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REGULAR ARTICLE

Allelopathic effect of β-cembrenediol and its mode of action: Induced oxidative stress in lettuce seedlings

Xia Ren[1,2], Zhiqiang Yan[1], Xiaofeng He[1], Xiuzhuang Li[1], Bo Qin[1]*

[1]Key Laboratory of Chemistry of Northwestern Plant Resources of CAS and Key Laboratory for Natural Medicine of Gansu Province, Lanzhou Institute of Chemical Physics, Chinese Academy of Sciences, Lanzhou, China, [2]University of Chinese Academy of Sciences, Beijing, China

ABSTRACT

β-cembrenediol ((*1S,2E,4R,6R,7E,11E*)-2,7,11-cembratriene-4,6-diol), shown to be one of the most important allelochemicals of tobacco in previous studies, effectively inhibited the root and stem growth of receptor plants and the inhibitory effects was concentration-dependent. However, its mechanism of action remains unclear. Seedlings of lettuce (Tai Yuan Sun) were treated with β-cembrenediol to clarify its mode of action. Results showed that β-cembrenediol significantly inhibited the seedling growth and reduced fresh weight of *L. sativa*. The compounds effectively affected cell mitotic index and caused cell death. Exposure to β-cembrenediol induced overproduction of reactive oxygen species (ROS). Moreover, increased content of hydrogen peroxide, malondialdehyde, and proline, and decreased in chlorophyll content indicated lipid peroxidation and induction of oxidative stress. These results suggested that β-cembrenediol caused oxidative damage through enhanced generation of ROS, as indicated by increased lipid peroxidation, disruption of membrane integrity and impacted mitosis, ultimately resulted in growth inhibition of the receptor plant.

Keywords: β-Cembrenediol; Phytotoxicity; Lettuce; Oxidative stress; Reactive oxygen species

INTRODUCTION

Allelopathy is a natural ecological phenomenon which involves the release of plant-produced secondary products (allelochemicals) into the environment by volatilization, root exudation, decomposition and/or leaching (Pan et al., 2015). It influences the growth and development of neighboring plants or itself (Cruz-Ortega et al., 2007; Babula et al., 2009; Rial et al., 2014; Kimura et al., 2015; Pan et al., 2015). Many aspects of plant physiological and biochemical processes, such as the cell cycle, phytohormone metabolism, reactive oxygen species generation and plant photosynthesis could thus be affected (Weir et al. 2004; Gniazdowska and Bogatek., 2005; Ding et al.,2007; Babula et al., 2009; Ding et al., 2016). Recent findings showed that allelochemical stress was similar in action to that of pathogens (biotic stress) or herbicides (abiotic stress), in that it increases the concentration of reactive oxygen species (ROS) in plant cells (Golisz et al., 2007; Su et al., 2016). The ROS subsequently leads to oxidative damage, increases the degree of membrane lipid peroxidation, and ultimately results in the death of cells (Ye et al., 2006; Yan et al., 2015).

The tobacco plant, a member of the Solanaceae family, is an important cultured and economic crop over the world. Extracts of this plant have been reported to have a wide range of bioactivities, such as anti-cancer, anti-inflammatory, anti-invasive and anti-microbial (Sayed et al., 2008; Olsson et al., 1993; Ferchmin et al., 2009; Eterović et al., 2013). In recent years, the plant has attracted an increasing attention for its strong allelopathy. It is considered that the homogeneity of both allelochemicals and chemical compositions of tobacco is the major reason for its allelopathic effect (Zhang et al., 2011). Various known allelopathic secondary metabolites including diterpenoids, alkaloids, flavonoids, coumarins, lignans and aliphatics, are synthesized during plant growth and development (Xia et al., 2014). A crude extract of tobacco rhizospheric soil significantly inhibited the seed germination as well as root and shoot growth of lettuce and tobacco (Chen et al., 2012; Yu et al., 2014). In earlier studies, it was determined the specific allelochemicals of the extract from rhizosphere soil of tobacco and studied their biotoxicity (Ren et al., 2015). By showing inhibitory effects against lettuce and tobacco seedlings and being detected

***Corresponding author:**
Bo Qin, Key Laboratory of Chemistry of Northwestern Plant Resources of CAS and Key Laboratory for Natural Medicine of Gansu Province, Lanzhou Institute of Chemical Physics, Chinese Academy of Sciences, Lanzhou 730000, China. E-mail: bqin@licp.cas.cn

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in the root zone soils of tobacco, β-cembrenediol was recognized as an important and main allelochemical (Ren et al., 2015). However, the detailed mechanism of action for the phytotoxicity of β-cembrenediol remained unknown. Hence the aim of this study was to elucidate the phytotoxic action of β-cembrenediol in *L. sativa* seedlings by examining changes in seeding growth and oxidative stress, measured in terms of ROS-induced lipid peroxidation, hydrogen peroxide generation and chlorophyll and proline content. To existent knowledge, this is the first report on such a study of the phytotoxicity of β-cembrenediol.

