Pak. J. Bot., 50(1): 371-380, 2018.

BIOASSAY EVALUATION OF THE POTENTIAL ALLELOPATHIC EFFECTS OF GARLIC (*ALLIUM SATIVUM* L.) ROOT EXUDATES ON LETTUCE AND CUCUMBER

HAIYAN DING[1], IMRAN AHMAD[2], AHMAD ALI[1], ABDUL REHMAN KHAN[3] AND ZHIHUI CHENG[1*]

[1]*College of Horticulture, Northwest A&F University, Yangling, Shannxi 712100, China.*
[2]*Department of Horticulture, The University of Agricultural, Peshawar, Pakistan*
[3]*Horticulture Department, Nuclear institution for agriculture & biology, Punjab 38000, Pakistan*
[*]*Corresponding author's email: chengzh@nwsuaf.edu.cn; Tel:+862987091332. Fax: +862987082613*

Abstract

Two experiments were carried out to assay the allelopathic effects of garlic root exudates on seed germination and seedling growth of lettuce and cucumber. In the first experiment, a hydroponic method was adopted to collect garlic root exudates to simulate the natural secretion. Both the primary aqueous solution and different concentrations (10%, 12.5%, 25%, 50% and 100%) of the three different organic extracts of petroleum ether (PE), ethyl acetate (EA) and methanol (MA) were tested for lettuce. The results indicated that the primary aqueous solution showed a significant suppressive effect on germination and root length, but a stimulative effect on antioxidant enzyme activities in lettuce. The three organic extracts showed dose-dependent effects, and the frequency of inhibition amplified with increasing concentration of exudate extract. During comparative intensity evaluation of three different organic extracts, the activity of PE extract demonstrated the highest allelopathic reaction for seed germination and root morphology. The second experiment was conducted to eliminate the microbial effects, which might degrade the allelochemicals in the root exudates. Then, a biological test under an aseptic condition was directed to verify the allelopathic effect of garlic root exudates on cucumber. The results exhibited that garlic root exudates had a significant inhibitory effect on the germination of cucumber, but a dose-dependent effect on the growth of cucumber seedlings, thus confirming the results of the first experiment. These results provided a theoretical basis for further research into the identification of putative allelochemicals in garlic root exudates.

Key words: Garlic (*Allium sativum*); Root exudates; Bioassay; Allelopathy.

Introduction

Allelopathy refers to a plant rhizosphere phenomenon in which different organic chemicals called allelochemicals are released from the donor plant species by diversified mechanisms regarding degradation of plant residues, leaching, volatilization and root exudation (Inderjit &Vivanco, 2006). The potential role of these chemical compounds from root exudates is a principle investigative approach to historical allelopathic research (Oburger *et al.*, 2013; Xu *et al.*, 2013). The heterogeneous influence of allelochemicals on the growth of other bioassay test species has been investigated on the basis of mode of action in certain allelopathic crop species (Kato-Noguchi & Peters, 2013). Currently, plant ecologists mainly study the harmful interaction among plants and suggest that direct and indirect mechanisms of interspecific interference in allelopathy would be different from other plant growth models in crop-specific activity, chemical structure, and effectiveness level, associated with the induction of biological and non-biological changes (Hierro & Callaway, 2003).

Plants produce numerous secondary metabolites, and some of them show an allelopathic effect on neighboring plants, ranging from phytotoxicity to organogenesis induction. The action of these substances towards plant growth and germination may occur through a variety of mechanisms, including germination reduction (Khan & Cheng, 2010), inhibited seedling growth rate (Liu *et al.*, 2011), decreased roots and hypocotyls mitotic activity, suppressed hormonal and photosynthetic action (Zhang *et al.*, 2010), minimized ionic uptake amount, seized protein formation and targeted antioxidative mechanisms (Cheema *et al.*, 2013; Ding *et al.*, 2016).

However, allelopathy was implicated in the autotoxicity phenomenon and multi-cropping system in vegetable production under protected agriculture (Liu *et al*., 2011). In the multi-cropping system, root exudates of target plants play a significant role in influencing themselves or the plants nearby.Root exudates primarily represent the frequent direct source of certain plant allelochemicals distributes into rhizosphere soil environment. Root exudation performs various functions in the internal plant structure, including enzymatic mucilageformation, ion secretion, free osmotic oxygen and water regulation, especially achieving a diversified role in the distribution of primary and secondary metabolites (Oburger *et al.*, 2013).

Garlic (*Allium sativum* L.) perceived an excellent previous crop in multi-cropping systems, especially considering due to insecticidal, antiviral and sterilization effects, which have provoked the interest of researchers (Iciek *et al.*, 2009). Its root exudates can significantly inhibit the growth of mycelia in pepper blight (Khan & Cheng 2010; Khan *et al*., 2011). Intercropping of various vegetables with garlic can expand the microbial population density and may alter the internally biochemical traits of growing medium (Xiao *et al.*, 2012; Ahmad *et al.*, 2013a, b; Liu *et al.*, 2014). Furthermore, it also increases the chlorophyll content, photosynthetic rate and antioxidant enzyme activity, which reduces the obstacles of receptor plants (Xiao *et al.*, 2013;Wang *et al.*, 2014).

At present, chemical-mediated plants that possess strong allelopathic influence have drawn the attention of investigators, but their synergistic effect has been explored only in cereal crops such as wheat-rice allelopathic interaction (Kato-Noguchi, 2011a; Kato-Noguchi, 2011b; Kato-Noguchi *et al.*, 2011; Kato-Noguchi *et al.*, 2012). Nonetheless, a few detailed studies involving the direct allelopathic effectiveness of garlic root exudates on donor vegetables have been conducted. Correspondingly, this allelopathy phenomenon of garlic root exudates and its potential role in the bioassay studies of leafy vegetables have not been well demonstrated in the literature. Furthermore, the isolation and identification of specific allelochemicals from root exudates is an important study, but theirs discovering procedure and SOPs for required agents are very complex and challenging to do. An unspecified large number of allelochemicals in plants produce a synergistic effect on donor plants instead of releasing a single particular compound; therefore, not all chemicals have been recognized as a potential allelopathic agent (Chon & Kim, 2002; Tchone *et al.*, 2006). Moreover, the complex soil environment and microbial effects make it harder to isolate and identify the allelochemicals in root exudates, the biological test under controlled condition was still widely used to explore the phytochemical biological activity of phytochemical substances (Rehman *et al.*, 2017). Therefore, in-depth studies are necessary to search and distinguish different compounds according to their polarity classification through standard procedures.

Thus, two different methods were adopted to investigate the allelopathic influence of garlic root exudates in this study. First, to simulate the natural secretion in the field, we used a hydroponic culture method to collect garlic root exudates, and a biological test method was used to determine the allelopathic inhibitory or stimulatory influence of three kinds of organic solvents for lettuce seed and germination bioassay indexes. Second, to eliminate the microbial effects, which may degrade the allelochemicals in the root exudates, a biological test under aseptic conditions was conducted to confirm the allelopathy of garlic root exudates on cucumber. This research aimed to detect the direct influence of garlic root exudates on donor plants, screen the most sensitive component of garlic root exudate extraction and provide theoretical rationalization and technical guidance for further research on identifying allelochemical-profiles of garlic root exudates.

Materials and Methods

Experiment 1

Plant material and collection of garlic root exudates: Bulbs of garlic cv. Gailiang were provided by College of Horticulture, Northwest A&F University, Yangling, Shaanxi, China. Seeds of lettuce (*Lactuca sativa* L.) were provided by Nongcheng Seed Technology Centre of Northwest A&F University.

