

## Effects of allelochemicals from *Ageratina adenophora* (Spreng.) on its own autotoxicity

X. Z. ZHU<sup>1</sup>, J. GUO<sup>2</sup>, H. SHAO<sup>3</sup> and G. Q. YANG<sup>2\*</sup>

<sup>1</sup>College of Biotechnology, Jiangsu University of Science and Technology, Zhenjiang, Jiangsu 212018, China  
E. Mail: zxz412@sina.com

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### ABSTRACT

We investigated the autotoxic effects of 4-allelopathic compounds [dis(2-ethylhexyl) phthalate (DEHP), dibutyl phthalate (DBP), amorpho-4,7(11)-dien-8-one (DTD), 6-hydroxy-5-isopropyl-3,8- dimethyl-4a,5,6,7,8,8a- hexahydraphthalen-2(1H)-one (HHO)] from the invasive Weed species *Ageratina adenophora* and their biochemical mechanisms. The allelochemicals DEHP and DBP were extracted from the roots, while, DTD and HHO (the main allelopathic compounds) were isolated from the leachates of *A. adenophora*. In bioassay, the allelochemical DBP from the root exudates was inhibitory to seed germination and seedling growth of *A. adenophora*, but the DEHP was not autotoxic. Both allelochemicals i.e. DTD and HHO from leachates of *A. adenophora* also inhibited either germination or seedling growth of *A. adenophora* at different concentrations. The application of DEHP and DBP increased the activities of superoxide dismutase (SOD) enzyme. Whereas DTD had no significant effects on SOD activities and HHO inhibited the SOD activities. All these four allelochemicals did not influence the activity of peroxidase (POD). Thus two allelochemicals (DEHP and DBP) from the root exudates of *A. adenophora* caused the lipid peroxidation and damage to cell membrane of *A. adenophora* leaves.

**Key words:** Allelopathy, *Ageratina adenophora*, allelochemicals, autotoxicity, antioxidant enzymes, leachates, root exudates, seed germination, seedling growth

### INTRODUCTION

*Ageratina adenophora* Sprengel (also named *Eupatorium adenophorum*), a native plant from Mexico and Costa Rica, is a new and one of the most destructive alien weed (2,8) in Asia and Australia to South Africa. It causes native species extinction, livestock diseases and economic damages in Southwest China. It is also allelopathic to other plants (15). Allelopathy plays an important role in exotic plants invasion (4). Its chloroform extract inhibits germination and seedling growth of *Allium cepa*, *Raphanus sativus*, and *Cucumis sativus* (3). Its allelopathic effects could occur through natural leaching of its allelochemicals from the aerial tissues by precipitation (rain, dew, mist, snow) (12). Its aqueous leaf leachates significantly inhibits the germination and seedling growth of other plant species [*Brassica rapa* (21), *Chloris gayana*, *Ixeridium gracile* and *Mariscus cyperinus* (23)]. Yang *et al.* (17,18) isolated, purified and identified two primary

\*Correspondence author; <sup>2</sup>College of Horticulture and Plant Protection, Yangzhou University, Yangzhou, Jiangsu 225009, China. Phone (Office): +86-514-87979246, E. Mail: gqyang@yzu.edu.cn

<sup>3</sup>Key Laboratory of Biogeography and Bioresource in Arid Land, Xinjiang Institute of Ecology and Geography, Chinese Academy of Sciences, Urumqi 830011, China

allelochemicals (sesquiterpenes) of *A. adenophora*, 9-Oxo-10, 11-dehydroageraphorone (DTD) and 9b-Hydroxyageraphorone (HHO) and tested their bioactivity and physiological effects. They also reported three sesquiterpenes and two esters as the potential allelochemicals from the root exudates of *A. adenophora* (19).

Autotoxicity is often observed in continuous cropping in vegetable and fruit crops (6,14,20). Here we assumed that autotoxicity may influence or regulate the invasive process of alien species. Till now, allelopathy research on *A. adenophora* has mostly focussed on direct phytotoxicity that alters the interspecific interference between *A. adenophora* and native plant species. Little is known about the autotoxic effects of its allelochemicals in determining intraspecific interactions. Autotoxicity may influence the seed germination or seedling growth in the vicinity of mature parent plants and may play a role in the invasion of *A. adenophora*. This study aimed to examine the autotoxicity of two main allelochemicals from the leachates and two allelopathic esters from its root exudates and to determine their effects on enzymatic antioxidant activities in leaf.

