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Effect of exogenous calcium on growth, nutrients uptake and plasma membrane H^+-ATPase and Ca^{2+}-ATPase activities in soybean (*Glycine max*) seedlings under simulated acid rain stress

Chanjuan Liang[a,b,c,*], Bingjie Zhang[b]

[a] State Key Laboratory of Food Science and Technology, Jiangnan University, Wuxi 214122, China
[b] Jiangsu Key Laboratory of Anaerobic Biotechnology, School of Environmental and Civil Engineering, Jiangnan University, Wuxi 214122, China
[c] Jiangsu Cooperative Innovation Center of Technology and Material of Water Treatment, Jiangnan University, Wuxi 214122, China

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ABSTRACT

Calcium (Ca) is one of essential elements for plant growth and development, and also plays a role in regulating plant cell physiology and cellular response to the environment. Here, we studied whether calcium played a role in enhancing tolerance of plants to acid rain stress by hydroponics and simulating acid rain stress. Our results show that acid rain (pH 4.5/pH 3.0) caused decreases in dry weight biomass, chlorophyll content and uptake of nutrients elements (NO_3^-, P, K, Mg, Zn and Mo) and an increase in membrane permeability of root. However, all parameters in soybean treated with exogenous calcium (5 mM) and acid rain at pH 4.5 were closed to the control levels. In addition, exogenous calcium (5 mM) alleviated the inhibition induced by pH 3.0 acid rain on the activity of plasma membranes H^+-ATPase and the expression of *GmPHA1* at transcriptional level, being benefiting to maintaining uptake of nutrients (NO_3^-, P, K, Mg, and Zn), and then lower the decrease in dry weight biomass and chlorophyll content. After a 5-day recovery (without acid rain stress), all parameters in soybean treated with acid rain at pH 3.0 and exogenous calcium were still worse than those of the control, but obviously better than those treated with acid rain at pH 3.0. Higher activity of plasma membrane H^+-ATPase in soybean treated with acid rain at pH 3.0 and exogenous calcium was good to uptake of nutrients and promoted the recovery of soybean growth, compared with soybean treated with acid rain at pH 3.0. In conclusion, exogenous calcium could alleviate the inhibition caused by acid rain on soybean growth by increasing the activity of plasma membrane H^+-ATPase for providing driving force to nutrient absorption, and its regulating effect was limited by intensity of acid rain. Furthermore, the application of exogenous calcium can be one of ways to alleviate the damage caused by acid rain to plants.

1. Introduction

Acid rain is one of the most widespread pollutions because of emissions of the two major acidic pollutants such as sulfur dioxide (SO_2) and nitrogen oxides (NO_x). Since the 1970s, acid rain has remained in the public spotlight in both Europe and the United States and recently has emerged as an important problem in other regions such as Southeast Asia, particularly China (Menz and Seip, 2004). Acid rain severely inhibits plant growth and productivity by reducing photosynthetic rate, varying stomatal conductance, decreasing chlorophyll content, destroying membrane integrity, and causing disorder of intracellular homeostasis and accumulation of reactive oxygen species (Chen et al., 2013; Ramlall et al., 2015; Yi et al., 2014). Actually, nutrient elements are essential to maintain physiological metabolisms for plant growth and development. Hence, negative effects of acid rain on morphology and growth of plant root can be one of main reasons for inhibiting plant growth, biomass accumulation and final yield by disturbing water metabolism, nutrient uptake and hormone synthesis in plants (Ericsson, 1995; Russell, 1979; Zheng et al., 2016). In response to acid rain stress, plants have developed complex sensing and signaling mechanisms to adapt to such stress. Previous study shows that the increase in root hair of rice seedlings under simulated acid rain stress is one of adaption mechanism to acid rain by promoting the uptake the K, Na, and Ca in roots. In addition, plasma membrane H^+-ATPase plays a role in plant tolerance to acid rain by pumping excessive intracellular H^+ out of cell to provide energy for nutrient absorption (Zhang et al., 2016, 2017). Zhu et al. (2009) also found a high regulation of various plasma membrane H^+-ATPase in rice is helpful to adapt to low pH.

* Corresponding author at: State Key Laboratory of Food Science and Technology, Jiangnan University, Wuxi 214122, China.
E-mail address: liangchanjuan@jiangnan.edu.cn (C. Liang).

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However, plant tolerance to acid rain by self-regulation is very limited, especially in some sensitive species. Thus, increasing tolerance of crops to acid rain stress is crucial to reduce losses in agricultural production, especially currently food security has been aggravated by population growth, environmental deterioration and climate change.

Application of mineral nutrients, such as calcium, has been shown to play an important role in plants response to abiotic stresses. Many studies have proved that calcium plays an important role in enhancing plant resistance to extremely temperature, drought, salinity, and heavy metals (Cramer et al., 1985; He et al., 2012; Nayyar et al., 2005; Wang, 2010). The influences of calcium on the tolerance of plants to abiotic stresses include: maintaining the selectivity and integrity of membranes to make the molecular of lipids combine more closer (Jones and Lunt, 1967; Yu et al., 1998); increasing antioxidant enzymes activities to scavenge excessive accumulation of reactive oxygen species for avoiding oxidative damage (Carafoli and Crompton, 1978; Jiang and Huang, 2001); being regarded as a second messenger that plays an important role in signal transduction in plant physiological and biochemical metabolism (Bush, 1995; Hepler 2005; Dolatabadian et al., 2013). However, little is known to clarifying the role of Ca in regulating adaptation of plants to acid rain stress. Owing to the fact that acid rain has been seriously affected the agricultural production, it is should be informative to know the roles of calcium in regulating tolerance in plants to acid rain stress.