MATERIALS AND METHODS

Chemicals

All reagents were of A.R. grade and used without purification. β-Cembrenediol was isolated according to previous procedures (Ren et al., 2015). It was characterized (Fig. 1), by ^{1}H and ^{13}C NMR performed on a Bruker AM-400BB instrument (Bruker, Karlsruhe, Germany) with TMS as internal standard, operating at 400 MHz.

Chemical treatment

Lettuce (*L- sativa*) seeds cv. 'Tai Yuan Sun', purchased from Gansu Academy of Agriculture Sciences, were soaked in 10% hypochlorite for 7 min, washed 5 times with distilled water and then transferred to a Petri dish (ξ = 9 cm) with filter paper (Qualitative, Whatman-Xinhua, Hangzhou, China). They were germinated in a growth chamber with a photoperiod of 16/8 h day/night at 22°C. After 48 h, seedlings of similar size were used for all experiments.

β-Cembrenediol was dissolved in dimethylsulfoxide (DMSO) at 800 μM as a stock solution and diluted with distilled sterile water to 400 μM, 200 μM, 100 μM, 50 μM. Equivalent volumes of DMSO were added to the control group. The final DMSO concentration did not exceed 0.1 % (v/v). Pre-germinated lettuce seedlings were transferred into a 6-well plate (NUNC, Shanghai, China) with three seedlings per well and three wells in each treated group and 1.0 mL of treatment solution was applied on it. The plates were then incubated in a growth chamber with a photoperiod of 16/8 h day/night at 25°C. The experiment was performed using three Petri dishes as three replicates. After 48 h, seedlings were harvested and their root and shoot lengths were measured.

Mitotic index (MI)

Lettuce seedlings were treated as described above. The root tips were fixed, macerated, stained and squashed as described previously by Yan et al (2015) with some modifications. After treatment with β-cembrenediol, roots of lettuce seedlings were collected and immediately fixed in freshly prepared Carnoy's fluid (ethanol/acetic acid, 3/1, v/v) for 24 h, then hydrolyzed for 2 min in 1M HCl at 60°C. Schiff's reagent (Leagene, Beijing, China) was used to stain the chromosomes for 30 min followed by rinsing in distilled water (three to four times). Root tips (about 1.0 mm) were cut off and squashed to separate the cells. Cells were observed using an inverted microscope (Shanghai, Shanghai, China) equipped with a digital camera (Panasonic, Osaka, Japan) and 1000 cells were analyzed per treatment with three repeats. The MI was calculated as percentages between the number of cells in mitosis and the total number of cells observed. The proportions of cells in prophase, metaphase, anaphase and telophase were also calculated.

Cell death

Cell death was evaluated by staining with Evans blue. After 48 h of β-cembrenediol treatment, distal fragments of root tips (1.0 cm) of lettuce seedlings were cut and stained with 0.25% (w/v) Evans blue (Solarbio, Beijing, China) for 1h at room temperature and then washed with distilled H_2O for 30 min. The stained roots were extracted using N, N-dimethylformamide (1.0 mL) for 24 h at 30°C in the dark, and absorbance of the released Evans blue was measured at 600 nm by a spectrophotometer (Shimadzu Corp., Kyoto, Japan).

ROS production

ROS production was assayed according to the procedure of Pan et al (2015) with modifications. 2', 7'-Dicholrofluorescein diacetate (DCFH-DA) was used to determinate reactive oxygen species. After the treatment with the β-cembrenediol at 25°C for 48 h, the lettuce seedlings were washed with distilled water five times and transferred into a dye solution of DCFH-DA (20 μmol/L, 1% DMSO) and stained for 20 min at room temperature in the dark. The samples were then soaked in distilled water to remove residue dyes and the roots were excised to for observation under a fluorescence microscope (Olympus FV1200, excitation 488 nm and emission > 520 nm).

Hydrogen peroxide

Hydrogen peroxide content was measured according to the procedure of He et al (2005). The roots and shoots of lettuce seedlings were homogenized in cold acetone (1.0 mL) in an ice bath, followed by centrifugation at 8000 g for 10 min. Then, the supernatant was mixed with 0.1 ml 20% titanium reagent and 0.2 ml 17 mol ammonia solutions. The supernatant was replaced by acetone in control group. The mixture was centrifuged at 4000 g at 25°C for 10 min and the supernatant was discarded. The sediment was dissolved in 1.0 mL 1 mol sulfuric acid and the absorbance of the solution was measured at 415 nm. Absorbance values were calibrated to a standard curve generated with known concentrations of H_2O_2.