## MATERIALS AND METHODS

### Extraction of aqueous leachates and allelochemicals isolation

For the experiments, we used leaves and shoots of healthy and mature plants of *A. adenophora* (without flowers and seeds) collected in July 2013 from Kunming City, Yunnan Province, China. All the plant materials were brought immediately to our laboratory in Yangzhou University to conduct extraction experiments. The DTD (amorpho-4,7(11)-dien-8-one) and HHO (6-hydroxy-5-isopropyl-3,8-dimethyl-4a,5,7,8, a-hexahydraphtalen- 2(1H)-one) were extracted as per the method of Yang *et al.* (17). The fresh aerial plant parts (4 Kg) were extracted with 20 L distilled water three times (24 h per extraction) at room temperature. Afterwards, the extracts were filtered through 4-layers of cheesecloth and Whatman (No.1) filter paper. Followed by evaporation in vacuo, the residues (2 L) were decanted and extracted by n-hexane in a separating funnel. N-hexane fractions were combined, dried using anhydrous  $Mg_2SO_4$ , filtered and the solvent was dried in vacuo at room temperature. The residues were re-dissolved in 20 ml methanol and the volume was reduced to 3 ml under a flow of  $N_2$ . The solution was applied to a silica gel (200-300 mesh; 4 × 80 cm) column chromatography eluted with a gradient consisting of mixtures of n-hexane/ chloroform/EtOAc to increase polarity. Fractions of 30 mL were collected and combined to form 7 fractions according to the similarity of  $R_f$  in TLC. The second fraction contained one major compound and other minor components, which were finally purified by Recycling Preparative HPLC to yield colourless solid of DTD ( $R_t$  16.5 min, 195 mg). Recycling Preparative HPLC was done with JAI LC-9201, with column of GS-310 (21.5×300mm), with MeOH/H<sub>2</sub>O (80:20 V/V) as the mobile phase at a flow rate of 5 ml/min. After twice purification with silica gel column chromatography eluted with chloroform/EtOAc (3:1, V/V), HHO (53 mg) as yellow oil was isolated from the fifth fraction.

The other two allelochemicals (DBP and DEHP) from root exudates of *A. adenophora* were purchased from Sigma Chemical Co., St. Louis, MO, USA. The chemical structures of the four allelochemicals were shown in Fig.1.

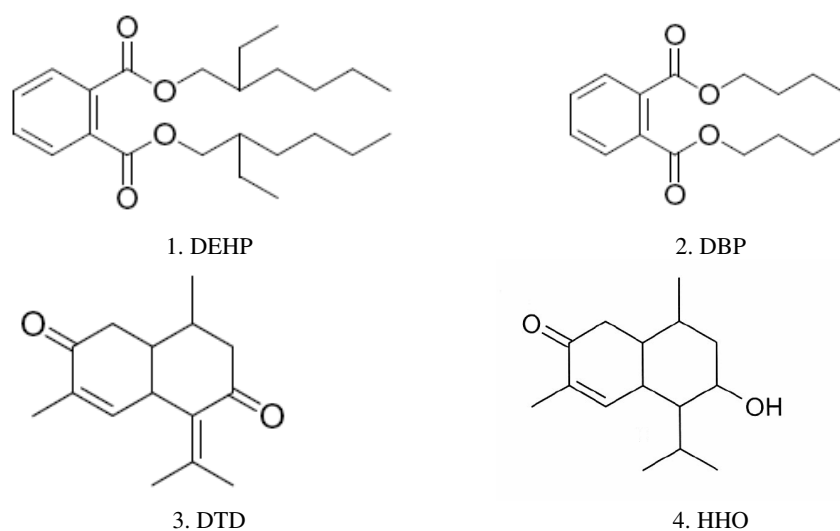


Figure 1. Structures of allelopathic compounds from leaves and root extraction of *Ageratina adenophora*: 1. dis(2-ethylhexyl) phthalate (DEHP); 2. dibutyl phthalate (DBP); 3. amorpha-4,7(11)-dien-8-one (DTD); 4. 6-hydroxy-5-isopropyl-3,8-dimethyl-4a,5,6,7,8,8-hexahydraphthalen-2(1H)-one (HHO).

