and counting RBCs"). A 10 μL aliquot of cell suspension was mixed with 4 μL AO/EB dye in

0.5 mL Eppendorf tube. Cell activity and morphology were observed under blue excitation light with a LEICA DM300 fluorescence microscope. Dead cells fluoresce orange, while living cells give green fluorescence. RBC viability = (the number of viable cells/number of total cells) × 100%. Each treatment was repeated five times.

Cell morphology observations

Nuclei: Roots were treated for 48 h and the RBC suspensions were made according to the method described in. Modified phenolic dye (Sigma) was used at 0.05% (w/v) in deionized water for 5 min to observe the cells under a bright-field microscope. The cells showing nuclear abnormalities (fragmentation, marginalization, malformation, or dispersion) were counted. We examined and photographed 100 cells per treatment group. Each treatment was repeated thrice.

The nuclear abnormality rate = (Number of cells with abnormal nuclei/Total cell number) x 100%.

Transmission electron microscopy (TEM) observation: RBCs were prepared as described above RBCs were fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4 at 4°C overnight. After post-fixation in OsO_4 and dehydration in ascending concentrations of ethanol infiltration and embedding were performed in Spurr Resin. Ultrathin sections were picked up onto 200 mesh copper grids, stained with lead citrate and uranyl acetate and observed under a Tecnai G2F20S-TWIN Transmission Electron Microscope (FEI Company, Hillsboro, OR, USA).

TUNEL assay

After treated with volatile oil from *C. ambrosioides* and its two main components for 48 h, RBCs were coated on polylysine slides, allowed to dry and then fixed in 4% PFA for 20 min. Endogenous peroxidase activity in the RBC sections was blocked with 0.3% H_2O_2 in methanol for 30 min. Sections were then blocked with 1% BSA for 30 min at room temperature. At the end of incubation, sections were washed with PBS and nicked DNA was detected using the TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling) assay kit (Nanjing Jiancheng Bioengineering Institute) as per the manufacturer's instructions. Sections were viewed under a fluorescence microscope (Nikon Instech Co. Ltd., Kawasaki, Kanagawa, Japan).

Statistical analyses

Quantitative data were described as the means ± SD and analyzed using one-way ANOVA. Statistical analysis was performed with PASW software, version 18.0 (Chicago, IL, USA). $P < 0.05$ was considered significant. The figures were drawn using Microsoft Excel 2007 (Microsoft, USA).

RESULTS AND DISCUSSION

Root length

Previous studies have shown that the allelochemicals released from the plants can enter the soil through many ways and affect the underground root systems of plants in the

ecosystem. The study of Wang *et al.* (37) showed that monoterpenoid volatiles from *Ambrosia trifida* L. have an allelopathic effect on other plants through the soil. The volatile terpenes released from the leaves of *Salvia leucophylla* form a "terpenoid cloud" around the plant and are brought into the soil by rain (27). Seedling growth is inhibited when adsorbed terpenes are dissolved in the cutin of seedlings in contact with soil colloids in hot weather 29). The components of *C. ambrosioide* volatile oil from various areas are different, but all contain α-terpinene and cymene (5,39,44). In our study, the volatile oil, α-terpinene and cymene were inhibitory to maize root elongation (Fig. 1). There were significant differences in root lengths between the controls and treatment groups, except for the α-terpinene 12 h treatment. This inhibitory effect was more pronounced with increasing treatment time and increasing volatile concentration. The inhibitory effects of the volatile oil on root length was highest, followed by cymene and the mixture. α-terpinene had the lowest inhibitory effect.