Lipid peroxidation

Lipid peroxidation was determined by measurement of the formation of malonyldialdehyde (MDA) according to Hodges et al (1999), which is a product of lipid peroxide decomposition content. The roots and shoots of lettuce seedlings were homogenized in 3.0 mL trichloroacetic acid (TCA, 10%, w/v) in an ice bath, followed by centrifugation at 8000 g for 10 min. Then, 1.0 mL of the supernatant was added to 2.0 mL of thiobarbituric acid (TBA, 0.6%, w/v) in 10% TCA. The mixture was incubated in boiling water for 30 min and the reaction stopped by placing the reaction tubes in an ice bath. After centrifugation at 10000 g for 10 min, the absorbance of the supernatant was measured at 440, 532 and 600 nm, and the levels of MDA were calculated by the following formula:

$$MDA = [6.452(A532-A600)-0.56A450] \bullet V_T/V \bullet W$$

[MDA] represents the concentration of MDA expressed in μM/g. A450, A532 and A600 are the absorbance values at 450, 532 and 600 nm, respectively. V_T and V represent total volume of the extracting solution and the volume used in measurement, respectively. W is the fresh weight of the lettuce tissues used.

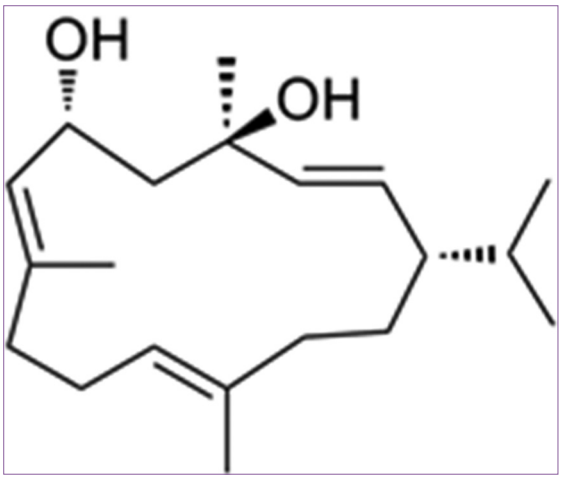

Fig 1. Structure of β-cembrenediol.

Free proline

After treated with β-cembrenediol, about 100 mg samples (lettuce roots and shoots) were used to determined the concentrations of the free proline. The concentrations of the free proline were measured by kites test methods (Comin biotechnology Co. Ltd, Suzhou, China).

Chlorophyll content

After 48 h of β-cembrenediol treatment under a 16/8 h day/night photoperiod at 25°C, fresh lettuce leaves (about 50mg) were homogenized in 4.0 mL of 80% acetone and centrifuged at 2000 g for 5 min. The absorbance of the supernatant was measured at 663 and 645 nm and the results were recorded according to Wellburn (1994).