### Bioassays

The bioassays were conducted in laboratory, Yangzhou University. For autotoxic bioassay, *A. adenophora* was selected as receptor plant to determine the phytotoxic effects of its own allelochemicals. Two layers of Whatman No. 1 filter paper were put in 9 cm petri dishes, which were impregnated with the allelochemicals dissolved in 1 ml MeOH. Pure MeOH applied to filter paper was used as control. After MeOH evaporated completely, 2 ml distilled water was added to each petri dish. The final concentrations of allelochemicals applied were 2, 10, 50 and 250 mg/L. Fifty seeds of *A. adenophora* were placed on two sheets of filter paper. Then all petri dishes were sealed with Parafilm and kept in a growth chamber (25°C, 75% relative humidity, 10 h L: 14 h D photoperiod). Thereafter, if needed 1 ml distilled water was added to maintain sufficient moisture if necessary. All experiments were conducted at 25°C, as maximum germination of *A. adenophora* seeds occurred at this temperature (9). Germinated seeds were counted daily. After 10 d incubation, growth parameters (number of germinated seeds, length of primary shoots and roots, and the biomass of seedlings) were measured. The experiment was done in completely randomized design with 5 replicates.

### Greenhouse Experiment

The greenhouse experiment was conducted at Yangzhou University, to assess whether soil influences the autotoxic effects of four test allelochemicals. Soil was collected from the non-invaded area in South China to avoid residual allelochemicals in the soil. Fresh soil was dried at room temperature and sieved (2 mm) to remove gravels etc.

Then the soil was used to fill 52 pots (upper diameter 14 cm, lower diameter 10 cm, height 12 cm), with 1 kg fresh soil in each pot. Pots were impregnated with the allelochemicals dissolved in MeOH. Pure MeOH was used as control treatment. After MeOH evaporated completely, 300 ml distilled water was added to each pot to make allelochemical solutions final concentrations of 10, 50 and 250 mg/L. Fifty seeds of *A. adenophora* were sown 1-2 cm below the surface in each pot for germination trials (4 allelochemicals  $\times$  3 concentrations). The remaining pots were used as controls (4 replicates) and treated with equivalent volumes of distilled water. All the glass house experiments were conducted in completely randomized design with four replications. Then all pots were kept in greenhouse (25 °C and 10-h-light/14-h-dark, using mercury lamps) at Yangzhou University i.e. similar conditions under which *A. adenophora* and native species occur. Distilled water was added to maintain a constant moisture regime of c. 50% field soil water capacity. All pots were randomly arranged. Germinated seeds were counted daily. After 10 d incubation, growth parameters (length of primary shoots and roots) were measured.

#### **Determination of lipid peroxidation and enzymatic antioxidant activity**

From bioassay of physiological effects, two-months-old healthy *A. adenophora* seedlings were selected and transplanted in 20 ml flat-bottom tubes. They were irrigated with 1/4 strength Hoagland's solution (5) and placed in the growth chamber [14 h/L:10 h/D photoperiod at 25 °C] for 2 days. Then two layer of Whatman No. 1 filter paper was put in another series of 20 ml flat-bottom tubes, which were impregnated with different concentrations of 4-test allelochemicals contained in 0.5 ml MeOH. Pure MeOH applied to filter paper was used as the control treatment. After the EtOAc was evaporated, 15 ml of Hoagland's solution was added to all tubes to make the final concentrations of allelochemicals to 10, 50 and 250 mg/L. 52 *A. adenophora* seedlings (4 allelochemicals  $\times$  3 concentrations  $\times$  4 replicates + control  $\times$  4 replicates) were transferred into those tubes (1 seedling per tube). At 48 h after treatment, the leaves of *A. adenophora* were sampled. All experiments were conducted in completely randomized design and treatments were replicated 4-times.

Leaves of *A. adenophora* seedlings were collected, weighed (0.5g) and immediately frozen in liquid nitrogen and stored at -70 °C until extraction. Frozen tissues were quickly homogenized in 10 ml of 50 mM sodium phosphate buffer containing 0.1 mM EDTA at pH 8. All manipulations were performed at 0-4 °C. The homogenate was centrifuged at 12,000 g for 20 min and the supernatants were stored at -70 °C until enzyme activity were determined. Superoxide dismutase (SOD) activity was assayed by its ability to inhibit the photochemical reduction of nitroblue tetrazolium (13). Peroxidase (POD) activity was measured at 460 nm using guaiacol as the hydrogen donor (13).

**Statistical analysis:** The data obtained was analyzed for variation using the analysis of variance (ANOVA) procedure (version 6.12; SAS Institute, Cary, N.C., USA). Multiple comparisons of mean values were based on Fisher's protected least significant difference (PLSD).