The method tracked for the collection of garlic root exudates was of Khan *et al*., 2011 with little modification. A total of 1000 uniform fresh and healthy garlic bulbs were sterilized in 0.1% potassium permanganate ($KMnO_4$) for 10 minutes, rinsed several times with distilled water and then sown in sterilized and moistened perlite medium kept at 20°C for ten days. While during culture period's garlic plants were uniformly irrigated with distilled water for every 2 or 3 days to allow the perlite wet. After rooting (approximately 5 cm root length), the bulbs were gently removed from the growth medium avoiding any root damage, rinsed to remove perlite particles and then transplanted on perforated thermo-pore plates. A self-designed, simple hydroponic culture system equipped with electric ventilators (220 V, 80 W) was established to facilitate seedling growth through continuous ventilation. In each system, 20 garlic bulbs were arranged in a square layout into a flat-surfaced, 15-L capacity, rectangular plastic tub (40 cm× 30 cm× 12 cm). Since rich nutrients are present in garlic bulbs, no exogenous nutrition was provided, and only distilled water was supplied. The experiment was conducted at room temperature, and the water level was maintained to 10 L per tub; every ten days the root exudates were collected during culture for 30 days in the growth chamber, and then the total root fresh weight of garlic (1000 garlic bulbs) was recorded as 4900 grams.

Preparation of primary aqueous solution and three different polar extracts: After vacuum filtration and through a filter membrane (aqueous phase, 0.25 µm), an aqueous solution called primary aqueous solution was obtained. Then, the garlic root exudates were extracted by a continuous trapping system as adopted by Khan *et al.* (2011) in which primary aqueous solution was passed drop-by-drop through an active carbon column to trap the exudates. The carbon was washed individually with petroleum ether (PE), ethyl acetate (EA) and methanol (MA), respectively, according to the degree of polarity of the solvent, and then the volume was made up to 1 L of each extract solution. After filtering with filter membrane (organic phase, 0.25 µm), the mother liquors of the three extracts were obtained.

Seed bioassay for evaluating primary aqueous solution: A lab bioassay method was performed to check the allelopathic bioactivity of the garlic root exudates for lettuce seedling growth. For that purpose, the seeds of lettuce were sterilized in hot water at 55°C for 15 minutes and then rinsed three times with sterilized distilled water. Fifty lettuce seeds were sown in culture dishes (90 mm) with a layer of Whatman No.18 filter paper. Firstly, 5 ml primary aqueous solution of garlic root exudates were added to the culture dishes, then 2 ml of the same primary aqueous solution was poured into the culture dishes on alternate days during culture. For control, only double distilled water was used in the same pattern. The growing dishes were placed in an incubator maintained the photoperiod of 12 h light and 12 h dark at 22°C, the optimal temperature of lettuce germination, and the experiment was replicated five times. The number of germinated seeds in each dish was counted daily, and the root length, shoot length, fresh weight of root and shoot were measured after 10 days of incubation.

Seed bioassay estimation for three extracts of garlic root exudates at different concentration: Five concentration treatments with 10%, 12.5%, 25%, 50% and 100% dilutions of mother liquors, which corresponded to 0.49 g/ml, 0.6125 g/ml, 1.225 g/ml, 2.45 g/ml and 4.90 g/ml of fresh weight of garlic root were designed for each of the extracted garlic root exudates. Initially, 5 ml primary aqueous solution of garlic root exudates were added to the culture dishes symmetrically, then 50 seeds of lettuce were sown in culture dishes (90 mm) and 2 ml distilled water was poured into the culture dishes on alternate days during culture. Bioassay of all three extracts was the same as the above aqueous solution.

Experiment 2

Plant material and collection of garlic root exudates: Fresh and uniform sized garlic bulbs sterilized by 75% ethanol for one minute, rinsed five times with distilled water, then sterilized by soaking in 4% sodium for 30 min, and finally again rinsed 5 time with distilled water. Different numbers of garlic bulbs (0, 2, 4, 6, 8, 10, respectively) were sown in 100 ml agar medium (5‰ w/v) of each tissue culture vessel (7 cm * 11 cm, 340 ml), each treatment with 10 replications. The vessels were kept under aseptic conditions at 22 to yield a rooting culture for 30 days.

Seed germination bioassay: Cucumber seeds sterilized in the same way as garlic bulbs; twenty seeds were sown in the agar medium culture after uprooting the garlic bulbs and added 20 ml fresh agar medium in each tissue culture vessel. The vessels incubated in a culture chamber under a photoperiod of 12 h light and 12 h dark at 22 °C, the optimal temperature of cucumber germination, and the experiment was replicated five times. The number of germinated seeds in each vessel was counted daily, and the root length, shoot length, fresh weight of root and shoot were measured after 10 days of incubation.

Data collection on plant growth: Final germination percentages recorded after seven days,and root and shoot length measured with a ruler and weight balance after ten days, respectively. The allelopathic reaction index (RI) of each indicator was adopted to analyze the allelopathy of garlic root exudates on lettuce (Bruce-Williamson & Richardson, 1988) using the following formulae:

$$RI=1-C/T \text{ (when } T\geq C)$$

$$RI=T/C-1 \text{ (when } T<C)$$

Here, RI refers to the extent or intensity of allelopathy; T stands for the treatment value, and C refers to the control value for a particular investigated parameter. A positive RI value refers to a stimulatory effect and vice versa.

Data collection on the activities of antioxidative enzymes: For the assay of antioxidative enzymes, the leaf tissue (0.5 g) was homogenized in 10 ml of 200 mM chilled phosphate buffer (pH 7.8) containing 1.0% (w/v) soluble polyvinyl pyrrolidone (PVP) in an ice bath. The homogenate was centrifuged at 20,000 g for 20 min at 4 °C, and the supernatant was used for assaying antioxidant enzymatic activities.

Superoxide dismutase (SOD) activity was determined according to (Ali *et al.*, 2014). The reaction mixture contained 100 µl of enzyme extract, 100 µl of 1.3 l M riboflavin and 3 ml of the SOD buffer. The reaction was carried out at 25 °C under fluorescent lamps (40 W) for approximately 8 minutes until the reaction mixture color turned dark, an indication of the formation of formazane as a result of nitrobluetetrazolium (NBT) photo reduction. The same reaction mixture, while kept in the dark without illumination, was taken as a control, and the mixture lacking leaf extract was taken as blank. Absorbance was recorded at 560 nm using a UV-visible spectrophotometer (UNIC 2100). A unit of SOD was defined as the amount of enzyme that inhibits the NBT photo reduction by 50% in comparison with the sample lacking the plant extract.

The activities of peroxidase (POD), catalase (CAT) and phenylalnineammonialyase (PAL) were determined following the method of Wang (Wang *et al.*, 2015). The reaction mixture of POD comprised 0.1 ml enzyme extract, 2.9 ml phosphate buffer (pH 7.0), methyl catechol and 0.3% H_2O_2. A change in absorbance was recorded at 485 nm for 3 min with a spectrophotometer. The POD activity was recorded as OD 485 nm $min^{-1}g^{-1}$. The reaction mixture of CAT comprised 0.1 ml enzyme extract, 1.9 ml of 200 mM phosphate buffer (pH 7.0), 1 ml 0.3% H_2O_2, and the enzyme activity was assayed by measuring the reduction of H_2O_2 at 240 nm for 3 min with a spectrophotometer. The activity of CAT was recorded as OD 240 nm $min^{-1}g^{-1}$. The reaction mixture of PAL comprised 0.3 ml enzyme extract, 2.7 ml of borate buffer solution (pH 8.8), 1 ml 0.02 M phenylalanine, water bath at 37 °C for 1 hour. Absorbance was read at 290 nm using a UV-visible spectrophotometer.