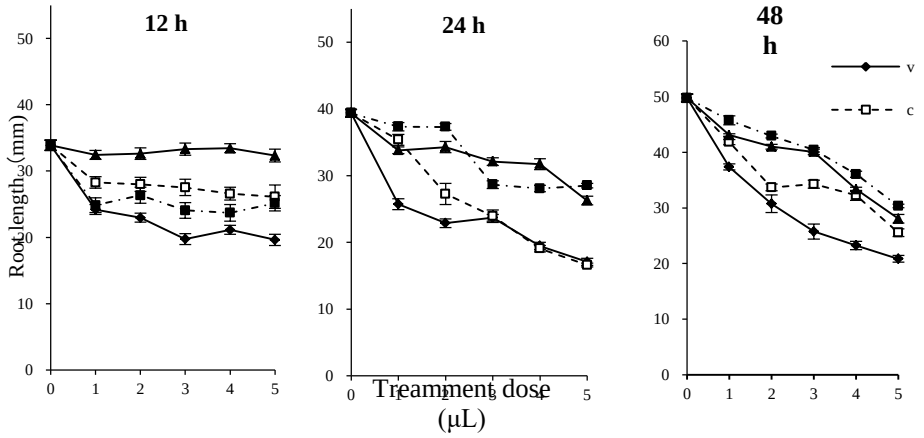

Figure 1. Effects of volatile oil from *C. ambrosioides* and its two main components on maize seedlings roots length
Note: V: Volatile oil; C: Cymene; AT, α-terpinene; M: Mixture of α-terpinene and cymene. 0, 1, 2, 3, 4 and 5 represent the amount of *Chenopodium ambrosioides* volatile oil and its two main components (μL).

RBC numbers and Pectin methyl esterase activity

Plant roots release more RBCs and mucilage to adhere or repel nematodes, bacteria, spores and other pathogenic microorganisms, as their guard against environmental stress caused by bacteria, heavy metals and other secondary metabolites (6,28,36). In our study, volatile oil, α-terpinene and cymene influenced the release of maize RBCs. RBC numbers decreased significantly when the maize radicles were exposed to volatile oil for 12, 24 and 48 h (Fig. 2), while the numbers for the other treatments were slightly higher than control. The number of maize root tip RBCs was 675, which was much lower than α-terpinene group (5569), cymene group (4431) and two main sub-mixing group (4031) when treated with 5 μL volatile oil.

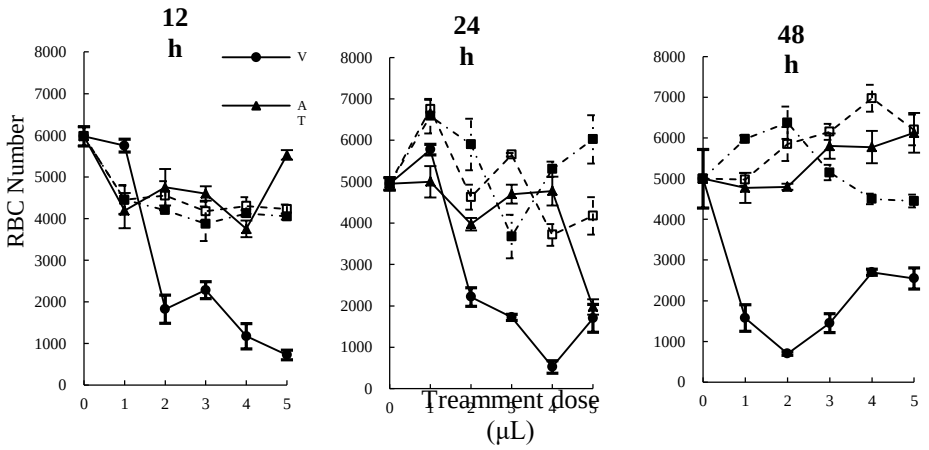

Figure 2. Effects of volatile oil from *C. ambrosioides* and its two main components on RBC number in maize seedlings root tip

PME catalyses the de-methylesterification of homogalacturonans by converting the methoxyl groups into carboxyl groups and releasing both methanol and protons (24) and the de-esterified polygalacturonic acid will change the cell wall properties under Ca^{2+}, making cell separation easier. The change in PME activity in the root cap was found closely related to *rcpme1* expression (41). In this study, after exposure to the volatile oil, α-terpinene, cymene and the mix of α-terpinene and cymene for 24 h, PME activity increased in all experimental groups than control group (Fig. 3a). In the 3 μL treatments, PME activity reached a maximum in all groups; the PME activity of volatile oil, α-terpinene, cymene and the mixture treatments were 9.31, 5.69, 4.71 and 5.45 μmol H^+ root $cap^{-1}\cdot h^{-1}$, respectively.

