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BRIEF COMMUNICATION

Effects of cadmium and lead on the growth and the activity of peroxidase and superoxide dismutase of blueberry plantlets *in vitro*

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Abstract

To study the effects of cadmium and lead on the growth of blueberry (*Vaccinium ashei* Reade) plantlets *in vitro* and on the activity of peroxidase (POD) and superoxide dismutase (SOD) of blueberry shoots, Cd^{2+} and Pb^{2+} were added separately to a cultivation medium. The results show that 0.01 mM Cd^{2+} significantly inhibited the growth of blueberry shoots, and the height and fresh mass of the shoots were significantly lower than those of the control; 0.05 mM Cd^{2+} significantly inhibited the proliferation of the shoots; the inhibitory effect on the growth and proliferation of blueberry *in vitro* was enhanced with the increase of Cd^{2+} concentration. Also Pb^{2+} (0.10 - 1.00 mM) significantly decreased the proliferation of the shoots, but it did not decrease significantly the shoot height and fresh mass. After 20 d of Cd or Pb treatments, the POD and SOD activities of the shoots increased with the increase of their concentrations, and when the concentration was 1.00 mM, the POD and SOD activities were significantly higher than in the control; the effect of Pb^{2+} on POD and SOD activities was generally stronger than that of Cd^{2+}. The upregulation of activities of antioxidative enzymes played an effective role in acclimatization to these stresses, especially to Pb stress.

Additional key words: antioxidants, heavy metals, *Vaccinium ashei*.

In recent years, due to the impact of human activities, a large number of heavy metals such as cadmium and lead have been released into the soil, resulting in the increasingly serious pollution in the soil (Chen *et al.* 2014). Cd and Pb in soil not only affect the yield and quality of plants, but also accumulate in animals and human bodies through the food chain, threatening their health (Xiao *et al.* 2015, Tóth *et al.* 2016). Therefore, the content of Cd and Pb in contaminated soil and the tolerance mechanisms of plants to Cd and Pb stress attract great attention.

Blueberries (*Vaccinium* spp.) are perennial berry fruit trees of *Ericaceae* family. Blueberry fruits are popular around the world due to their nutritional value and elevated content of bioactive phenolic compounds (Kalt *et al.* 2007, 2020). Although many species of blueberries are native to North America, several of them especially highbush (*V. corymbosum* L.), lowbush (*V. angustifolium* Ait.), and rabbiteye (*V. ashei* Reade) blueberries are commercially cultivated in many countries in Europe, South America, Asia, Australia, and New Zealand (Strik 2005, Strik and Yarborough 2005). Soil is one of the main limiting factors that make it difficult to expand the cultivation scope of blueberry. Blueberry cultivation requires high soil quality; loose and acid soil rich in organic matter and with a low heavy metal content (Markus and Mcbratney 1996). However, there are few reports about the effect of heavy metal stress on the growth of blueberry.

In this paper, the effects of cadmium and lead stress on the growth and antioxidant enzyme activities in blueberry (*V. ashei* cv. Garden blue) grown *in vitro* were studied, in order to provide scientific basis for promotion of blueberry growing area, as well as to give theoretical basis for revealing the tolerance mechanisms of plants to cadmium and lead.

The sterile shoots of blueberry were provided by the Plant Biotechnology Laboratory at Huzhou University. The shoots were multiplied on a woody plant medium (Lloyd and McCown 1980) with zeatin (1.5 mg dm^{-3}), agar (5 g dm^{-3}), and sucrose (20 g dm^{-3}), pH 5.2. Blueberry shoot cultures were kept at a temperature of 25 ± 2 °C, a 12-h photoperiod, and an irradiance at the culture level of 50 μmol m^{-2} s^{-1} provided by cool white fluorescent lamps. Stock solutions of 10 mM Cd^{2+} or Pb^{2+} were prepared using $Cd(NO_3)_2 \cdot 4\,H_2O$ and $Pb(NO_3)_2$, respectively. For cadmium

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Abbreviations: POD - peroxidase; SOD - superoxide dismutase.