## RESULTS AND DISCUSSION

### Bioassay

The allelochemical DBP from root exudates of *A. adenophora* was inhibitory to its seed germination (Fig. 2). Meanwhile, the other allelochemical (DEHP) did not influence the *A. adenophora* germination (Fig. 2). While both allelochemicals (DTD and HHO) from the leachates of *A. adenophora* inhibited the germination at high concentrations (250 mg/L), but had little effects at lower concentrations (Fig. 2). The fresh weight of seedlings was significantly reduced by DBP at all test concentrations and reduced by DTD and HHO at high concentrations of allelochemicals, but not affected by DEHP (Fig. 3). For shoot length of seedlings, DBP exhibited significant inhibitory effects and DTD was slightly inhibitory, but DEHP and HHO showed no significant effects (Fig. 4). For the root length, only DBP was most inhibitory and the other three allelochemicals did not show any effects except that DTD showed stimulatory effects at 2 mg/L and HHO showed inhibitory effects at 250 mg/L (Fig. 5).

The two allelopathic esters from root exudates of *A. adenophora* (DEHP and DBP) had quite different effects. DBP inhibited the seed germination and reduced the seedling growth significantly; however, DEHP did not influence *A. adenophora* at all. While, the allelopathic effects of two allelopathic sesquiterpenes from *A. adenophora* (DTD and HHO) leachates depended on their concentrations. At high concentration of leachate, the germination and seedling growth of *A. adenophora* were decreased sharply. However, the leachate at low concentration had little influence on germination and seedling growth of *A. adenophora*. The leachates showed autotoxic effects in a dose-dependent manner.

### Greenhouse Experiment

The allelochemical DEHP from root exudates of *A. adenophora*, did not affect its shoot height (Fig. 6). Meanwhile, the other three, DBP, DTD and HHO, showed significantly inhibitory effects only at 10 mg/L concentration and did not influence the *A. adenophora* shoot heights at other concentrations (Fig. 6, Table 1). In root length, all 4 allelochemicals showed phytotoxic effects on seedling growth, which depended on their concentrations (Fig. 7, Table 1). The concentrations of allelochemicals significantly influenced the allelopathic effects of 4 compounds and indicated that autotoxicity really occurred (Table 1).

### Enzymatic activities

Two allelochemicals from root exudates, i.e. DEHP and DBP, significantly enhanced the SOD activities in leaves of *A. adenophora* (Fig. 8). The activities of SOD increased by 107%, 128% and 117% at three concentrations of DEHP, i.e. 10, 50 and 250 mg/L, respectively. And SOD activities increased by 24%, 55% and 45% at three concentration levels of DBP (10, 50 and 250 mg/L), respectively. There were no significant differences in SOD activities, when treated with DTD, an allelopathic compound from *A. adenophora* leachate. However, when treated with the other allelopathic compound HHO from *A. adenophora* leachate, SOD activities decreased at all