Data collection on malondialdehyde content: Methane dicarboxylic aldehyde (MDA) content was determined by thiobarbituric acid reaction with slight modifications (Shi *et al*., 2014). The extraction was obtained by the same method as antioxidant enzyme activity. The reaction mixture comprised 1.5 ml extraction and 2.5 ml 0.5% thiobarbituric acid (TBA, w/v). The mixture was reacted at 100ºC for 20 min before being cooled in an ice bath. After centrifugation at 7888 g for 10 min, the absorbance of the supernatant was measured at 450 nm, 532 nm and 600 nm in a spectrophotometer. The MDA content was calculated using the following formula: MDA content ($mmo\cdot g^{-1}FW$)=[6.452*(OD532-OD600)-0.559*OD450]*8/(DW*1.5).

Statistical analysis: The experiment was accomplished according to a completely randomized design (CRD). The data are means of five independent repeats. Analyses of variance (ANOVA) was performed to determine significant differences ($p<0.05$) among treatments for different parameters, using the software SAS 8.1 (SAS Institute Inc, Cary, NC, USA).

Results

Effect of the primary aqueous solution of garlic root exudates on lettuce: The primary aqueous solution of garlic root exudates collected 30 days after hydroponic culture showed significant allelopathic effects on the germination rate and seedling growth of lettuce (Table 1). Both seed germination and radicle length showed a relatively more sensitive response to this aqueous solution. Significantly negative allelopathic effects on both the germination rate and root length were observed, and the reduction rates were 5.92% and 62.71%, respectively. In contrast, no significant effect was observed on shoot length and root fresh weight. However, a high stimulative effect (110.8%) was documented on fresh shoot weight of lettuce seedlings.

The activity of SOD and CAT was remarkably lower in control conditions, whereas the contents of MDA were relatively higher than primary aqueous solution treatment (Table 2); however, no significant difference was found for the activity of POD between the control and primary aqueous solution treatment.

Table 1. Effect of garlic root exudates primary aqueous solution on seed germination and seedling growth of lettuce.

Treatment	Germination rate (%)	Root length (cm)	Shoot length (cm)	Root weight (mg)	Shoot weight (mg)
CK	90.0 ± 0.6a	5.47 ± 0.32a	2.84 ± 0.05a	4.88 ± 0.10a	11.80b ± 0.21b
Primary aqueous solution	84.7 ± 1.2b	2.04 ± 0.11b	2.73 ± 0.08ab	4.83 ± 0.11a	24.80 ± 0.19a

Note: The data were presented as the mean ± standard error (n=5). Values within a column not followed by the same letter are significantly different at p≤0.05

Table 2. Effect of garlic root exudates primary aqueous solution on the activity of protective enzymes of lettuce.

Treatment	SOD ($U \cdot gFw^{-1} \cdot h^{-1}$)	POD ($U \cdot gFw^{-1}$)	CAT ($U \cdot gFw^{-1}$)	MDA ($mmol \cdot g^{-1}Fw$)
CK	172.70 ± 5.41b	1.10 ± 0.08b	7.93 ± 0.36b	1.95 ± 0.08a
Primary aqueous solution	241.86 ± 3.04a	1.20 ± 0.07a	10.42 ± 0.21a	1.60 ± 0.05b

Note: The data were presented as the mean ± standard error (n=5). Values within a column not followed by the same letter are significantly different at p≤0.05

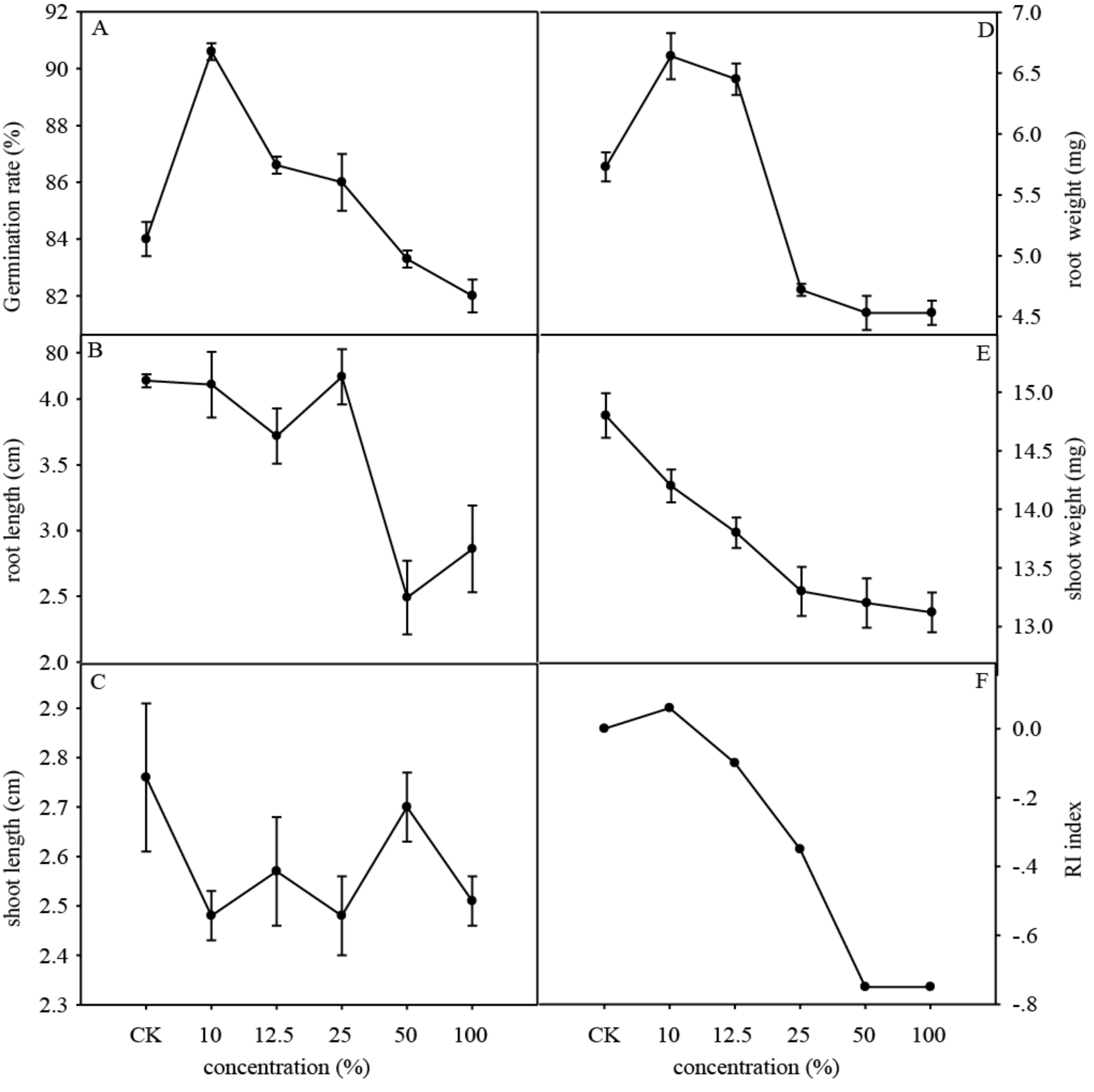

Fig. 1. Effect of petroleum ether extract of garlic root exudates on seed germination and seedling growth of lettuce. (A) germination rate, (B) root length, (C) shoot length, (D) root weight, (E) shoot weight and (F) RI index. Note: RI refers to the allelopathic reaction index, and a positive value represents promotion, a negative value represents inhibition. The data were presented as the mean ± standard error (n=5). Values within a column not followed by the same letter are significantly different at p≤0.05.

