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Full Length Research Paper

Effects of ginsenosides on the growth and activity of antioxidant enzymes in American ginseng seedlings

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American ginseng (*Panax quinquefolium* L.) cannot be replanted in the same soil consecutively. Inhibitory allelopathy has been reported to be one of the factors in its replant failure. Ginsenosides is the important allelochemicals of ginseng. However, the allelopathic effects of ginsenosides on American ginseng are not well known. This paper investigated the effects of ginsenosides on American ginseng seedling growth, the activity of antioxidant enzymes and the content of malondialdehyde (MDA) in seedling radicles. Results showed that total ginsenosides, panaxadiol ginsenosides and ginsenosides-Rb group have inhibitory effects at higher concentrations but at low concentrations they have stimulatory effects on the growth of American ginseng seedling. The panaxatriol ginsenosides have stimulatory effects on the growth at various concentrations. All the ginsenosides treatments caused an increased MDA content. Under total ginsenosides treatment, catalase (CAT) activity was not detected. Both superoxide dismutase (SOD) and peroxidase (POD) activities increased at low concentrations, and they decreased at high concentrations, under panaxadiol ginsenosides treatment, CAT activity was not detected. Both SOD and POD activities decreased, under panaxatriol ginsenosides treatment, CAT activity was not detected. SOD activities significantly increased while POD activities significantly decreased, under ginsenosides-Rb group treatment, the SOD, CAT and POD activities increased at low concentrations while decreased at high concentrations. The phytotoxicity of ginsenosides is one of many possible factors contributing to ginseng replant failure.

Key words: American ginseng (*Panax quinquefolium* L.), allelopathy, ginsenosides, replant failure.

INTRODUCTION

American ginseng (*Panax quinquefolium* L.) is a herbaceous perennial plant (*Araliaceae* family) originating from Eastern North America (Barbara et al., 2006) and is highly valued as a medicinal herb in China. It was not commercially cultivated until the 1980s. A major cultivation area of American ginseng is Jilin Province in China. Its yield and quality is greatly reduced due to replant failure, if replanted in the same soil consecutively. Currently ginseng is mainly cultivated in forestry lands, hence, large forestry area have been cut to grow ginseng. It has become sharp conflict between the ginseng industry and forestry officials. Researchers believe that ginseng replant failure was due to soil deterioration and accumulation of pathogenic fungi. But American ginseng still cannot be cultivated on the same plot after soil improvement and sterilization (Zhang et al., 2008). Recently, inhibitory allelopathy has been reported to be one of many possible factors contributing to ginseng replant failure (Zhao et al., 2005a, b; Chen et al., 2006a, b; He et al., 2009; Li et al., 2008, 2009; Bi et al., 2010). Ginsenosides are biologically active compounds produced by ginseng plants. There are at least 30 different ginsenosides in American ginseng (Andreea et al., 2009). Ginsenosides can be released into the soil by ginseng plants. Ginsenosides can act as allelopathic stimulators of the growth of the important ginseng pathogen, *Pythium irregulare* while inhibitors of an antagonistic non-pathogenic fungus, *Trichoderma hamatum* (Nicol et al., 2002, 2003; Yousef, 2006). Nothing is known about the allelopathic effects of ginsenosides on American ginseng. Recently, the role of

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the antioxidation systems the plant in response to environmental stress has received wide attention ((Bowler et al., 1992; Walker and Mckensie, 1993; Jagtap and Bhargava, 1995; Shalata and Tal, 1998; Prasad et al., 1999; Yu et al., 2003; Ali et al., 2005a, b; Ali et al., 2008; Agrawal and Mishra, 2009). Few studies have investigated the role of the antioxidant enzyme activities in ginsengs' responding to allelochemicals. This paper mainly investigated the effects of four ginsenoside mixtures-total ginsenosides, panaxadiol ginsenosides, panaxatriol ginsenosides and ginsenosides-Rb group, on the growth of American ginseng seedlings and on the activity of antioxidant enzymes in American ginseng seedling radicles. The outcome of which will contribute to alleviating the ginseng replant failure and improving the yield and quality of ginseng.

METHODS AND MATERIALS

Ginsenoside solution

Total ginsenosides, panaxadiol ginsenosides, panaxatriol ginsenosides and ginsenosides-Rb group extracted from the root of Jilin *P. ginseng* were provided by Doctor Chun-hong Zhang from Jilin Agricultural University in powder form with 98.2% purity (HPLC analysis). These were first dissolved in distilled water. All solutions were filtered through a 0.45 µm hydrophilic polypropylene membrane and prepared their 12.5, 25, 50 and 100 mg/L concentrations for use in subculture mediums. In control only sterile distilled water was used in the subculture mediums.