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stress treatment 0.01, 0.05, 0.10, 0.50, and 1.00 mM Cd^{2+} were added to the above mentioned medium, and 0.01, 0.05, 0.10, 0.50, and 1.00 mM Pb^{2+} were added to medium for lead stress treatment; distilled water was used as the control, and all the final media were adjusted to pH 5.2. Each bottle with medium was inoculated with five sterile blueberry shoots and the shoot cultures were maintained in the same condition as mentioned above. After 10 and 20 d of culture, blueberry shoots were sampled triply for each treatment for enzyme assay. The height, fresh mass, and multiplication coefficient were examined after 75 d of culture.

Vaccinium shoots (0.5 g) were ground to a slurry with a mortar and pestle with 4 cm^3 of phosphate buffer (pH 7.0) containing 1 % (m/v) of polyvinylpyrrolidone (PVPP) under cooling condition (ice bath). The homogenates were centrifuged at 14 000 *g* and 4 °C for 10 min, and the supernatants were kept at 4 °C prior to use for peroxidase (POD) and superoxide dismutase (SOD) assays. Protein content was determined by the method of Lowry (Lowry *et al.* 1951), slightly modified by Peterson (1977), using bovine serum albumin as a standard.

Peroxidase activity was measured according to Pan *et al.* (2006) with minor modifications. The determination was based on the change of absorbance at 420 nm caused by decomposition of H_2O_2. One unit of POD activity (U) was defined as the amount of POD required to consume 1.0 μg of the substrate by 1.0 mg of the tissue protein in 1 min in the reaction system at 37 °C.

Superoxide dismutase activity was determined by the ferricytochrome-*c* assay method using xanthine/xanthine oxidase as the source of superoxide radicals (McCord and Fridovich 1969). One unit SOD activity (U) was defined as the quantity of SOD required to produce a 50 % inhibition of reduction of nitrite by 1.0 mg tissue protein in 1.0 cm^3 of a reaction solution by measuring the change of absorbance at 550 nm.

The data were analyzed by one-way analysis of variance (*ANOVA*) using *SPSS 19.0*. Significant differences between data sets were tested by the least significant difference (LSD) test at $P < 0.05$ and $P < 0.01$.

Firstly, the effects of Cd and Pb stresses on the growth and proliferation of blueberry shoots *in vitro* were studied. Fig. 1*A* showed that the height of blueberry shoots treated with 0.01 mM Cd^{2+} was significantly ($P < 0.05$) lower than that of the control, and the height gradually decreased with the increase of Cd^{2+} concentration. When the concentration of Cd^{2+} reached 0.05 mm, the difference between the shoot height of the treatment and control was highly significant ($P < 0.01$). The height of the shoots after exposure to 1.00 mM Cd^{2+} decreased by 48 %. The effect of Pb^{2+} stress with the same concentration was different from that of Cd^{2+}. There was no significant difference in shoot height between 0.01 - 1.00 mM Pb^{2+} treatment and control ($P > 0.05$).

The fresh mass of blueberry shoots decreased when Cd^{2+} was added to the medium. Already 0.01 mM Cd^{2+} could significantly ($P < 0.01$) decrease the fresh mass of the shoots, and the inhibitory effect was more obvious with the increase of the concentration of Cd^{2+}. The fresh mass of the shoots after exposure to 1.00 mM Cd^{2+} was only 50 % of the control (Fig. 1*B*). The ability of blueberry shoots to endure Pb^{2+} stress was stronger than Cd^{2+}. Lower concentration of Pb^{2+} (≤ 0.50 mM) had no significant effect on the fresh mass of the shoots. The fresh mass of the shoots treated with 1.00 mM Pb^{2+} was significantly higher than that of the control ($P < 0.05$). Actually, 1.00 mM Pb^{2+} had no significant effect on the height of the shoots, but the shoots were stronger.

There was no significant difference in the proliferation coefficient of blueberry shoots after exposure to 0.01 mM Cd^{2+} when compared with the control ($P > 0.05$), however, the proliferation ability decreased with the increase of Cd^{2+} concentration. After treatment with Cd^{2+} higher than 0.05 mM, the proliferation coefficient of the shoots was significantly lower than that of the control ($P < 0.05$), and there was highly significant difference ($P < 0.01$) between the shoots treated with Cd^{2+} higher than 0.10 mM and the control. When Cd^{2+} concentration reached 1.00 mM, the proliferation coefficient was 1.00, indicating that the proliferation ability of the shoots was stopped. The effect of Pb on the proliferation ability of blueberry shoots was less than that of Cd. Although the proliferation coefficient after treatment of Pb^{2+} higher than 0.10 mM was significantly lower than control ($P < 0.01$), there was no significant difference among 0.10 - 1.00 mM Pb^{2+} treatments. When the concentration of Pb^{2+} reached 1.00 mM, blueberry shoots still had multiplication ability about 3 times (Fig. 1*C*).