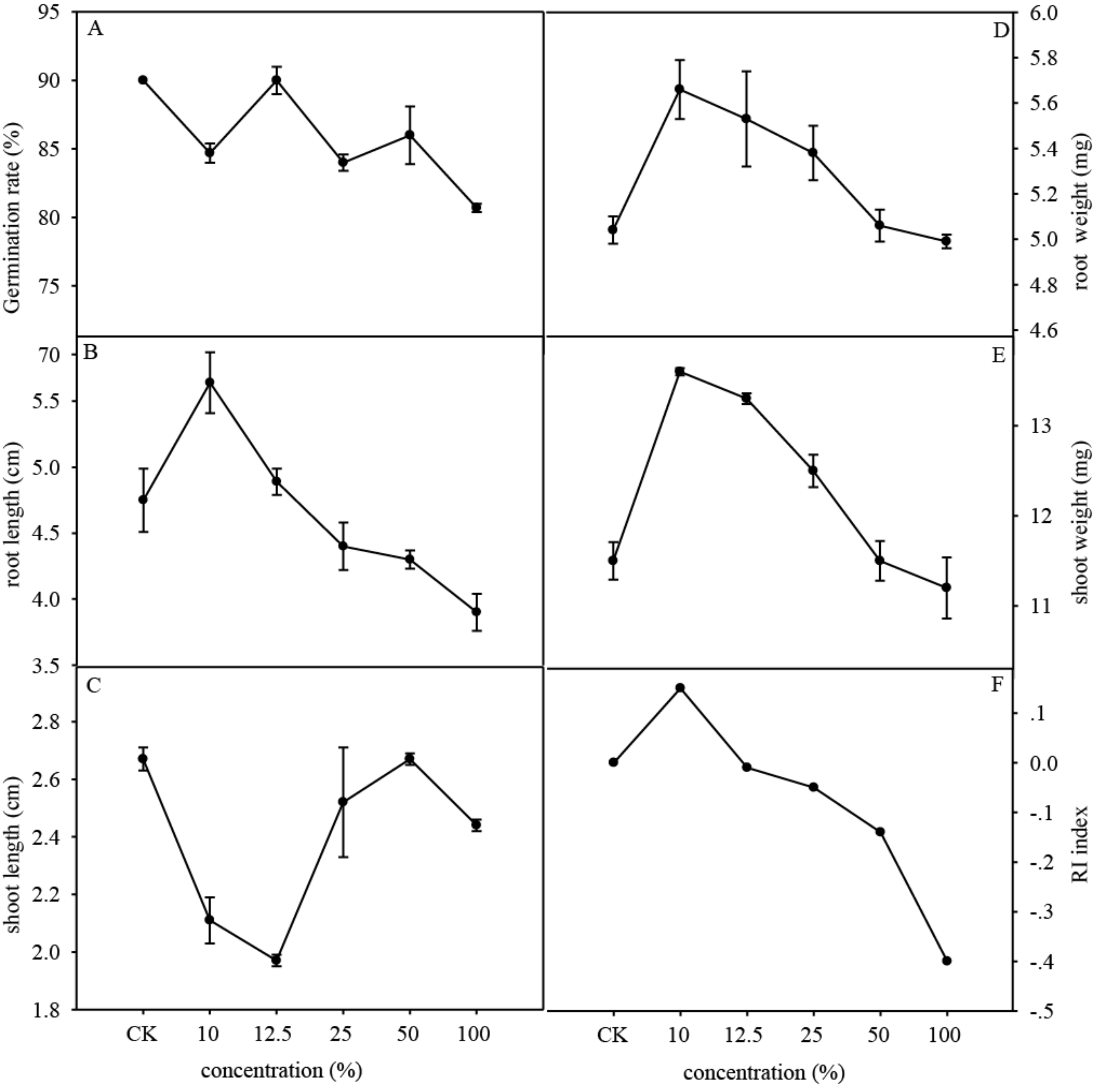

Fig. 2. Effect of ethyl acetate extract of garlic root exudates on seed germination and seedling growth of lettuce. (A) germination rate, (B) root length, (C) shoot length, (D) root weight, (E) shoot weight and (F) RI index. Note: RI refers to the allelopathic reaction index, and a positive value represents promotion, a negative value represents inhibition. The data were presented as the mean ± standard error (n=5). Values within a column not followed by the same letter are significantly different at $p \leq 0.05$.

Effect of petroleum ether extract of garlic root exudates on lettuce: The effect of petroleum ether (PE) extract on seed germination, shoot and root growth of lettuce varied significantly at different concentrations (Fig. 1). The germination rate was lower than the control at 100%, and 50% concentrations and the reduction rates were 2.38% and 0.80%, respectively. However, it was higher than the control at 25% concentration, and the germination rate was highest at 10% concentration (90.66%). The root length of lettuce decreased with the increased concentrations. It was significantly lower than the control at concentrations of 50% and 100%, and the reduction rates were 39.86% and 30.92%, respectively. Additionally, petroleum ether extract showed a more allelopathic response towards root length and shoot, root fresh weight; the reduction rate increased with the increasing concentrations. Interestingly, a lower concentration (10%) accelerated the growth promotional activity for the reaction index (RI) of petroleum ether extract.

Effect of ethyl acetate extract of garlic root exudates on lettuce: The germination and the root growth inhibition of lettuce was higher in the presence of ethyl acetate extract at a higher (100%) concentration relative to the control. However, the germination rate was lower than the control except at a 12.5% concentration. A similar inhibitory trend was observed regarding root length, shoot weight and root weight. In the case of shoot elongation, ethyl acetate extract always demonstrated positive allelopathy response and growth activity compared with the control (Fig. 2).