Germinating tests for American ginseng seeds

American ginseng seeds were from Ji'an county in Jilin province of China. Two layers of Xinhua filter paper were placed in a 12 cm diameter Petri dish, Thirty disinfected seeds were placed in each dish. 5 ml diluents of each of the ginsenosides above (12.5, 25, 50 and 100 mg/L, respectively) were added into the culture dishes, while the control received 5 ml sterile distilled water. The Petri dishes were placed in a 18°C incubator for germination under a light condition of 12 h/d. Both the filter papers and the diluent were changed every 24 h. When all the seeds in the control had germinated, the lengths of roots were measured by a vernier caliper, and the fresh weights of roots were measured by an analytical balance.

American ginseng embryo culture

First of all, some cracked physiologically after-ripped seeds are obtained and shelled. Then, they are washed with tap water before placing them on a clean bench. Then they were sterilized on the surface with 2% sodium hypochloride for 8 to 10 min, and washed with sterile water three to four times. Unnecessary sterile water was absorbed by sterile filter paper. The embryos were excised and placed on sterilized half strength Murashige Skoog mediums (pH = 6.0). Then the flasks were placed in an illuminated incubator at 22˚. After compounding leaves (composed of three leaf-lets) sprouted from the seedlings after 6 weeks' culture, we add various ginsenosides solutions into mediums at concentrations of 12.5, 25, 50 and 100 mg/L, respectively. The control received a certain amount of sterile water. 12 h after the ginsenosides treatments, the antioxidant enzyme activities in American ginseng radicles were tested.

Enzyme assays

Frozen American ginseng roots (0.5 g) were crushed into a fine powder in a mortar and pestle under liquid N_2. The soluble protein was extracted by homogenizing the roots in 1 ml of respective extraction buffer. The homogenate was centrifuged twice at 19 000 g for 20 min at 4°C. The supernatants were used for the enzyme assays described subsequently. Protein content was determined according to the Bradford (1976) method with bovine serum albumin as the standard.

SOD activity was measured according to Madamanchi et al. (1994). Crude extract was added to a reaction solution (3 ml) containing 50 mM sodium phosphate buffer, pH 7.8, 0.1 mM ethylenediamine tetraacetic acid (EDTA), 13 mM methionine, 2 µM riboflavin and 75 µM nitroblue tetrazolium chloride (NBT). The reaction was started by exposing the mixture to cool white fluorescent light at a photosynthetic photon flux density of 50 µmol·/m^2/s for 15 min. One unit of SOD activity was defined as the amount of enzyme required to cause 50% inhibition of the reduction of NBT as monitored at 560 nm. The specific SOD activity was expressed as U·/mg protein.

CAT activity was assayed in a reaction solution (3 ml) composed of 50 mM sodium phosphate buffer, pH 7.0, to which 30% (w/v) H_2O_2 (0.3 ml) was added. The reaction was started by adding the reaction solution to 10 µl of crude extract and the activity was determined by spectra photometric method at 240 nm (Aebi, 1984). One unit of CAT activity was defined as 0.01 change of absorbance per minute. The specific CAT activity was expressed as U•/min/mg protein.

POD activity using guaiacol as a substrate, was assayed by the method of Chen and Wang (1989) in a reaction mixture (3 ml) containing 0.05 ml enzyme solution, 2.75 ml of 50 mM phosphate buffer (pH 7.0), 0.1 ml of 1% H_2O_2 and 0.1 ml of 4% guaiacol. The increase in absorbance at 470 nm due to the guaiacol oxidation was recorded for 2 min. One unit of enzymatic activity was defined as the amount of the enzyme that caused a change of 0.01 in absorbance per minute. The specific POD activity was expressed as U•/min/mg protein. MDA content was determined by the thiobarbituric acid (TBA) reaction (Zhang et al., 2004). One gram of American ginseng roots was homogenized in 10 ml 5% trichloroacetic acid, the mixture was centrifuged at 4000 g for 15 min, 2 ml of the supernatant was mixed with 2 ml of 0.6% TBA. It was heated at 100°C for 30 min followed by quick cooling over ice, and further centrifuged at 4000 g for 15 min. The absorbance was measured in the supernatant at 450, 532 and 600 nm, respectively. MDA content was calculated according to the following formula: $C\ (mmol/L)_{MDA} = 6.45\times(A_{532} - A_{600}) - 0.56\times A_{450}$, where A_{450}, A_{532} and A_{600} are the absorbency of solution at 450, 532 and 600 nm, respectively.