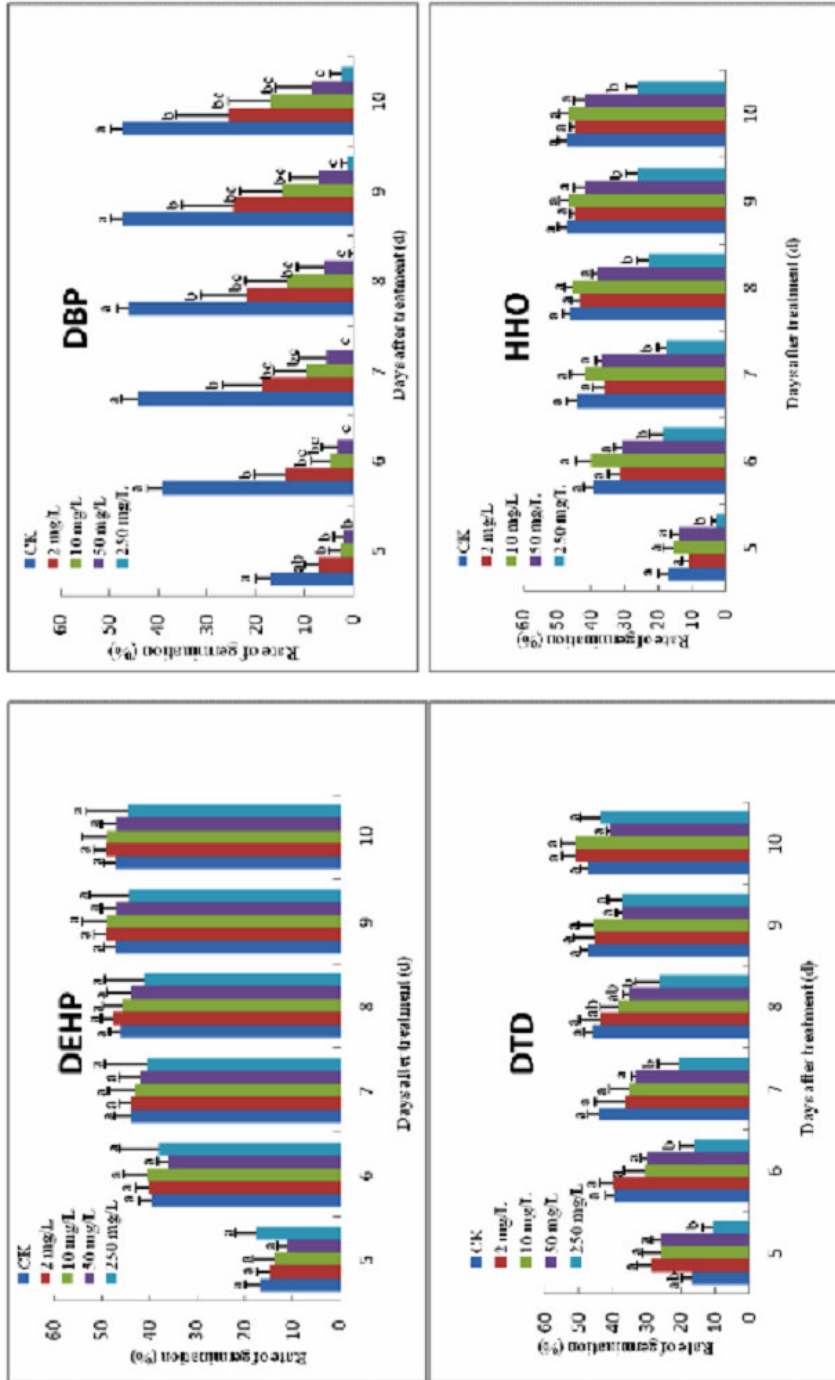


Figure 2. Effects of various concentrations of 4-test allelochemicals from *A. adenophora* on their seed germinations (laboratory bioassay). Mean  $\pm$  SE. Bars with different letters within the same day after treatment indicates significant differences within each treatment at  $P < 0.05$  (Fisher's PLSD test).

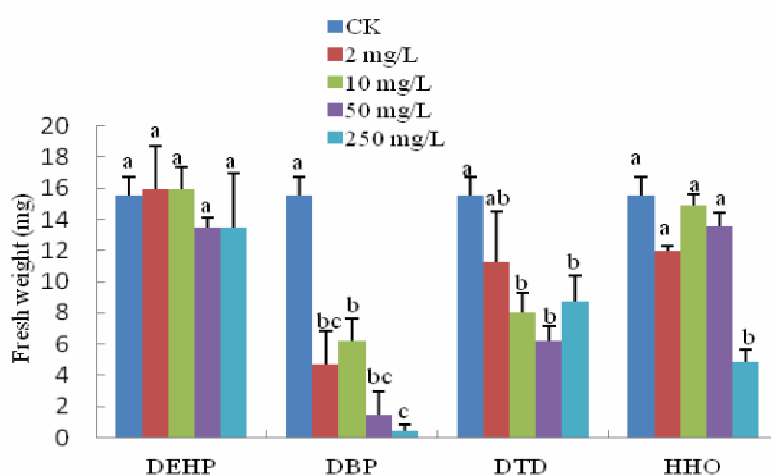


Figure 3. Effects of various concentrations of 4-test allelochemicals from *A. adenophora* on their seedling fresh weight (laboratory bioassay). Mean  $\pm$  SE. Bars with different letters within the same allelochemical indicates significant differences within each treatment at  $P < 0.05$  (Fisher's PLSD test). DEHP (dis(2-ethylhexyl) phthalate), DBP (dibutyl phthalate), DTD (amorpha-4,7(11)-dien-8-one), HHO (6-hydroxy-5-isopropyl-3,8- dimethyl-4a,5,6,7,8,8a-hexahydraphthalen- 2(1H)-one).