Heavy metal pollution of soil has become a global environmental problem to be solved urgently (Ali *et al*. 2013, Wiafe *et al*. 2019). The tolerance and adaptability of plants to heavy metal pollution have been widely concerned by scientists and technologists. Under heavy metal stress, plants often show a series of physiological and metabolic changes such as growth inhibition (Chandrasekhar and Ray 2019, Murtaza *et al*. 2019, Xu *et al*. 2019), cell cycle progression inhibition (Hendrix *et al*. 2018) but increase in photosynthetic rate (Xu *et al*. 2019). Our results showed that the height and fresh mass of blueberry shoots decreased significantly after treatment of Cd^{2+} higher than 0.01 mM (Fig. 1*A*,*B*), and the proliferation of the shoots was inhibited significantly after exposure to Cd^{2+} higher than 0.05 mM (Fig. 1*C*). With the increase of Cd^{2+} concentration, the inhibition effect on the growth and proliferation of the shoots became more and more significant (Fig. 1*A*-*C*). When the concentration of Pb^{2+} reached 0.10 mM, the proliferation of blueberry shoots was significantly affected, however, the inhibition was less than that of the same concentration of Cd^{2+} (Fig. 1*C*). Lead at concentration 0.01 - 1.00 mM did not significantly decrease the height and fresh mass of the shoots (Fig. 1*A*,*B*), suggesting that the effect of Pb^{2+} on the growth of the shoots was less than the effect on the proliferation capacity. Our results and previous results from *Salix* (Xu *et al*. 2019) and wheat (Murtaza *et al*. 2019) indicate that the toxicity of Cd^{2+} to plants is greater than Pb^{2+}, and the tolerance threshold of plants to Pb is higher than Cd, that is to say, the tolerance of plants to lead is stronger than to cadmium. Probably Pb might be