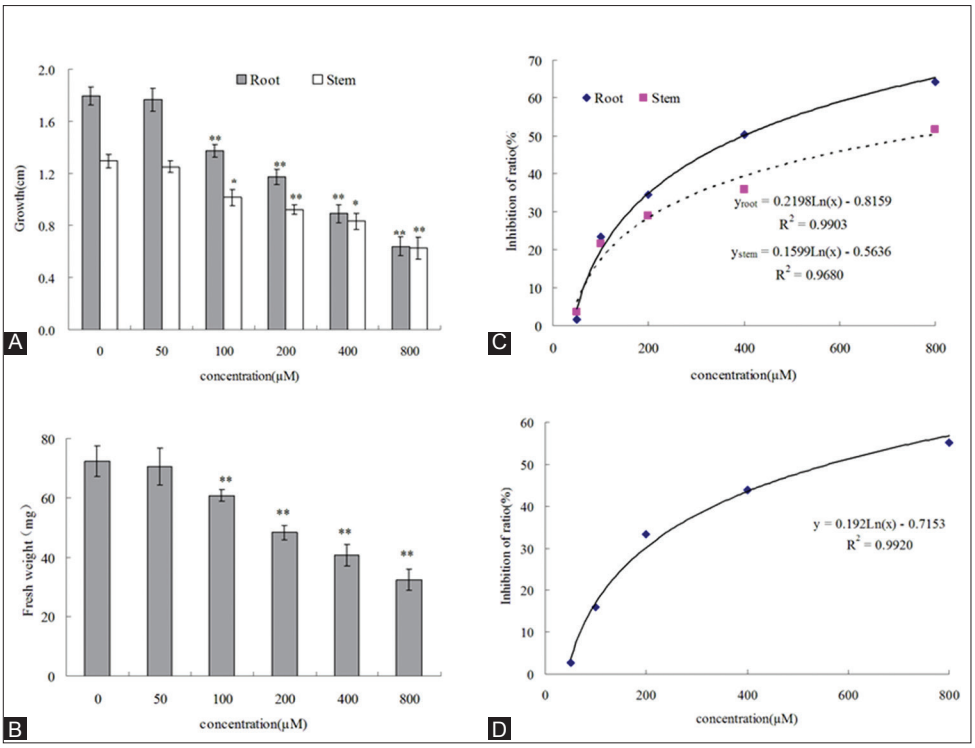

Fig 2. Root and shoot elongation (A), the fresh weight (B), the inhibition ratio of growth (C) and fresh weight (D) of lettuce (*Lactuca sativa*) cv. 'Tai Yuan Sun' after treated by β-cembrenediol for 48 h. Values are presented as mean percentage of the control (±SD). Means significantly lower than the DMSO controls are indicated with one asterisk (*) ($p < 0.05$) or two asterisks (**) ($p < 0.01$).

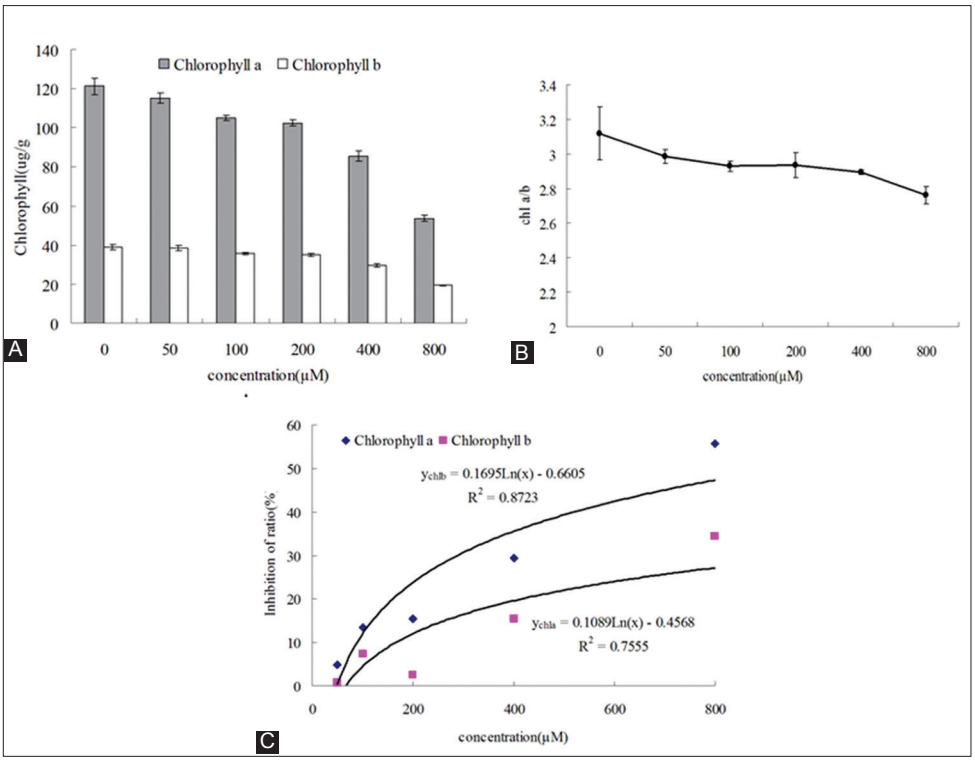

Fig 3. Effects of β-cembrenediol on chlorophyll levels (A), root/shoot ratio (B) and the inhibition ratio of chlorophyll (C) of lettuce (*Lactuca sativa*) cv. 'Tai Yuan Sun' seedlings. Values are presented as mean percentage of the control. Means significantly lower than the DMSO controls are indicated with an asterisk (*) ($p < 0.05$) or double asterisks (**) ($p < 0.01$).