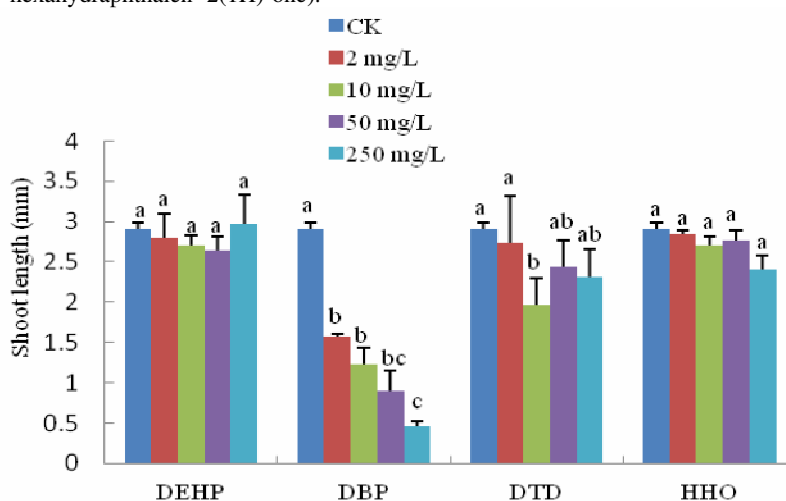


Figure 4. Effects of various concentrations of 4-test allelochemicals from *A. adenophora* on their seedling shoot lengths (laboratory bioassay). Mean  $\pm$  SE. Bars with different letters within the same allelochemical indicates significant differences within each treatment at  $P < 0.05$  (Fisher's PLSD test). DEHP (dis(2-ethylhexyl) phthalate), DBP (dibutyl phthalate), DTD (amorpha-4,7(11)-dien-8-one), HHO (6-hydroxy-5-isopropyl-3,8- dimethyl-4a,5,6,7,8,8a-hexahydraphthalen- 2(1H)-one).

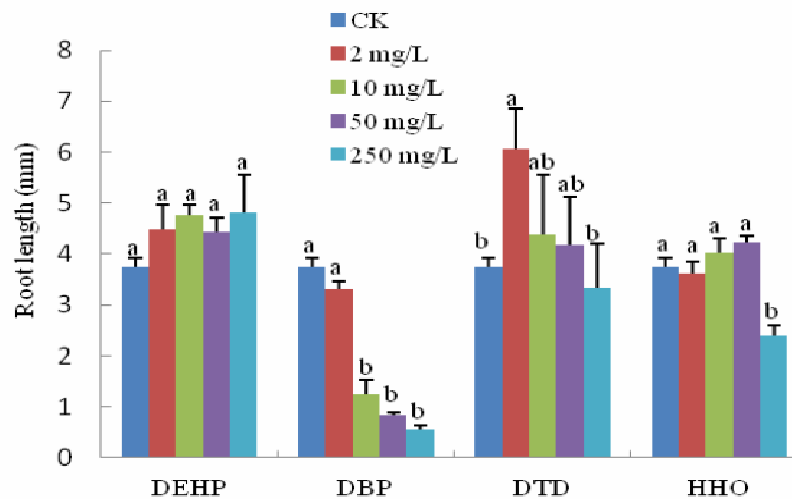


Figure 5. Effects of various concentrations of 4-test allelochemicals from *A. adenophora* on their seedling root lengths (laboratory bioassay). Mean  $\pm$  SE. Bars with different letters within the same allelochemical indicates significant differences within each treatment at  $P < 0.05$  (Fisher's PLSD test). DEHP (dis(2-ethylhexyl) phthalate), DBP (dibutyl phthalate), DTD (amorpha-4,7(11)-dien-8-one), HHO (6-hydroxy-5-isopropyl-3,8- dimethyl-4a,5,6,7,8,8a-hexahydraphtalen- 2(1H)-one).