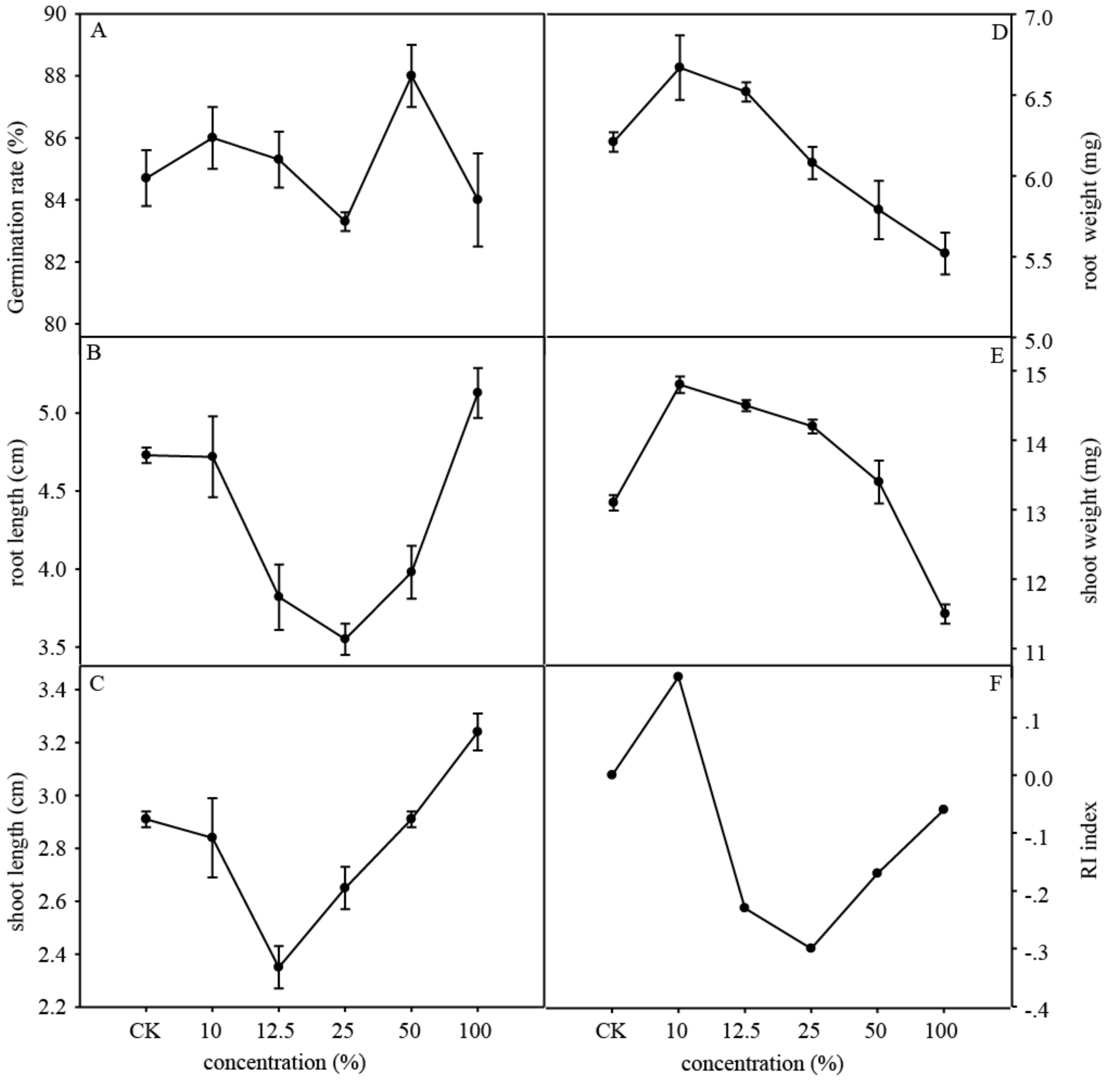

Fig. 3. Effect of methyl alcohol extract of garlic root exudates on seed germination and seedling growth of lettuce. (A) germination rate, (B) root length, (C) shoot length, (D) root weight, (E) shoot weight and (F) RI index. Note: RI refers to the allelopathic reaction index, and a positive value represents promotion, a negative value represents inhibition. The data were presented as the mean ± standard error (n=5). Values within a column not followed by the same letter are significantly different at $p \leq 0.05$.

Effect of methyl alcohol extract of garlic root exudates on lettuce: The allelopathic potential was verified among all the tested concentrations along with the control. The treatments with the methyl alcohol extract derived from garlic root exudates did not show a significant difference in lettuce seed germination (Fig. 3). It showed the highest value at 100% concentration and lowest values at 12.5% concentration for root length and shoot length. In contrast, the highest value of both root fresh weight and the shoot fresh weight were noted at low concentration (10%). Root and shoot fresh weight measurements especially showed a high degree of inhibition by these extracts at 100% concentration.

Effect of different number of garlic root exudates on cucumber under aseptic condition: To dispel the potential effect of microbes on garlic root exudates, another experiment was carried out for verifying the results under aseptic conditions (Fig. 4). It showed that the germination of cucumber was inhibited by 3.03%, 3.03%, 7.07%, 9.09% and 13.13% in the five treatments while comparing to the control. However, for the growth of cucumber seedlings, all treatments with a different number of garlic bulbs showed better performance in shoot length and fresh weight. Also, garlic root exudates exhibited a significant dose-dependent effect on root growth of cucumber. Root length showed the highest levels when treated with two garlic bulbs; 70.77% higher than that of the control, but the other treatments were lower than the control, and treatment with ten garlic bulbs reached the lowest value (3.52 cm). The trend was same as root weight and root length. Moreover, the reaction index (RI) also exhibited a dose-dependent trend in the treatments, which refers to the extent or intensity of allelopathy of garlic root exudates. A positive RI value appeared in treatments with 2, 4, and 6 garlic bulbs, but a negative value was observed in treatments with 8 and 10 garlic bulbs.

Discussion

Allelopathy unveils a prominent influence in defining the dynamics and structure of a plant community; and its role has been observed in several crop species (Alias *et al.*, 2006). It was previously demonstrated that garlic root exudates have the significant influence on both microbes and plants (Khan *et al.*, 2011; Xiao *et al.*, 2013; Wang *et al.*, 2014; Wang *et al.*, 2015). Therefore, they might be used as an alternative strategy for relieving continuous cropping obstacles for vegetables.

The present investigation revealed that a primary aqueous solution of garlic root exudates inhibited the lettuce seed germination and negatively affected root length. However, it significantly promoted shoot weight thus contributing to a higher biomass as compared with the controls (Table 1). The allelopathic effect of garlic root exudates primary aqueous solution showed similar effects to CCC (chlormequat chloride), which belonged to the category of plant hormones. This speculation might be due to the presence of allelochemicals containing secondary metabolites in garlic root exudates, which include phenolic compounds that can adjust hormone levels in donor plants by regulating the expression of related genes and act as scavengers to eliminate ROS in plants (Pandhair & Sekhon, 2006; Oracz *et al.*, 2012).

Moreover, a primary aqueous solution could also have other less known aspects, such as modulation of cellular redox state, signal transduction and post-translational modification of proteins (Iciek *et al.*, 2009). It was further suggested that garlic and garlic-derived organic-sulfur compounds exert antioxidant action by scavenging reactive oxygen species (ROS) and improving the cellular antioxidant enzyme activities by increasing glutathione levels in the cells (Iciek *et al.*, 2009). In our study, the presence of a primary aqueous solution of garlic root exudates improved the activity of protective enzymes in lettuce seedlings and reduced the cell membrane peroxidation damage in lettuce (Table 2). Our results were parallel with the findings of Yu *et al.* (2003) which indicated that SOD activity significantly increased when exposing the cucumber roots to these phytotoxic compounds (Yu *et al.*, 2003). Moreover, Oracz *et al.* (2007) also reported that the sunflower extract significantly increased SOD and CATactivities of mustard seeds during germination (Oracz *et al.*, 2007). Certain chemicals and their derivatives such as phenolics, terpenoids, and alkaloids are the primary entities of various secondary metabolites viewed as potential inhibitors for seed germination, seedling growth and enzyme activity (Siddiqui, 2007). SOD, POD, CAT and other protective enzymes inplants act synergistically to eliminate accumulated ROS. Regarding the allelopathic potential of garlic root exudates on lettuce seeds, the main antioxidant enzymatic changes were examined in this study. The optimum concentrations of a primary aqueous solution of garlic root exudates significantly up-regulated activities of SOD, POD and CAT, suggesting that the garlic root exudate (in primary aqueous solution) in suitable concentrations can be helpful in eliminating the accumulation of ROS to keep the plants stable. However, we found conflicting interpretation with some finds where garlic intercropping decreased the activity of protective enzymes and MDA content (Wang *et al.*, 2015). These contrary results may be because of distinct soil biological characteristics that might modify the allelochemicals response and therefore describe the alterations due to changing the substrate (Alias *et al.*, 2006).