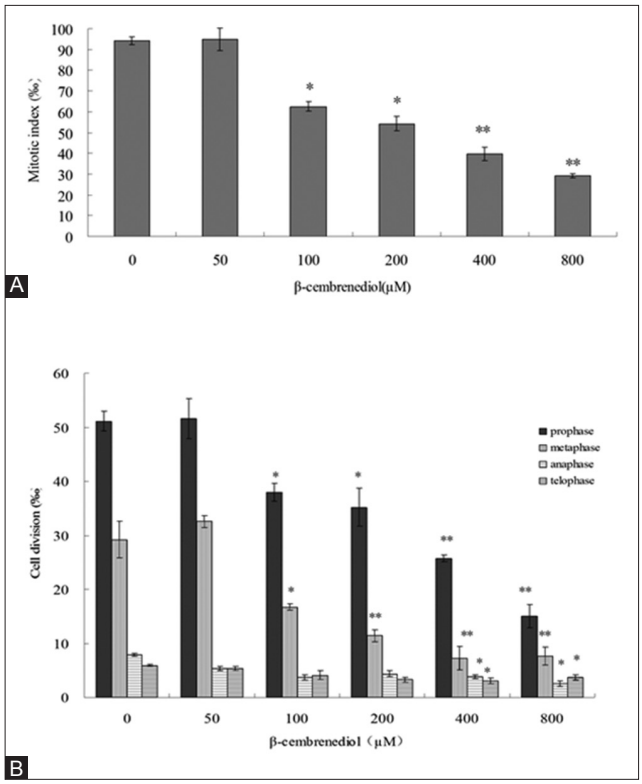

Fig 4. Mitotic index (A) and mitosis process (B) in lettuce (*Lactuca sativa*) cv. 'Tai Yuan Sun' root tips after treatments with β-cembrenediol for 48 h. The results presented are mean of three replicates ± SE, * and ** represent the significant difference at $p < 0.05$ and 0.01 respectively as compared to control.

Statistical analysis

All data were subject to an analysis of variance using SPSS Statistics 18. Each value was expressed as the mean ± standard error (SE). The significant differences between the treatments and control were calculated using a one-way analysis of variance (ANOVA) followed by a Fisher's least significant difference (LSD) test.

RESULTS

Effects of β-cembrenediol on the growth and fresh weight of *L. sativa* seedlings

β-Cembrenediol significantly inhibited the growth and fresh weight of *L. sativa* seedlings. The logarithm models between in concentration and ratio of root and shoot inhibition were of good quality (R^2 ranged from 0.9680 to 0.9920) to showed the inhibitory effects were concentration-dependent (Fig. 2B and D). As shown in Fig. 2A, β-cembrenediol inhibited the root and shoot elongation of lettuce seedlings. When the concentration of β-cembrenediol up to 200 μM, root and shoot lengths were inhibited by more than 30% in contrast to the control. Further, β-cembrenediol significantly reduced the fresh weight of *L. sativa* seedlings. The fresh weights of lettuce seedlings treated with β-cembrenediol at 50-800 μM were reduced by 2.65%-56.1% compared to the control.

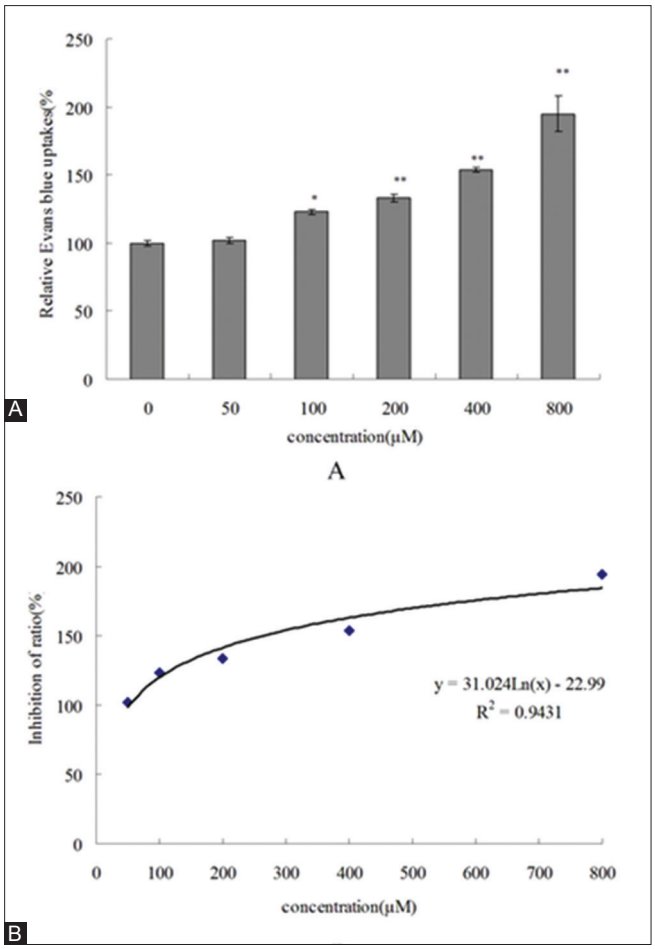

Fig 5. Relative Evans blue uptake (A) and the inhibition ratio (B) in lettuce (Lactuca sativa) cv. 'Tai Yuan Sun' roots after treated with β-cembrenediol for 48 h. The results presented are mean of three replicates ±SE, * and ** represent the significant difference at $p < 0.05$ and 0.01 as compared to control.