Statistical analysis

All experiments were performed in a completely randomized manner with five replicates. All enzymatic assays involved five sample replicates per treatment. The data were subjected to one-way analysis of variance, and treatment means separated from the control at $P< 0.05$ or 0.01 applying Dunnett's test. Statistical analysis was done with SPSS 11.0 for Windows statistical software package.

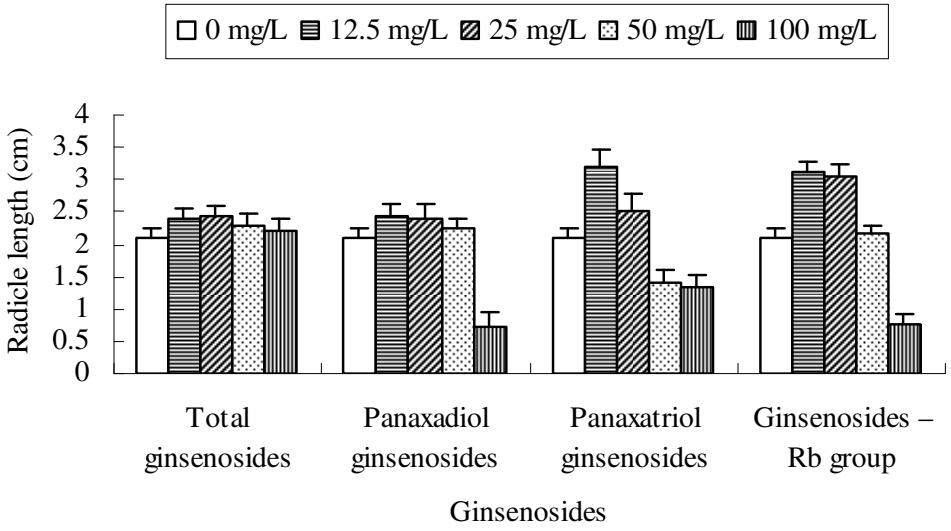

Figure 1. Effects of ginsenosides on American ginseng seedling radicle length.

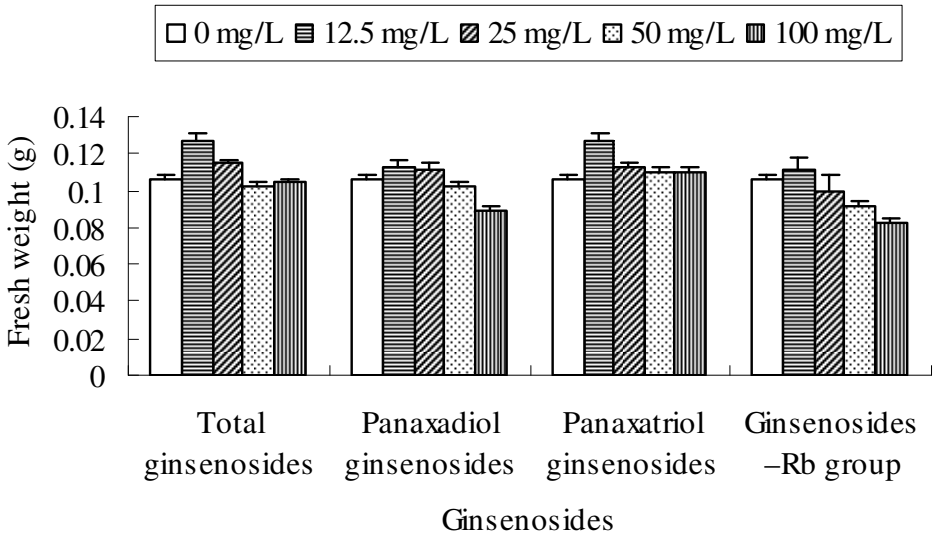

Figure 2. Effects of ginsenosides on American ginseng seedling fresh weight.