To investigate the allelopathic intensity of garlic root exudates, we used different solvents with different polarities (PE, EA and MA). By comparing the RI values, we can summarize that the intensity order of the three different polarity extract of garlic root exudates was PE (petroleum ether extracts) >MA (methanol extracts) >EA (ethyl acetate extracts). The possible reasons for these differences might be that different solvents may contain different profiles of allelochemicals in different concentrations. It was demonstrated that the allelopathic chemicals possessed a certain level of hydrophilic and hydrophobic property due to easily dissolve in polar water in appropriate diffusion and might permit the allelochemicals to easily cross the biological membranes and reduce the chance of being transported away from the site (Alias *et al.*, 2006). In this study, the PE extracts showed the strongest inhibitory effect on lettuce, which might possess the certain hydrophobicity to meet the requirement. It can be speculated that the main target site of garlic root exudates was the biological membranes of cells in lettuce, but this needs to be further corroborated. In recent years, some organic sulfides, organic acids, phenols and organic ester compounds have been found in garlic and identified as allelochemicals (Ding & Cheng, 2014). However, it was reported that plant roots can also secrete organic acids, amino acids, fatty acids, flavonoids compounds, nucleotides and some other secondary metabolism compounds, mainly produced by the acetic acid or the shikimic acid pathway or a combination of these two pathways (Singh & Duke, 2003).

In this study, according to the polarity of the three organic solvents, PE extracts possibly contained lipophilic and poor water solubility substances such as lipin, essential oils, sterols, waxes, etc. MA extracts possibly contain poor lipophilic substances, such as glycosides, alkaloids, amino acids, flavonoids, phenolic acids tannin, etc. Moreover, EA extracts possibly contain moderate lipophilic substances, such as lactones, flavones, quinines and weak base alkaloids (Chen, 2004). Thus, the lipophilic and moderate lipophilic substances were probably the major constituents of garlic root exudates, which provided us a clue for identifying the allelochemicals in garlic root exudates in a subsequent study. Ni *et al.* (2011) used a similar method to investigate the effects of extracts of three plants species (*Artemisia annua*, *Conyza canadensis* and *Erigeron annuus*) on blue algae and theyfound dominant suppressive effects. Moreover, further separation of PE and EA extracts according to water-solubility and acidity or alkalinity showed that fatty acids and terpenoids in *A. annua*, terpenoids and organic acids in *C. canadensis* and *E. annuus* might be the main anti-algal active constituents (Ni *et al.*, 2011).

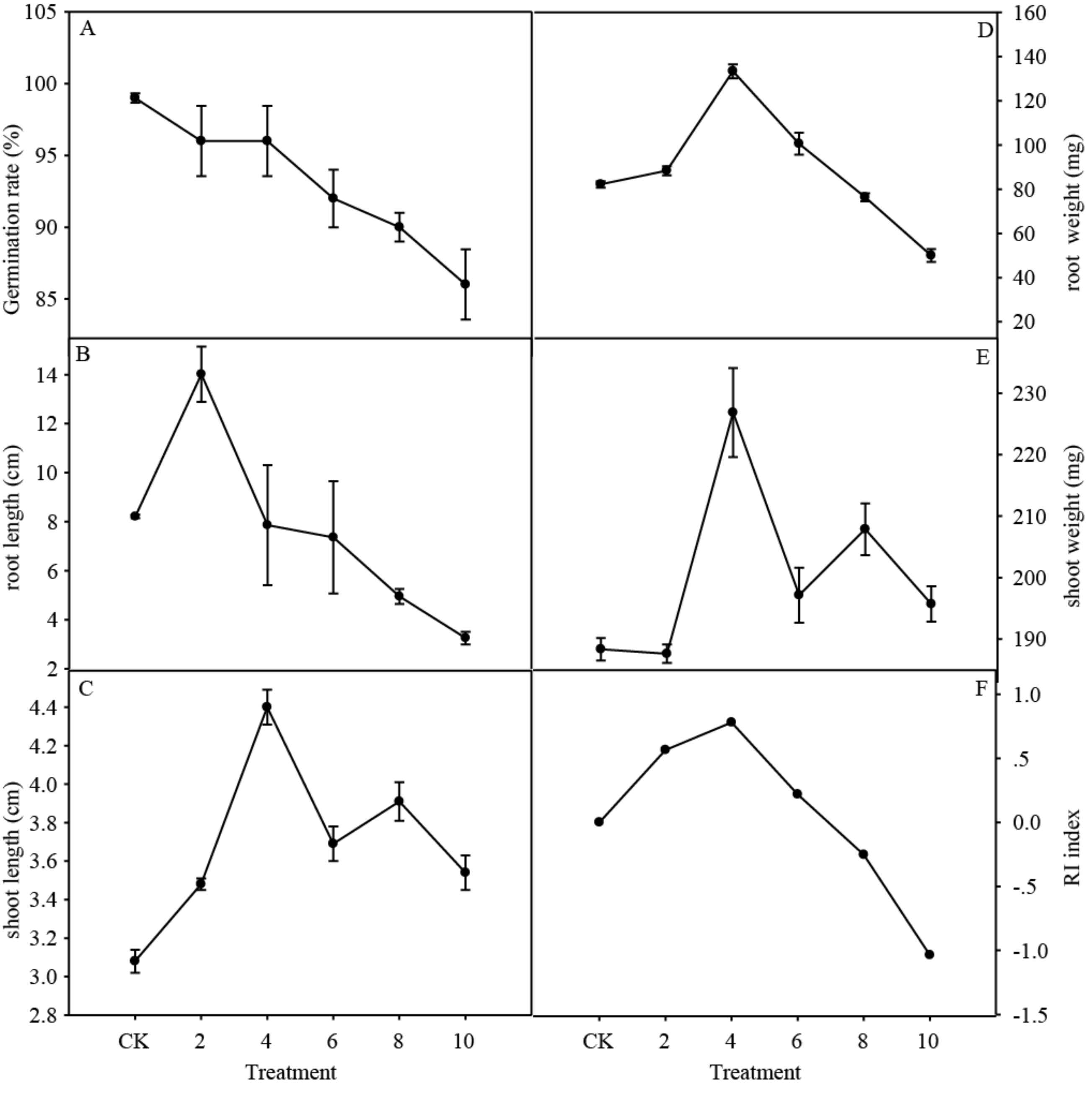

Fig. 4. Effect of different concentration of garlic root exudates on seed germination and seedling growth of cucumber under aseptic conditions. (A) germination rate, (B) root length, (C) shoot length, (D) root weight, (E) shoot weight and (F) RI index. Note: RI refers to the allelopathic reaction index, and a positive value represents promotion, a negative value represents inhibition. The data were presented as the mean ± standard error (n=5). Values within a column not followed by the same letter are significantly different at $p \leq 0.05$.