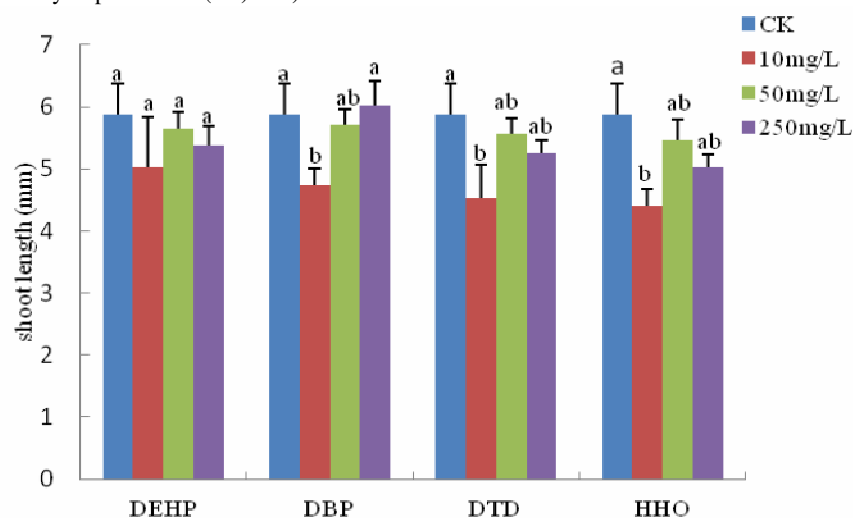


Figure 6. Effects of various concentrations of 4-test allelochemicals on shoot lengths of *A. adenophora* seedlings in soil (Glass house study). Mean  $\pm$  SE. Bars with different letters within the same allelochemical indicates significant differences within each treatment at  $P < 0.05$  (Fisher's PLSD test). DEHP (dis(2-ethylhexyl) phthalate), DBP (dibutyl phthalate), DTD (amorpha-4,7(11)-dien-8-one), HHO (6-hydroxy-5-isopropyl-3,8- dimethyl-4a,5,6,7,8,8a-hexahydraphtalen- 2(1H)-one).

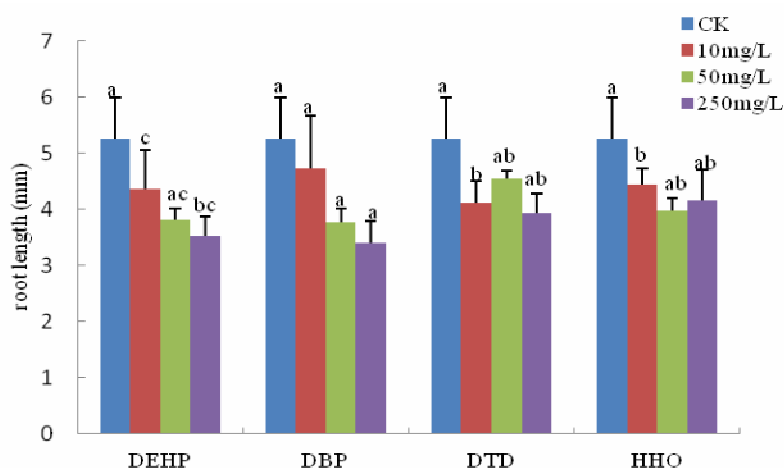


Figure 7. Effects of various concentrations of 4-test allelochemicals on root lengths of *A. adenophora* seedlings in soil (Glass house study). Mean  $\pm$  SE. Bars with different letters within the same allelochemical indicates significant differences within each treatment at  $P < 0.05$  (Fisher's PLSD test). DEHP (dis(2-ethylhexyl) phthalate), DBP (dibutyl phthalate), DTD (amorpho-4,7(11)-dien-8-one), HHO (6-hydroxy-5-isopropyl-3,8-dimethyl-4a,5,6,7,8,8a-hexahydrophthalen-2(1H)-one).

Table 1. ANOVA of growth indexes of *A. adenophora* seedlings from Figs. 6, 7. A means four allelochemicals from *A. adenophora* (DEHP, DBP, DTD and HHO), and B means concentration of allelochemicals. Freedoms of A, B, and AxB are 3, 3, and 9, respectively

Growth indexes	Source of variance	F-value	P-value
Shoot height	A	2.602	0.1164
	B	22.16	0.0002
	AxB	0.283	0.9763
Root length	A	1.236	0.3525
	B	8.263	0.0059
	AxB	0.79	0.6265

concentration levels (Fig. 8). On the contrary, all 4- allelochemicals had no significant effects on the activities of POD at all test concentrations (Fig. 9).

The terpenoid allelochemicals could cause inhibitory effects on the receptor plants in some way, such as the monoterpene on the oxidative metabolism of mitochondria (1) and the asparagines synthetase (11). Exposed to the four allelochemicals (DEHP, DBP, DTD and HHO), the POD activity in leaves of *A. adenophora* did not change with their increasing concentrations. These findings do not agree with previous literature (7), indicating no increased production of peroxides and active oxygen species (AOS) in allelochemical-treated root tips. The activity of SOD increased when treated with DEHP and DBP, showing that SOD could not scavenge the excess active oxygen with the relative high dose treatment and  $H_2O_2$  might accumulate in the roots of test plants (10). These results suggest that those two esters allelochemicals (DEHP and DBP) are associated with