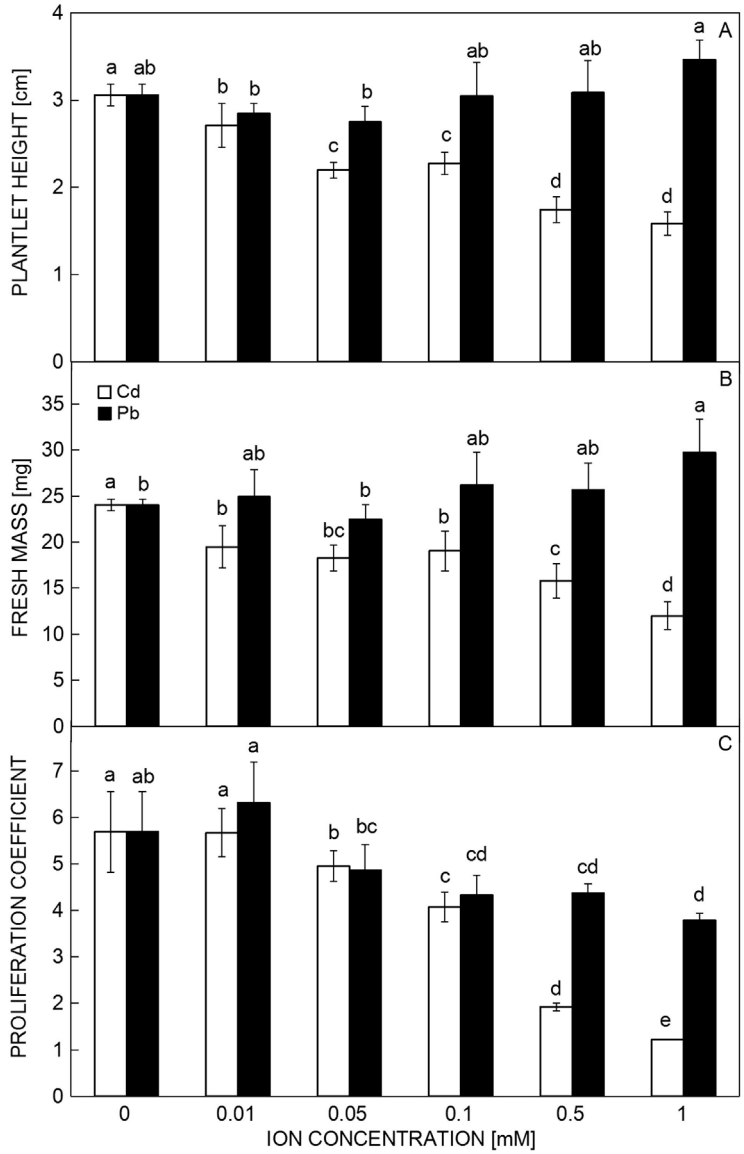

Fig. 1. The effect of Cd and Pb stress on height (*A*), fresh mass (*B*), and proliferation (*C*) of blueberry shoots. Means ± SEs, $n = 3$; different letters indicate significant differences at $P < 0.05$.

used as a "trace element" in blueberry culture medium, as low concentration of Pb might be beneficial to the growth of blueberry shoots.

Heavy metal stresses in plants have been documented from the aspects of physiology, biochemistry (Romero-Puertas *et al*. 2019), genetics (Zorrig *et al*. 2019), and molecular biology (Akbudak and Filiz 2019, Zhang *et al*. 2020). In the present study, the activities of POD and SOD in blueberry shoots after exposure to Cd and Pb were investigated. After 10 d of treatment with Cd^{2+} or Pb^{2+}, the POD activity of blueberry shoots showed an increasing trend with the increase of their concentration (Fig. 2*A*). When Cd^{2+} concentration was 1.00 mM, POD activity was significantly ($P < 0.05$) higher than in control, while POD activity of the shoots was significantly higher than in control ($P < 0.05$) when the concentration of Pb^{2+} was higher than 0.01mM, and there was highly significant increase ($P < 0.01$) after treatment with Pb^{2+} higher than 0.10 mM (Fig. 2*A*). After 20 d of exposure the POD activity of blueberry shoots also increased with the increase of the concentration of Cd or Pb (Fig. 2*B*). POD activity after 20-d exposure to 0.10 mM Cd^{2+} was significantly higher than in the control ($P < 0.05$), and there was highly significant difference ($P < 0.01$) between 1.00 mM Cd^{2+} and the control. The POD activity of the shoots after 20-d exposure to 0.01 mM Pb^{2+} was significantly higher than in the control ($P < 0.05$), and the difference between 1.00 mM Pb^{2+} and the control was highly significant ($P < 0.01$) (Fig. 2*B*).

After 10 d of culture, 0.01 - 1.00 mM Cd had no significant effect on SOD activity of blueberry shoots (Fig. 2*C*). The SOD activity of the shoots treated with 1.00 mM Pb^{2+} was significantly higher than that of the control ($P < 0.05$), while other concentrations of Pb^{2+} had