Though, some investigators question whether the root exudates would be degraded by microbes so that there is no impact or concentrations present in the collected root exudates. Thus, we also did another experiment to justify the results of the first experiment under aseptic conditions to avoid interruption by microbes. The results confirmed the conclusion of the first experiment, which showed that garlic root exudates revealed basic 'promotion at lower and suppression at higher' dose-dependent effects according to the RI value, in both normal and aseptic conditions. Thus, the allelopathy of plant root exudates strengthens with increased concentrations because of the accumulation of allelochemicals; root exudates are stable and hardly degraded by microbes under normal conditions. A similar effect was previously reported in other plants (Bogatek *et al.*, 2006; Pramanik *et al.*, 2001). These results were further confirmed by Zhou *et al*. (2007), who studied root exudates of two garlic cultivars and observed positive effects on lettuce seedling growth at lower concentrations (0.1 and 0.2 g/ml) but exhibited inhibitory effects at high concentrations (0.4 and 0.6 g/ml). Han *et al.* (2013) also reported that lower concentrations of decomposed garlic stalk indorsed the lettuce growth and productivity, but higher concentrations inhibited its growth (Han *et al.*, 2013). A possible reason could be that appropriate concentration of garlic root exudates could promote the absorption of mineral elements and enhance the assimilation of nutrients (Xiao *et al.*, 2013). Moreover, the exudates can take part in the biosynthetic pathway of chlorophyll and promote its content in plants consequently high photosynthates, more energy and dry matter addition in the tested pepper plants (Shi *et al.*, 2009). Moreover, it cannot be overlooked that garlic root exudates may regulate the contents of endogenous hormones, which possess potential influence on the growth of lettuce (Oracz *et al.*, 2012). However, high concentrations of allelochemicals in garlic root exudates may cause irreversible damage to the cell membrane system, leading to ROS burst and membrane peroxidation. Thus, it can be inferred that higher concentrations of garlic root exudates induced stress conditions in lettuce plants due to the high concentrations of allelochemicals.

A multitude of methods have been used for collecting plant root exudates, such as soil, sand and another media, hydroponic culture systems or a successive root exudate collection system, and each system has its benefits and limitations (Cheng & Xu, 2013). The biological assay is widely used for detecting the allelopathic effect of plants, and the delay or reduction of seed germination and root-shoot inhibition are typically the first visible symptoms (Dayan *et al.*, 2000; Wu *et al.*, 2007). In the present study, garlic root exudates were collected by hydroponic culture, which is convenient and proven reliable in the extraction of garlic root exudates (Khan *et al*., 2011). Moreover, seed germination is usually crucial stage during seeding establishment; the delay or reduction of seed germination and inhibition of root and shoot growth represent the main symptoms of phytotoxic stress (Oracz *et al.*, 2007; Hubbard *et al.*, 2012).

Conclusions

Our study showed that the allelopathic effects of garlic root exudates showed significant differential responses and played a direct or indirect role in overall growth indexes under laboratory conditions. Both garlic root exudates and its organic extracts had a remarkable allelopathic effect on lettuce, especially the petroleum ether (PE) extract, which exerted the strongest inhibitory allelopathic potential at critical concentrations. Moreover, garlic root exudates revealed a basic ‘promotion at lower and suppression at higher’ dose-dependent effect on both lettuce and cucumber. These findings may suggest an approach to collect and isolate garlic root exudates and provide a clue about the identification of active components in the three extracts of garlic root exudates, especially the PE extract.

Acknowledgements

This work was supported by the National Natural Science Foundation of China under Grant (No. 31171949 and No. 31471865). We would like to thank the said agency for financing the project.

References

Ahmad, I., Z.H. Cheng, H.W. Meng, T.J. Liu, M.Y. Wang, M. Ejaz and H. Wasila. 2013a. Effect of pepper-garlicintercropping system on soil microbial and bio-chemical properties. *Pak. J. Bot.*, 45: 695-702.

Ahmad, I., Z.H. Cheng, H.W. Meng, T.J. Liu, M.Y. Wang, M. Ejaz, H. Wasila and A. Khan. 2013b. Effect of Intercropped garlic (*Allium sativum* L.) on chlorophyll contents, photosynthesis and antioxidant enzymes in pepper. *Pak. J. Bot.*, 45: 1889-1896.

Ali, A., M. Arshad, S.M.S. Naqvi, M. Ahmad, H. Sher, S. Fatima,A. Gul Kazi, A. Rasheed and A. Mujeeb-Kazi. 2014. Exploitation of synthetic-derived wheats through osmotic stress responses for drought tolerance improvement. *Acta. Physiol. Plant.*, 36: 2453-2465.

Alias, J.C., T. Sosa, J.C. Escudero and N. Chaves. 2006. Autotoxicity against germination and seedling emergence in *cistus ladanifer* L. *Plantsoil*., 282: 327-332.

Bogatek, R., A. Gniazdowska, W. Zakrzewska, K. Oracz and S. Gawronski. 2006. Allelopathic effects of sunflower extracts on mustard seed germination and seedling growth. *Biol. Plantarum*., 50: 156-158.

Bruce-Williamson, G. and D. Richardson. 1988. Bioassays for allelopathy: measuring treatment responses with independent controls. *J. Chem. Ecol.*, 14: 181-7.

Cheema, Z.A., M. Farooq, A. Wahid and S. Ambika. 2013. *Allelopathy*. Springer, Berlin.

Chen, Y. 2004. *The Constituents of Phytochemistry*. Chemical Industry Press,Beijing.

Cheng, Z.H. and P. Xu. 2013. Lily (*Lilium* Spp.) root exudates exhibit different allelopathies on four vegetable crops. *Acta Agric. Scand. B.*, 63: 169-175.

Chon, S.U. and J. Kim. 2002. Biological activity and quantification of suspected allelochemicals from alfalfa plant parts. *J. Agron. Crop Sci.*, 188: 281-285.

Dayan, F.E., J.G. Romagni and S. Duke. 2000. Investigating the mode of action of natural phytotoxins. *J. Chem. Ecol.*, 26: 2079-2094.

Ding, H.Y. and Z.H. Cheng. 2014. Research progress on garlic allelopathy and its utilization. *Chinavegetables.*, 9: 11-16.

Ding, H.Y., Z.H. Cheng, M.L. Liu, S. Hayat and H. Feng. 2016. Garlic exerts allelopathic effects on pepper physiology in a hydroponic co-culture system. *Biol. Open*., 5: 631-637.

Han, X., Z.H. Cheng, H.W. Meng, X. Yang and I. Ahmad. 2013. Allelopathic effect of decomposed garlic (*Allium sativum* L.) stalk on lettuce (*L. sativa* var. *crispa* L.). *Pak. J. Bot.*, 45: 225-233.

Hierro, J.L. and R. Callaway. 2003. Allelopathy and exotic plant invasion. *Plant Soil*, 256: 29-39.

Hubbard, M., J. Germida and V. Vujanovic. 2012. Fungal endophytes improve wheat seed germination under heat and drought stress. *Botany*., 90: 137-149.

Iciek, M., I. Kwiecien and L. Wlodek. 2009. Biological properties of garlic and garlic-derived organosulfur compounds. *Environ. Mol. Mutagen.*, 50: 247-265.

Inderjit, C.R.M. and J.M. Vivanco. 2006. Can plant biochemistry contribute to understanding of invasion ecology? *Trend. Plant Sci.*, 11: 574-80.

Kato-Noguchi, H. 2011a. Convergent or parallel molecular evolution of momilactone A and B: potent allelochemicals, momilactones have been found only in rice and the moss hypnum plumaeforme. *J. Plant. Physiol.*, 168: 1511-1516.

Kato-Noguchi, H. 2011b. The chemical cross talk between rice and barnyard grass. *Plant. Signaling & Behavior*., 6: 1207 1209.

Kato-Noguchi, H. and R. Peters. 2013. The role of momilactones in rice allelopathy. *J. Chem. Ecol.*, 39: 175-185.