RESULTS

The total ginsenosides have stimulatory effects on the seedling radicle length of American ginseng at various concentrations. But radicle length was not significantly higher than the control. Under panaxadiol ginsenosides, panaxatriol ginsenosides and ginsenosides-Rb group treatment, they have inhibitory effects at higher concentrations but at low concentrations they have stimulatory effects on the radicle length. At 12.5 mg/L, the radicle length significantly increased by 53.04 and 50.05% with panaxatriol ginsenosides and ginsenosides-Rb group treatment compared with the control. At 100 mg/L, the highest concentration, the reductions of American ginseng seedlings radicle length caused by panaxadiol ginsenosides, panaxatriol ginsenosides and ginsenosides-Rb group were 65.73, 35.78 and 63.53%, respectively. The results are shown in Figure 1.

Under total ginsenosides, panaxadiol ginsenosides and ginsenosides-Rb group treatment, they have inhibitory effects at higher concentrations while at low concentrations they have stimulatory effects on the fresh weight of American seedling. The panaxatriol ginsenosides have stimulatory effects on the fresh weight at various concentrations. The results are shown in Figure 2.

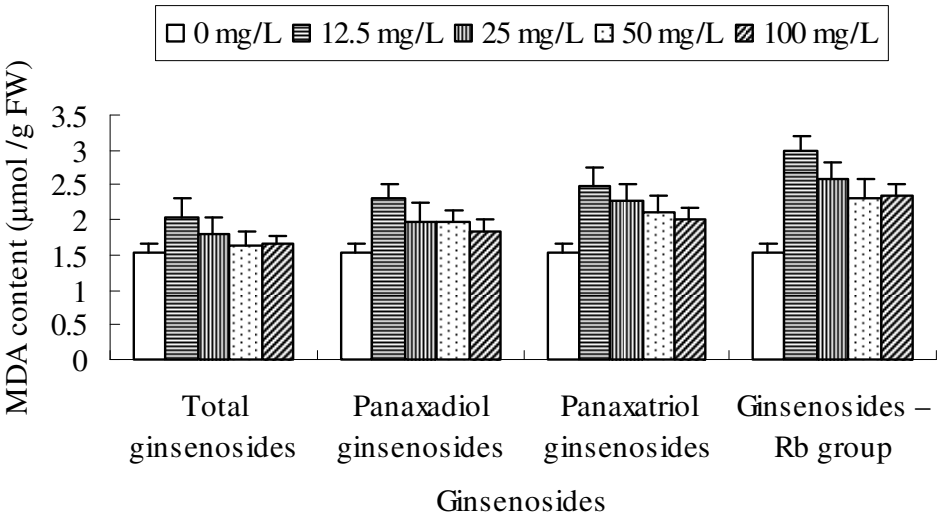

Figure 3. Effects of ginsenosides on MDA content in roots of American ginseng seedlings.

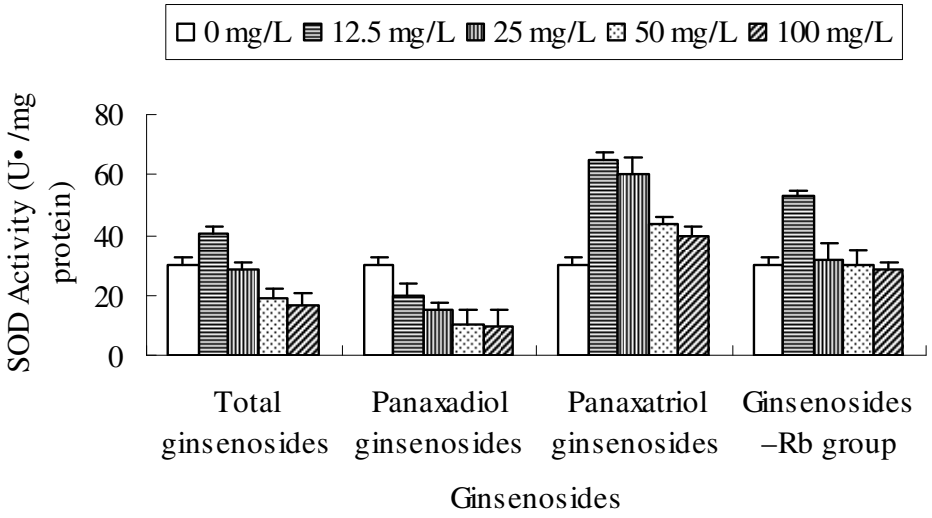

Figure 4. Effects of ginsenosides on SOD activity in roots of American ginseng seedlings.

The ginsenosides consistently increased MDA content in a concentration-dependent manner (Figure 3).