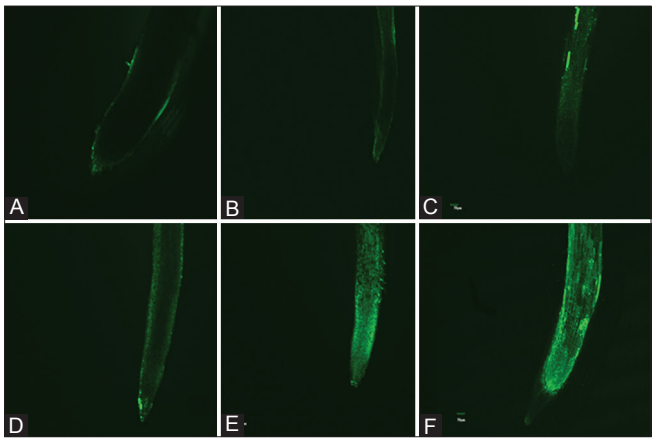

Fig 6. Effects of β-cembrenediol on ROS production in lettuce root tips. The lettuce were treated at the concentrations of 0 (A), 50 (B), 100 (C), 200 (D), 400 (E) and 800 (F) μM for 48 h, then stained with DCFH-DA. The bright green fluorescence shows the ROS.

Effects of β-cembrenediol on chlorophyll content

After β-cembrenediol treatment, the levels of chl *a*, *b* in *L. sativa* leaves both declined in a concentration-dependent manner according to the logarithmic models (R^2 ranged from 0.7555 to 0.8723) (Fig. 3). The contents of chl *a*, *b* were approximately 42.2 and 49.9% of the control after exposure to 800 μM β-cembrenediol, respectively. The chl a/b ratio was greatly reduced when treated with β-cembrenediol at 400 μM (Fig. 3).

Effects of β-cembrenediol on cell division in root tips of *L. sativa*

The mitotic index (MI) of cell division in *L. sativa* root tips was investigated with Schiff's reagent staining. β-Cembrenediol significantly decreased the MI in *L. sativa* root tip cells at concentrations of 100, 200, 400, 800 μM after treated for 48h (Fig. 4A). Moreover, the number of cells of each mitotic phase was reduced with increasing concentration of β-cembrenediol (Fig. 4B).

Effects of β-cembrenediol on cell death in root tips of *L. sativa*

As only dead cells can retain Evans blue, the higher Evans blue content in the plant indicated the drastic induction of cell death. Fig. 5 shows that the Evans blue uptake after 48h was increased significantly at concentrations of 100 μM and above.

Effects of β-cembrenediol on ROS production

Production of ROS in *L. sativa* roots was monitored by staining roots with 2', 7'-Dicholrofluorescein diacetate. Slight fluorescence was seen in the roots at low concentrations (≤100 μM). With increasing concentration, the fluorescence intensity increased significantly, suggesting that β-cembrenediol induced ROS in the root tips of lettuce (Fig. 6).

Effects of β-cembrenediol on H_2O_2 concentration and MDA content

A progressive increase in H_2O_2 content was found in roots and shoot under β-cembrenediol, thus confirming the oxidative stress of the by-product (Fig. 7A). After treated with β-cembrenediol, MDA level was increased in both roots and shoots, which indicated β-cembrenediol-treated could enhance lipid peroxidation (Fig. 7B).

Effects of β-cembrenediol on Proline accumulation

In plants treated with β-cembrenediol at a low concentration, proline level was the same as the control (Fig. 8), but the levels of proline significantly increased in both roots and shoots when treated with at 100 μM and above (Fig. 8).

DISCUSSION

Cembranoids are important secretion in surface of tobacco leaf and flower (He et al. 2016). β-Cembrenediol and its stereoisomer α-cembrenediol are the main cembranoids;