Kato-Noguchi, H., K. Ota and H. Kujime. 2012. Absorption of momilactone A and B by *Arabidopsis Thaliana* L. and the growth inhibitory effects. *J. Plant. Physiol.*, 169: 1471-1476.

Kato-Noguchi, H., Y. Yasuda and R. Sasaki. 2011. Anoxia tolerance and sugar level in roots and coleoptiles of aerobically germinated rice. *Plant. Prod. Sci.*, 14: 325-330.

Khan, M.A. and Z.H. Cheng. 2010. Influence of garlic root exudates on cyto-morphological alteration of the hyphae of *phytophthora capsici*, the cause of phytophthora blight in pepper. *Pak. J. Bot.*, 42: 4353-4361.

Khan, M.A., Z.H. Cheng, X.M. Xiao, A.R. Khan and S. Ahmed. 2011. Ultrastructural studies of the inhibition effect against *phytophthora capsici* of root exudates collected from two garlic cultivars along with their qualitative analysis. *Crop. Prot.*, 30: 1149-1155.

Liu, S.H., S.Q. Liu, Z.K. Zhang, H. Wei, Z.J. Huang and Y. Zhang. 2011. Inhibition effect of garlic root exudates on the genus allium. *Sci. Agri. Sinica*., 44: 2625-2632.

Liu, T.J., Z.H. Cheng, H.W. Meng, I. Ahmad and H.L. Zhao. 2014. Growth, yield and quality of spring tomato and physicochemical properties of medium in a tomato/garlic intercropping system under plastic tunnel organic medium cultivation. *Sci. Hortic-Amsterdam*., 170: 159-168.

Ni, L., X. Hao, S. Li,S. Chen, G. Ren and L. Zhu. 2011. Inhibitory effects of the extracts with different solvents from three compositae plants on cyanobacterium *Microcystis aeruginosas*. *Sci. China. Chem*., 54: 1123-1129.

Oburger, E., M. Demour, S. Hann, G. Wieshammer, M. Puschenreiter and W. Wenzel. 2013. Evaluation of a novel tool for sampling root exudates from soil-grown plants compared to conventional techniques. *Environ. Exp. Bot.*, 87: 235-247.

Oracz, K., C. Bailly, A. Gniazdowska, D. Come, D.F. Corbineau and R. Bogatek. 2007. Induction of oxidative stress by sunflower phytotoxins in germinating mustard seeds. *J. Chem. Ecol.*, 33: 251-264.

Oracz, K., A. Voegele, D. Tarkowska, D. Jacquemoud, V. Tureckova, T. Urbanova, M. Strnad, E. Sliwinska and G. Leubner-Metzger. 2012. Myrigalone a inhibits *Lepidium sativum* seed germination by interference with gibberellin metabolism and apoplastic superoxide production required for embryo extension growth and endosperm rupture. *Plant Cell. Physiol.*, 53: 81-95.

Pandhair, V. and B.S. Sekhon. 2006. Reactive oxygen species and antioxidants in plants: an overview. *J. Plant. Biochem. Biot.*,15:71-78.

Pramanik, M., T. Asao, T. Yamamoto and Y. Matsui. 2001. Sensitive bioassay to evaluate toxicity of aromatic acids to cucumber seedlings. *Allelopathy. J.*, 8: 161-169.

Rehman, S.U., R.Faisal, Z.K. Shinwari, N. Ahmad and I. Ahmad. 2017. Phytochemical screening and biological activities of *Trigonella incisa* and Nonea edgeworthii. *Pak. J. Bot.*, 49(3): 1161-1165.

Shi, D., Z. Liu and W.W. Jin. 2009. Biosynthesis, catabolism and related signal regulations of plant chlorophyll. *Yichuan*., 31: 698-704.

Shi, Y., Y. Zhang, H.J. Yao, J.W. Wu, H. Sun and H.J. Gong. 2014. Silicon improves seed germination and alleviates oxidative stress of bud seedlings in tomato under water deficit stress. *Plant Physiol. Biochem.*, 78: 27-36.

Siddiqui, Z.S. 2007. Allelopathic Effects of black pepper leachings on *Vigna mungo* (L.) hepper. *ActaPhysiol. Plant.*, 29: 303-308.

Singh, I. and S. Duke. 2003. Ecophysiological aspects of allelopathy. *Planta*., 217: 529-539.

Tchone, M., G. Baerwald, G. Annemueller and L. Fleischer. 2006. Separation and identification of phenolic compounds in jerusalem artichoke (*Helianthus tuberosus* L.). *Sci. Des. Aliments*., 26: 394-408.

Wang, M.Y., C.N. Wu, Z.H. Cheng and H.W. Meng. 2015. Growth and physiological changes in continuously cropped eggplant (*Solanum melongena* L.) upon relay intercropping with garlic (*Allium sativum* L.). *Front. Plant Sci.*, 6.

Wang, M.Y., C.N. Wu, Z.H. Cheng, H. Meng, M.R. Zhang and H.J. Zhang. 2014. Soil chemical property changes in eggplant/garlic relay intercropping systems under continuous cropping. *PLoSOne*., 9(10): e111040.

Wang, M.Y., C.N. Wu, F. Yang, Z.H. Cheng and H.W. Meng. 2015. Growth, chlorophyll content and combined output value in eggplant/garlic relay intercropping systems. *Pak. J. Bot.*, 47: 1727-1734.

Wu, H., J. Pratley, D. Lemerle, M. Anand D.L. Liu. 2007. Autotoxicity of wheat (*Triticum aestivum* L.) as determined by laboratory bioassays. *Plant Soil*., 296: 85-93.

Xiao, X.M., Z.H. Cheng, H.W. Meng, M.A. Khan and H.Z. Li. 2012. Intercropping with garlic alleviated continuous cropping obstacle of cucumber in plastic tunnel. *Acta. Agric. Scand. B.*, 62: 696-705.

Xiao, X.M., Z.H. Cheng, H.W. Meng, H.L. Liu, H.Z. Li and Y.X. Dong. 2013. Intercropping of green garlic (*Allium sativum* l.) induces nutrient concentration changes in the soil and plants in continuously cropped cucumber (*Cucumis sativus* L.) in a plastic tunnel. *PLoS One.*, 8(4): e62173.

Xu, N., M. Wei, C. Wang, W. Shi, F. Tian and X.F. Wang. 2013. Composition of welsh onion (*Allium fistulosum* L.) root exudates and their allelopathy on cucumber sprouts and *Fusarium oxysporum F.Sp cucumerinum*. *Allelopathy. J.*, 32: 243-256.

Yu, J.Q., S.F. Ye, M.F Zhang and W.H. Hu. 2003. Effects of root exudates and aqueous root extracts of cucumber (*Cucumis sativus*) and Allelochemicals, on photosynthesis and antioxidant enzymes in cucumber. *Biochem. Syst. Ecol.*, 31: 129-139.

Zhang, Y., M. Gu, K. Shi, Y.H. Zhou and J.Q. Yu. 2010. Effects of aqueous root extracts and hydrophobic root exudates of cucumber (*Cucumis sativus* L.) on nuclei DNA content and expression of cell cycle-related genes in cucumber radicles. *Plant Soil*., 327: 455-463.

Zhou, Y.L., Z.H. Cheng and H.W. Meng. 2007. Allelopathy of garlic root exudates on different receiver vegetables. *Chinese. J. Applied. Ecol*., 1: 81-86.

(Received for publication 9 January 2017)