With total ginsenosides treatment, only at a 12.5 mg/L concentration, the SOD activity significantly increased, 34.61% higher than the control. When the concentration hiked to a range of 25 mg/L to 100 mg/L, SOD activity decreased: in the experiment, a high concentration (100 mg/L) of ginsenosides treatment reduced the SOD activity by 45.57%, compared with the control (Figure 4). POD activity tended to decrease as total ginsenosides concentration increased. A significant increase in POD activity (89.99%) was observed at 12.5 mg/L concentration compared with the control. When the concentration increased to 100 mg/L POD activity was lower than the control (Figure 5). CAT activity, however, was too low to be detected in the present study. Both the SOD activity and POD activity were higher than the control at low or medium concentrations, indicating that American ginseng can make a positive self-protective effect at low to medium treatment concentrations, but lower than the control at medium or high concentrations, indicating that weakened clearance ability in SOD and POD, which in turn caused the accumulation of reactive oxygen species (ROS), as well as the lipid peroxidation of cell membranes in American ginseng roots.

With panaxadiol ginsenosides treatment, SOD and POD activities were all significantly lower than the control. In the experiment, a high concentration (100 mg/L) of panaxadiol ginsenosides reduced the SOD and POD activity by 69.68 and 84.61%, respectively

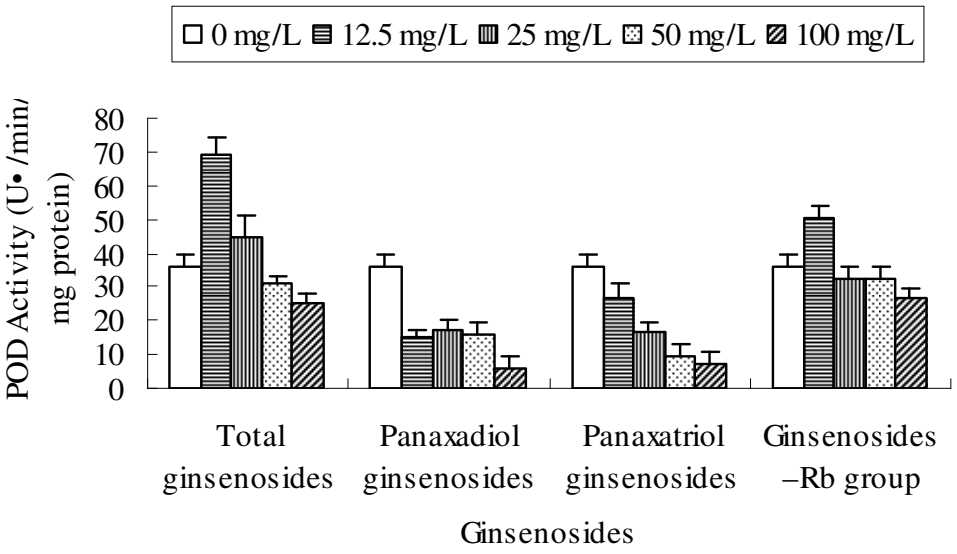

Figure 5. Effects of ginsenosides on POD activity in roots of American ginseng seedlings.

(Figures 4 and 5), CAT activity was still not detected in the present study. SOD and POD activities were all significantly lower than the control, indicating weakened clearance ability in antioxidant enzymes, making root cells unable to maintain the equilibrium of producing and clearing, hampering the self-protective mechanism of cells, disturbing the normal physiological metabolism in root cells, and inhibiting their growth. With panaxatriol ginsenosides treatment, SOD activity were higher than the control, the activity decreased as the treatment concentration increased. A significant increase in SOD activity (116.54%) was observed at 12.5 mg/L concentration compared with the control (Figure 4). Various concentrations of panaxatriol ginsenosides all had inhibitory effects on the POD activities, while the inhibitory power increased with treatment concentration, showing significant differences. In the experiment, a high concentration (100 mg/L) of panaxatriol ginsenosides reduced the POD activity by 79.88% (Figure 5). CAT activity was not detected yet. SOD activities were all significantly higher than the control, POD activities were all lower than the control, indicating weakened ability in scavenging of reactive oxygen species of POD and CAT, which in turn caused the accumulation of ROS, as well as the peroxidation of cell membranes in American ginseng roots as the level of oxidative stress exceeded the clearance rate. With ginsenosides-Rb group treatment, only at a 12.5 mg/L concentration, the SOD and POD activities were higher than the control, 75.79 and 37.94% higher than the control respectively (Figures 4 and 5). As for the impact on CAT, A significant increase in CAT activity (34.14%) was observed at 12.5 mg/L concentration compared with the control. When the concentration hiked to a range from 50 to 100 mg/L, CAT activity decreased while not significantly different compared with the control (Figure 6). The SOD, CAT and POD activities were higher than the control at low concentrations, indicating American ginseng can make a positive self-protective effect at low treatment concentrations, but lower than the control at medium or high concentrations, indicating weakened clearance ability in antioxidant enzymes, which in turn caused the accumulation of ROS, as well as the oxidative damage of cell membranes in ginseng roots.