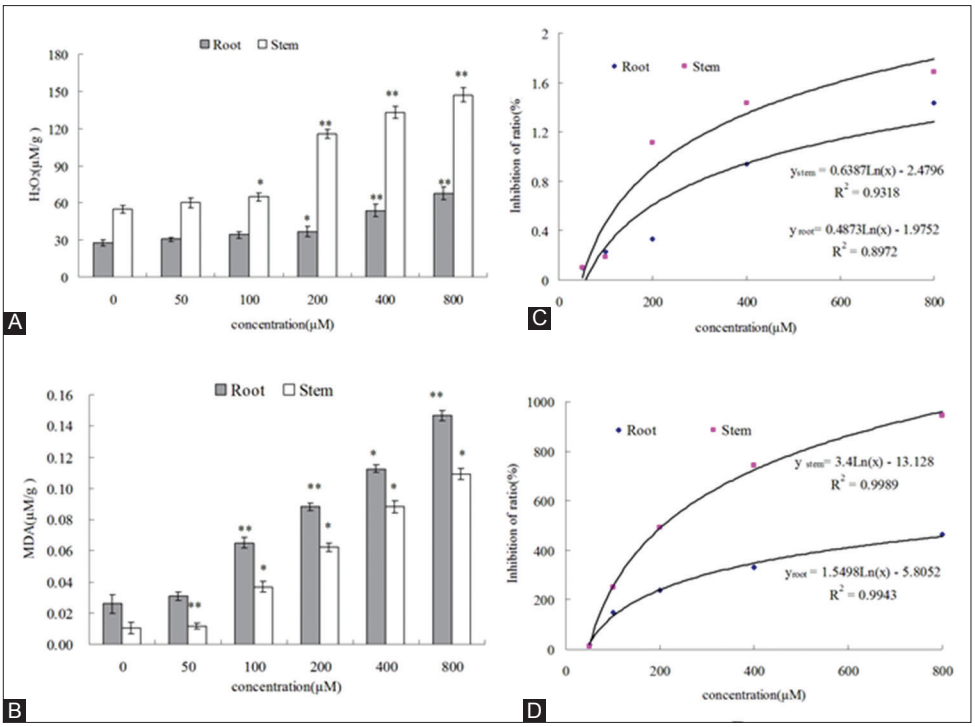

Fig 7. H2O2 content (A), MDA (B) content, the inhibition ratio of H2O2 (C) and MDA (D) in lettuce (*Lactuca sativa*) cv. 'Tai Yuan Sun' seedlings after treatments with β-cembrenediol for 48 h. The results presented are mean of three replicates ±SE. Means significantly lower than the controls are indicated with one asterisk (*) ($p < 0.05$) or two asterisks (**) ($p < 0.01$).

the two compounds were 60% of the total cuticular secreta of tobacco leaf and flower (Johnson et al.,1985). Recent studies indicated that β-cembrenediol played an allelochemical-affecting growth of *L. sativa* and tobacco itself (Ren et al., 2015). In this study, lettuce seedlings were used as a receptor to illustrate the action mechanism for the phytotoxicity of β-cembrenediol.

It was found that β-cembrenediol significantly inhibited the seeding growth and reduced fresh weight of *L. sativa*. Although the specific mechanism for the inhibitory effect of terpenes is still not completely understood, many researches have indicated that terpenoids (beta-pinene, alpha-pinene, and camphene) inhibited mitotic index and DNA synthesis in root apical meristem (Nishida et al., 2005). After treatment with β-cembrenediol, mitotic indexes and cells in different stage of mitosis decreased in roots. It could be attributed to the possible disturb a certain period of cell cycle and related changes such as DNA synthesis, and then can prevent cells from entering into mitosis (Planchais et al., 2000).

When exposed to stressful conditions, such as sub-optimal temperature, high light, salt, or pathogen infection, plants can generate more ROS (Yamamoto et al., 2003; Halliwell., 2006; Rhoads et al., 2006). These ROS's affect many different processes in plant, such as growth and development, programmed cell death (PCD) (Mittler et al., 2004). The generation and clearing of ROS play an important role in alleleptahy (Cheng and Cheng, 2015). Recent researches indicated that the ROS can be induced by allelochemicals in target plants and therefore cause oxidative damage (Cruz-Ortega et al., 2007; Shearer et al., 2012; Yan et al., 2015). In this research, the production of ROS in lettuce roots was investigated using a specific dye, DCFH-DA. After treatment, florescence was constantly strengthened with increasing concentration, indicating that β-cembrenediol generated ROS production in the root tips of *L. sativa*. Excessive ROS can cause oxidative damage to proteins, DNA, photosynthetic pigments and lipids, generate lipid peroxidation in the cell membranes, and induce programmed cell death (PCD) processes (Apel and Hirt., 2004; Sunohara et al., 2008; Goraya and Asthir, 2016). Various indicators (such as photosynthetic pigments, MDA, proline and H_2O_2 content) related to oxidative stress were evaluated in roots and shoots of *L. sativa* in order to study whether β-cembrenediol produces a similar response.

When treated with β-cembrenediol at higher concentrations, the level of MDA increased markedly in both lettuce roots and shoots, in agreement with the overproduction of ROS as shown with DCFH-DA. The lipid peroxidation can cause changes in the permeability and fluidity of the membrane lipid bilayer and can dramatically alter cell integrity (Dix and Aikens, 1993; Yan et al., 2015; Cheng and Cheng, 2015). The rates of dead cells in the