In this study, we aimed at (1) clarifying effect of exogenous Ca on growth and tolerance in plants under acid rain stress compared to single acid rain stress; (2) exploring the role of exogenous Ca in regulating nutrients absorption in plants to influence growth of plants under acid rain stress; (3) studying association of activities of plasma membrane H^+-ATPase and Ca^{2+}-ATPase with nutrients absorption in plants under single acid rain stress or the combined treatment of exogenous Ca and acid rain. These results can provide basic information for further clarifying the injured mechanism of acid rain on plants, and providing a new direction for finding ways to alleviate the damage caused by acid rain to plants.

2. Materials and methods

2.1. Plant material and growth conditions

Soybean seeds (Zhonghuang 25, Wuxi Seed Co., Ltd., China) were disinfected with $HgCl_2$ (0.1%) for 5 min and washed with distilled water. The disinfected seeds were germinated in an incubator at 25 ± 5 °C for about 5 days. When the radicle length was approximately 4 cm, the seedlings were transplanted into plastic pots (6.88 L) filled with distilled water. After the second true leaf had developed (about 10 days later), the seedlings were cultured in Hoagland's solution (pH7.0) in plastic pots (6.88 L) in a green house with a light intensity of 300 mol m^{-2} s^{-1} photosynthetically active radiation, temperature of 25 °C/20 °C (14 h/10 h), relative humidity of 70%/80% (day/night). After the 3rd true leaf had developed (approximately 30 d after germination), the soybean seedlings were treated with simulated acid rain and/or exogenous calcium.

2.2. Simulated acid rain and exogenous calcium treatment

Simulated acid rain was prepared by using a stock solution at pH 1.0 which was concentrated solution of H_2SO_4 and HNO_3 in a ratio of 3:1 (v/v) by chemical equivalents (Liang and Wang, 2013; Zhang, 1996). Then the stock solution was diluted to pH 4.5 and 3.0 as the spraying solution of simulated acid rain. Simulated acid rain solution (pH 4.5 and 3.0) was sprayed at 24 h intervals on the leaves of soybean seedlings till drops began to fall. As the control, the same amount of distilled water (pH 7.0) was applied to soybean leaves. For calcium treatment, the appropriate quantity of $CaCl_2$ (5 mM) was dissolving in nutrient solution (pH 7.0). In our preliminary experiments (data not shown in the manuscript), we studied the effect of different concentration of exogenous Ca (1, 5, 10 and 15 mM) on growth of soybean seedlings to clarify the dose-effect of Ca. We found that 5 mM Ca can promote obviously the growth of soybean seedlings, and then chose this concentration to do further experiments. In present experiments, There were six groups including the control (pH 7.0 distilled water and 0 mM exogenous Ca^{2+}), SAR 1 (pH 4.5 simulated acid rain), SAR 2 (pH 3.0 simulated acid rain), Ca (pH 7.0 distilled water and 5 mM exogenous Ca^{2+}), SAR 1 + Ca (pH 4.5 simulated acid rain and 5 mM exogenous Ca^{2+}) and SAR 2 + Ca (pH 3.0 simulated acid rain and 5 mM exogenous Ca^{2+}). After being treated for 5 days, half of the soybean seedlings were collected for analysis. The rest of soybean seedlings were cultured for another 5 days under the control conditions (without simulated acid rain and exogenous calcium), and then were collected for analysis. All treatments were done in triple biologically.

2.3. Determination of dry weight biomass

After harvesting, soybean seedlings were washed with deionized water. Then parts of aboveground and root of soybean seedlings were dried separately in a forced-air oven at 70 °C for about 48 h (reached a constant weight) for determination of dry weight.

2.4. Determination of chlorophyll content

The chlorophyll content was determined according to Erinle et al. (2016). Fresh leaves (0.1 g) were homogenized in 5 mL of extraction solution (80% acetone), and the pigment contents were determined by spectrophotometric method. Chlorophyll contents were calculated by the following equations:

$$\textbf{Total chlorophyll (a + b)(mg/gFw)} = \frac{(\mathbf{8.02 \times A663 + 20.20 \times A645}) \times \mathbf{V}}{\mathbf{1000 \times W}}$$

Where V = volume of the extract (mL); W = Weight of fresh leaves (g)

2.5. Determination of root permeability

Root permeability was determined with the conductivity method (Bajji et al., 2002). Electrical conductivity of the sample solution was measured by anLF-92 conduct meter (WTW GmbH, Weilheim, Germany). Membrane permeability was expressed by the ratio of the amounts of electrolytes released from the stressed or control tissues to total electrolyte amounts released after boiling (Liang et al., 2015):

$$\textbf{Root membrane permeability} = \mathbf{L_1/L_2 \times 100}\%$$

Where L1 = the amounts of electrolytes of soybean root before boiling; L2 = the amounts of electrolytes of soybean roots before boiling

2.6. Determination of nutrient content

The ammonium ($NH4^+$) concentration was determined by spectrophotometry according to Nessler's reagent method (Yuen and Pollard, 1954) with some modification. Fresh root samples (0.5 g) were homogenized in 0.3 mM H_2SO_4 and centrifuged (20,000 g) for 20 min. The supernatants (0.1 mL) was incubated in reaction mixtures (2.6 mL) containing0.1 mL of 10% (w/v) potassium sodium tartrate, 2.4 mL of distilled water, and 0.1 mL of Nessler's reagent for 5 min, and the absorbances were recorded at 425 nm. The ammonium concentration was calculated using a standard curve prepared with different concentrations of NH_4Cl.

Nitrates concentration was measured according to the method described by Cataldo et al. (1975). Fresh root samples (0.5 g) were collected and washed with deionized water for three times, and then put into graduated test tube with 20 mL deionized water. Test tubes blocked

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