DISCUSSION

The ginseng saponin (ginsenoside) is most important secondary metabolite in ginseng, with a content of about 6 to 10%. American ginseng during their growth excretes a small amount of ginsenosides through roots (Nicol et al., 2002). Ginseng is a perennial herb. A large number of the fibrous roots fall off from the large main root during the ginseng growth at the end of each year, Root decomposition will also release some ginsenoside into the soil. Although many studies have targeted the pharmacological properties of ginsenosides, little is known about their ecological role as compounds released into the soil by ginseng plants (Andreea et al., 2009). It may be possible that this tiny amount of ginsenosides may be one of the contributing factors to the problem of ginseng replant failure? This paper has investigated the *in vitro* effects of ginsenosides on the growth and the activity of antioxidant enzymes in American ginseng seedling. Experiments have shown that ginsenosides had allelopathy on American ginseng seedling which indicate that ginsenosides as the main ingredients of ginseng has a multi-purpose ecological role. These findings are incredibly consistent with the previous findings that

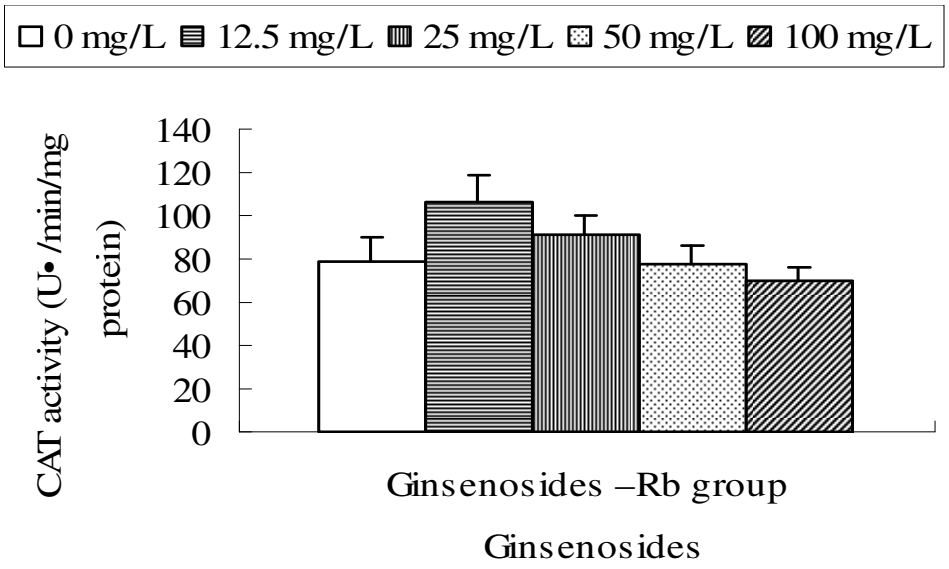

Figure 6. Effects of ginsenosides on CAT activity in roots of American ginseng seedlings.

medicinal plant allelochemicals show homology to its effective component (Kong, 1998; Guo, 2006).

Oxidative stress resulting from the cellular injury and plant growth inhibition due to generation of reactive oxygen species (ROS) has been also pointed as one of the mechanism of allelochemicals actions (Weir et al., 2004). Allelochemicals can damage the cell membranes through direct interaction with the constituents of plasma membranes or due to impairment of some metabolic function, necessary to maintain the membrane functions (Rice, 1984). All ginsenosides treatments increased the MDA content. Ginsenosides had different effects on antioxidant enzymes activities in American ginseng roots, respectively. The different changes of antioxidant enzymes activities might disturb the balance between production and scavenging of reactive oxygen species and lead to the accumulation of active oxygen. Excessive ROS can induce cell damage which in turn can induce ginseng seedling death. Ginsenoside is one of the many possible factors contributing to the replant failure of American ginseng. Besides, the deterioration of soil physicochemical properties, imbalance of soil microbial community and autotoxicity are all involved in the replant failure of American ginseng. More research is needed for further evaluation using pot and field experiments for better understanding of the autotoxicity potential of ginseng under field conditions.

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