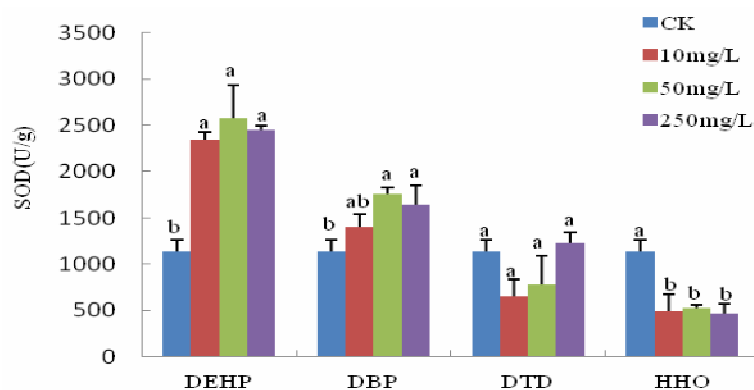


Figure 8. Effects of various concentrations of 4-test allelochemicals on activities of superoxide dismutase (SOD) in leaves of *A. adenophora*. Mean  $\pm$  SE. Bars with different letters within the same allelochemical indicates significant differences within each treatment at  $P < 0.05$  (Fisher's PLSD test). DEHP (dis(2-ethylhexyl) phthalate), DBP (dibutyl phthalate), DTD (amorpha-4,7(11)-dien-8-one), HHO (6-hydroxy-5-isopropyl-3,8- dimethyl-4a,5,6,7,8,8a-hexahydraphthalen- 2(1H)-one).

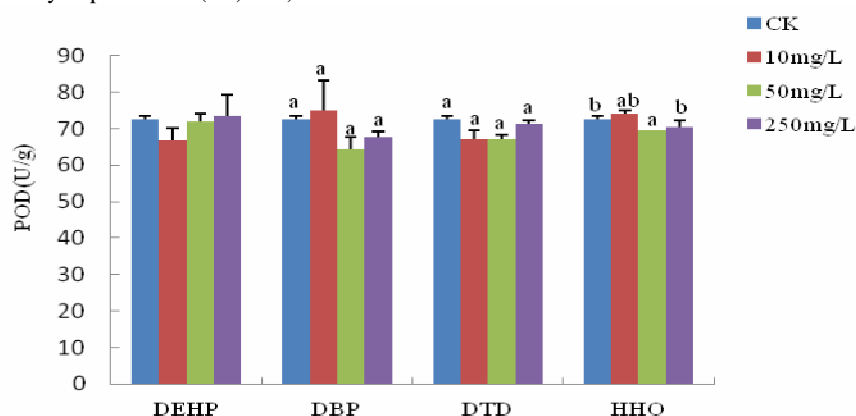


Figure 9. Effects of various concentrations of 4-test allelochemicals on activities of peroxidase (POD) in leaves of *A. adenophora*. Mean  $\pm$  SE. Bars with different letters within the same allelochemical indicates significant differences within each treatment at  $P < 0.05$  (Fisher's PLSD test). DEHP (dis(2-ethylhexyl) phthalate), DBP (dibutyl phthalate), DTD (amorpha-4,7(11)-dien-8-one), HHO (6-hydroxy-5-isopropyl-3,8- dimethyl-4a,5,6,7,8,8a-hexahydraphthalen- 2(1H)-one).

the production of AOS, whereas, increase in oxidizing enzymes is a secondary response to increase in free radicals. In addition, the allelochemical might directly inhibit the oxidizing enzymes in some way, rendering the plant vulnerable to oxidative damage (16).

The interactions between the *A. adenophora* leachate and natural soil on germination and seedling growth of *A. adenophora* itself, suggested that soil from different habitats altered the allelopathic effects observed in laboratory bioassay. The inhibitory

effects of DBP, DTD and HHO were reduced in soil. However, DEHP showed no phytotoxic effects in laboratory bioassay, but significantly inhibited the root length of *A. adenophora* seedlings in soil in glass house experiment. All results showed that in the soil, the autotoxic effects of allelochemicals from *A. adenophora* were eliminated or altered. The results were consistent with our previous study on allelopathic effects on *Brassica rapa* (24).

The two allelochemicals (DTD and HHO) from *A. adenophora* leachates are partially water-soluble, hence, they may be released by leaching in rains. The other two allelochemicals (DEHP and DBP) from root exudates of *A. adenophora*, could be released directly in to the soil. All 4-test allelochemicals were phytotoxic to *A. adenophora* seedling growth in laboratory bioassay or glass house experiments. The *A. adenophora* autotoxicity can play a role in regulating its population density. Our results were consistent with root box experiments of Yu & Ma 2006 (22). The growth of *A. adenophora* seedlings was significantly reduced when planted with their parental plants; but when parental plants were removed, the emergence of *A. adenophora* seedlings was significantly increased in field. When *A. adenophora* invades a new habitat, it can release allelopathic compounds to inhibit native species growth and development. However in its established mature monoculture population, those compounds could inhibit its own growth and development to regulate its population density.

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