The qRT-PCR assays showed that the relative expression of *rcpme1* was consistent with the observed change in PME activity and up-regulation of gene expression is most obvious in volatile oil treatments. Relative expression of the *rcpme1* gene at the 3 μL dose was highest of all the treatment groups after 24 h (Fig. 3b). Relative expression in volatile oil treatment was 13.5 at 3 μL, which was significantly (P<0.05) higher than α-terpinene group (2.6), while the relative expression in cymene and mixed treatments were under 1.6. These results showed that volatile oil has stronger effects on up-regulating *rcpme1* expression than α-terpinene and cymene.

In this study, the increase in PME activity was consistent with the increased expression of *rcpme1*, but was not consistent with the change in RBC quantity under stress induced by the *C. ambrosioides* volatile oil and its main constituents. PME activity in root cap did not reflect immediately change in RBC numbers. A large part of reason for this

may be due to the action of PME on the cell wall. The release of RBCs was indirectly affected by the dehydroesterification of pectin by PME, the release of H^+ and a decrease in environmental pH indirectly activate other cell wall-degrading enzymes (galactosidases and arabinosidases) that function at low pH. In addition, the release of RBCs is also affected by plant root apical meristem type, in vivo auxin levels, ethylene 12) and other factors.

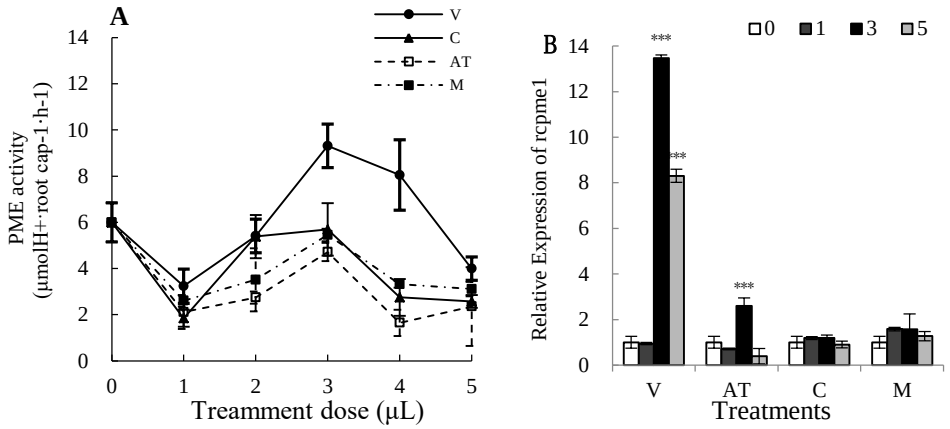

Figure 3. Effects of volatile oil from *C. ambrosioides* and its two main components on PME (pectin methyl esterase) activity and relative gene expression of *rcpme1* in maize root tip

RBC viability and morphology observations

The viability of maize RBCs was decreased by treatment with volatile oil for 12, 24 and 48 h (Fig. 4). Higher dose of volatile oil, α-terpinene and cymene caused lower RBC viability. Following exposure to 5 μL volatile oil for 48 h, RBC viability decreased to 70.2%, while in other treatment groups, RBC viabilities remained >90%. Exposure to α-terpinene, cymene and mixture of the two had little effect on RBC viability.

Allelochemicals such as chalcone induce nuclei breakdown, mitochondrial condensation, organelle disruption and chromatin fragmentation in Arabidopsis roots (10). Naphthoquinones are able to induces various structural, functional and enzymatic changes leading to apoptic-like cell death in tobacco BY-2 suspension cells (2). In our study, the nuclear morphologies of maize RBCs changed in response to treatment with volatile oil, α-terpinene and cymene and some abnormal characters such as lysis, marginalization and dispersion were observed (Fig. 5). As the treatment concentrations increased, the abnormality rate of maize RBCs increased (Fig. 6). Among the treatments, nuclear abnormality rate of volatile oil group was highest, (>70 %), when exposed at 5 μL dose. The effects of α-terpinene and cymene on RBC nuclear abnormality rate were relatively smaller than volatile oil. The nuclear abnormality rates of α-terpinene, cymene and the mixture were 15.00 %, 26.67 % and 14.00 %, respectively at 5 μL dose (Fig. 6).

There were numerous vacuoles in maize RBCs treated with volatile oil for 48 h and chromatin condensation and marginalization were observed under transmission electron microscopy (TEM) (Fig. 7). In control group, RBC morphology was intact, the organelles were clearly visible and the nuclei were regular.