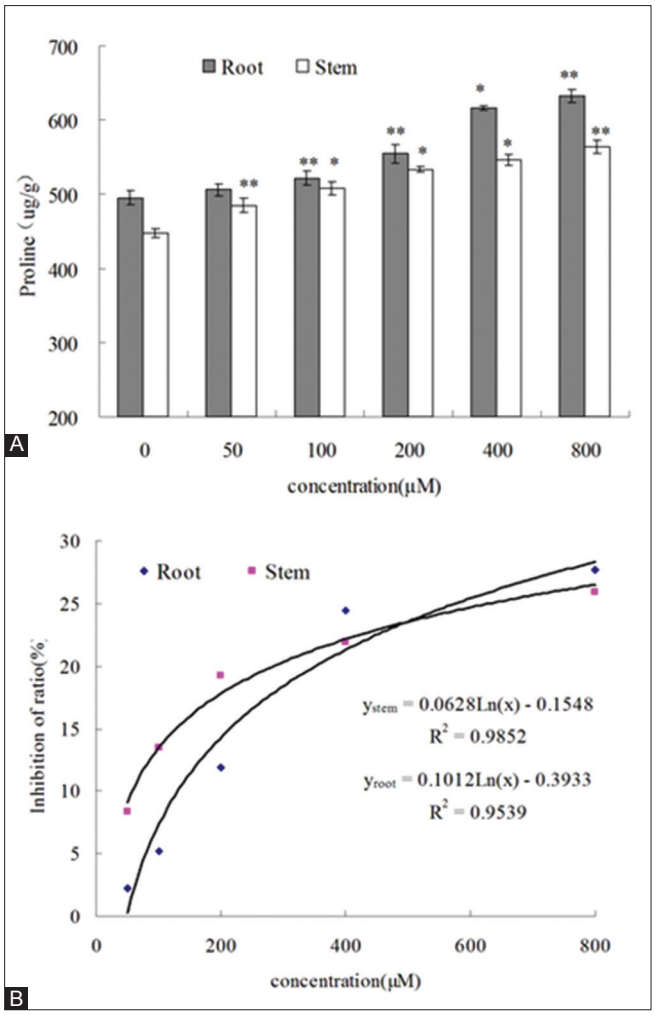

Fig 8. Proline content (A) and the inhibition ratio (B) in lettuce (*Lactuca sativa*) cv. 'Tai Yuan Sun' seedlings after treatments with β-cembrenediol for 48 h. The results presented are mean of three replicates ±SE, Means significantly lower than the DMSO controls are indicated with one asterisk (*) ($p < 0.05$).

β-cembrenediol-treated roots were also quantified. After treatment, the numbers of dead cells were obviously raised in root tips of *L. sativa*. These results suggested that β-cembrenediol induce the formation of ROS, and the excessive ROS strengthened lipid peroxidation, caused membrane disruption and leaded to cell death, which is consistent with many former researches (Yan et al., 2015; Cheng and Cheng, 2015). Proline accumulation in response to various conditions of stress, such as metals, salt, water and drought, has been proved in many plant species (Kishor et al., 2005). Proline can act as a transitory storage of organic nitrogen, stabilize proteins and membranes under stressful conditions, prevent protein aggregation during its folding or refolding, and scavenge ROS such as superoxide anions (Lee et al., 2013). After treatment with β-cembrenediol, the content of proline showed a significant increasing trend in lettuce seedlings, as well as ROS accumulation. These results are in accordance with reported researches that proline accumulation in response to oxidative stress, and the accumulation of proline was associated with ROS production under stress (Yang et al., 2009; Lv et al., 2011).

H_2O_2 acts as a signaling molecule when ROS accumulation in response to environmental stresses. H_2O content significantly increased after treatment with β-cembrenediol compared with the control. The accumulation of H_2O_2 further strengthens lipid peroxidation and the levels of oxidative stress in the target tissues (Singh et al., 2006). H_2O_2 disturbs the SH group-containing enzymes activities and inhibits photosynthetic activity at high concentrations (Takeda et al., 1995). Chlorophyll is the important pigment involved in photosynthesis in plant (Scott, 2008). Chlorophyll content has been considered as an indicator of oxidative stress (Guidi et al., 1997). It was founded that β-cembrenediol significantly decreased chlorophylls *a* and *b*. The decrease of chlorophyll might be explained by the inhibition of photosynthetic synthesis by β-cembrenediol. This assumption was in accordance with a previous study (Cheng and Cheng, 2015).

CONCLUSIONS

β-Cembrenediol have a significant inhibitory effect on lettuce root and shoot elongation through affected mitotic phases and induced oxidative damage through enhanced generation of ROS, strengthen the degree of membrane lipid peroxidation, increased the proline content and decreased chlorophyll content and subsequent cell death in lettuce roots.

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Authors' contributions

X.R. and B.Q. designed the research. X.R., Z.Q.Y., X.F.H., and B.Q. performed most of the experiments. X.R. and X.Z.L. conducted the data analysis. X.R wrote the

manuscript. All the authors contributed to improve the paper and approved the final manuscript.

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