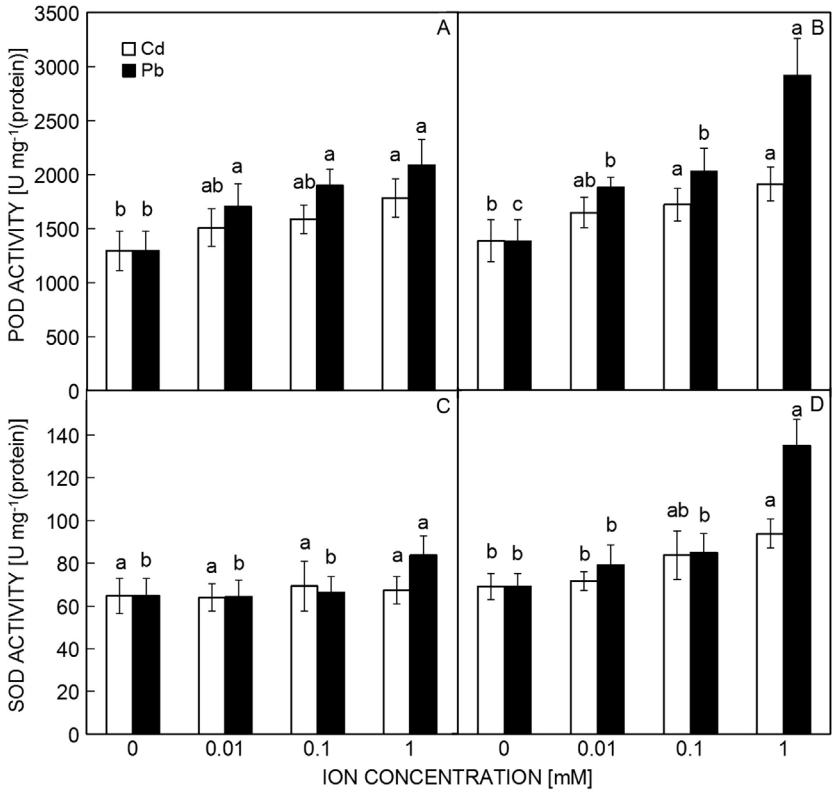

Fig. 2. The effect of Cd and Pb stress on peroxidase (POD) activity after 10 d (*A*) and 20 d (*B*), and superoxide dismutase (SOD) activity after 10 d (*C*) and 20 d (*D*) of exposure in blueberry shoots. Means ± SEs, $n = 3$; different letters indicate significant differences at $P < 0.05$.

no significant effect on SOD activity (Fig. 2*C*). After 20 d of treatment, the SOD activity under Cd or Pb stresses increased with the increase of their concentrations. The SOD activity of the shoots treated with 1.00 mM Cd^{2+} or Pb^{2+} was significantly higher than that of the control ($P < 0.01$), however, there was no significant difference between the other treatments and the control (Fig. 2*D*).

It is well known that ROS steady-state content is regulated by the interplay between different ROS-producing and ROS-scavenging mechanisms (Romero-Puertas *et al*. 2019). ROS production is a common feature in plants under heavy metal stress (Pellegrini *et al.* 2019). Pb and Cd, as nonredox heavy metals, can cause overproduction of ROS indirectly through the inhibition of antioxidative defense system or by stimulation of membrane bound NADPH oxidases (Pandey and Singh 2012, Chmielowskabąk *et al.* 2014). The ROS-scavenging mechanism involves enzymatic antioxidants such as superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT), and non-enzymatic compounds such as glutathione (GSH) and proline (Halliwell and Gutteridge 2015, Romero-Puertas *et al*. 2019). The present study demonstrated that the activity of POD and SOD of the shoots mostly increased when blueberry plants were exposed to Cd or Pb stress, except for the SOD activity after 10 d of exposure (Fig. 2). These results suggest that the increase in the activities of POD and SOD in blueberry shoots, and so ROS scavenging could improve their Pb and Cd tolerance. Fig. 2 also showed that the effect of Pb on POD and SOD activities was stronger than that of Cd; this result explains why the inhibitory effect of Pb on the growth and proliferation of blueberry was lower than that of the same concentration of Cd (Fig. 1).

Recently, the effects of cadmium and lead on the growth and development of plants have been well documented (Murtaza *et al*. 2019, Xu *et al*. 2019), however, most studies on heavy metal stress of plants had not been carried out under sterile culture conditions. Therefore, the previous results were inevitably affected by the role of microorganisms because some studies have shown that some bacteria can not only absorb heavy metals such as cadmium and lead, but also can fix them (Bravo *et al*. 2018, Pakdel *et al*. 2019). Our experiment was performed under completely aseptic culture condition, which excluded the influence of microorganisms on Cd^{2+} and Pb^{2+}, and truly reflected the influence of Cd and Pb stress on the growth of blueberry plantlets. It can provide a useful reference for the same kind of related research.

In conclusion, Cd stress significantly inhibited the growth and proliferation of blueberry shoots, whereas Pb stress only significantly restrained the shoot proliferation. The activities of POD and SOD in the shoots after exposure to Cd or Pb increased to mitigate the ROS accumulation caused by heavy metals. The results suggest that blueberry shoots exhibit higher tolerance to Pb than to Cd.

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