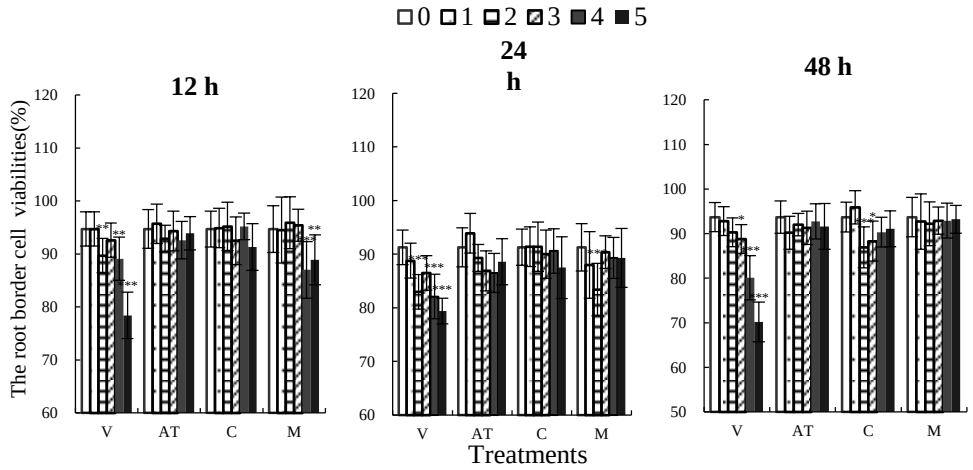

Figure 4. Effects of volatile oil from *C. ambrosioides* and its two main components on root border cell viability

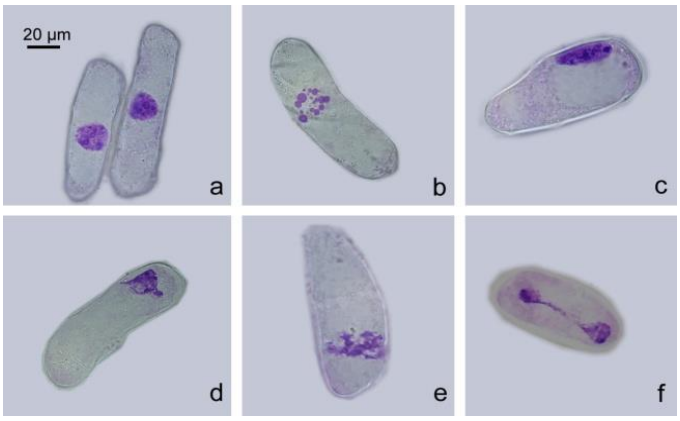

Figure 5. Effects of volatile oil from *C. ambrosioides* and its two main components on nuclear morphology of maize root border cell
Note: a, image of normal cell nuclei; b-f, images of deformed cell nuclei which show fragmentation, marginalization, malformation and dispersion, etc.

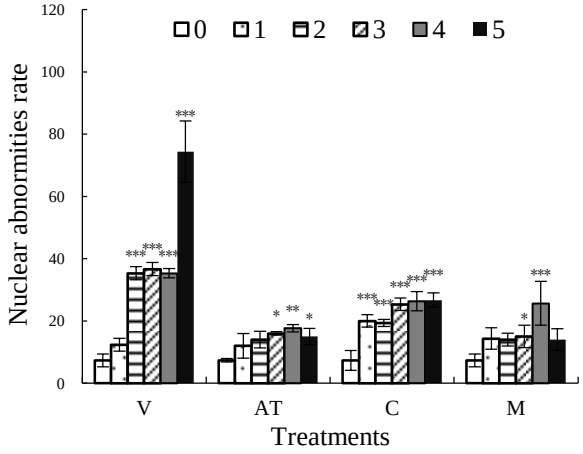

Figure 6. Effects of volatile oil from *C. ambrosioides* and its two main components on nuclear abnormality rates of maize maize root border cells

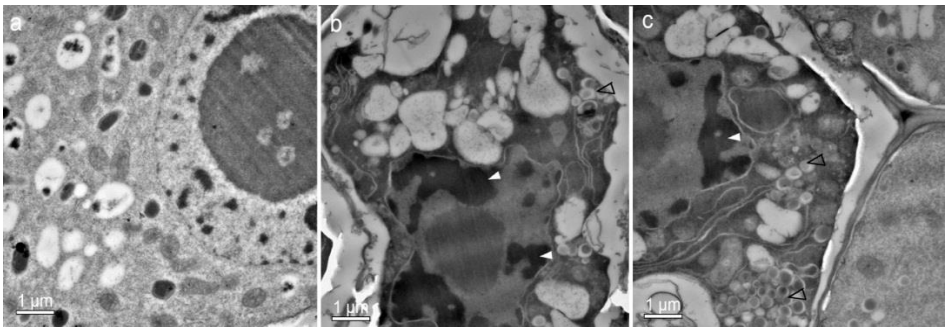

Figure 7. Transmission electron microscopy (TEM) of maize border cell treated with volatile oil from *C. ambrosioides*.
Note: **a** control groups, **b** and **c** were treated with *C. ambrosioides* volatile oil. White arrows indicate chromatin condensation; hollow black arrows indicate vacuolation. Treatment time was 48 h.

TUNEL assay was used to detect the DNA fragmentation as an indicator of apoptosis (44). We observed positive results for the volatile oil, α-terpinene, cymene and mixture when the RBCs were treated with 5 μL for 48 h (Fig. 8). The fluorescence intensities in α-terpinene and cymene treatments were higher than in volatile oil treatment and the mixture treatment was weakest. The activity in RBCs treated with *C. ambrosioides* volatile oil was lower than in cells treated with two main oil components. However, the positive reaction intensity observed in the TUNEL assay was higher in the α-terpinene and cymene treatments than in volatile oil, which may be because the volatile oil not only induced PCD in the RBCs, but also directly caused the necrosis of some RBCs. A weak a positive reaction in the TUNEL

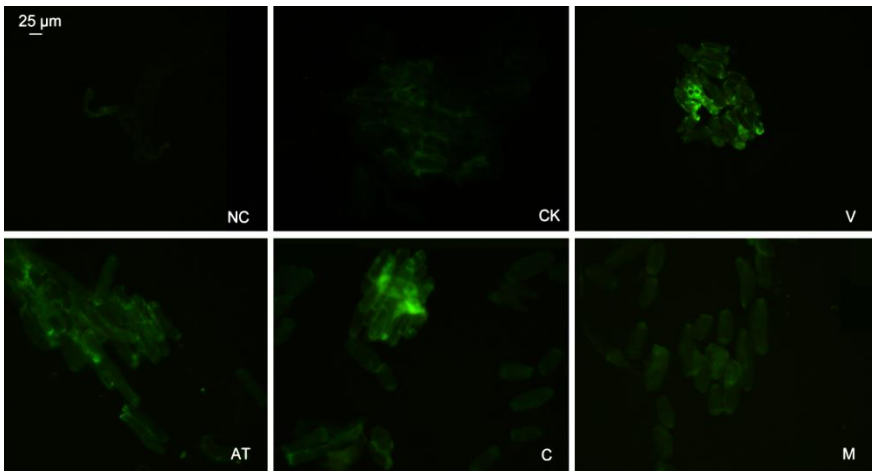

Figure 8. TUNEL assay of maize border cell treated with volatile oil from *C. ambrosioides* and its two main components.
Note: NC: Negative control. The treatment time was 48 h.

assay was observed in control group, suggesting that a small amount of cleaved DNA fragments are produced in RBCs during release from the apical root tip and the exposed 3'-OH termini become labeled with dUTP and fluoresce. Similar results have been seen in the TUNEL assay with colchicine-stressed lettuce (20,25,26,41,45). We suggest that one of the mechanisms accounting for the ability of *C. ambrosioides* to successfully invade new habitats is that it induces programmed cell death in RBCs through the release of volatile oil which removes the protective barrier established by RBCs, thus affecting the division and elongation of the root tip cells of the surrounding recipient plants, thereby inhibiting their growth.

CONCLUSIONS

C. ambrosioides volatile oil and its two main components, α- terpinene and cymene, possessed cytotoxic and genotoxic activity to maize RBCs. Exposure to these chemicals caused nuclear abnormalities, decreased the activity of the RBCs and induced programmed cell death and necrosis, thereby inhibiting the growth of young maize roots. The allelopathic toxicity of volatile oil on the RBCs was significantly greater than either α-terpinene or cymene individually.

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