Involvement of Antioxidative Defense System in Rice Seedlings Exposed to Aluminum Toxicity and Phosphorus Deficiency

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Abstract: Plants growing in acid soils may suffer both phosphorus (P) deficiency and aluminum (Al) toxicity. Hydroponic experiments were undertaken to assess the single and combination effects of Al toxicity and low P stress on seedling growth, chlorophyll and proline contents, antioxidative response and lipid peroxidation of two rice genotypes (Yongyou 8 and Xiushui 132) differing in Al tolerance. Al toxicity and P deficiency both inhibited rice seedling growth. The development of toxic symptoms was characterized by reduced chlorophyll content, increased proline and malondialdehyde contents in both roots and leaves, and increased peroxidase and superoxide dismutase activities in roots, but decreased in leaves. The stress condition induced more severe growth inhibition and oxidative stress in Yongyou 8, and Xiushui 132 showed higher tolerance to both Al toxicity and P deficiency. P deficiency aggravated Al toxicity to plant growth and induced more severe lipid peroxidation.

Key words: aluminum toxicity; phosphorus deficiency; rice; plant growth; oxidative stress

Acid soils that limit crop production have extended over 40% to 70% of the world's arable soils. Aluminum (Al) toxicity is considered to be one of the most serious abiotic stress factors limiting plant growth in acid soils, which alters nutrient availability in the rhizosphere, leads to accumulation of certain metabolites and affects the behavior of many key enzymes (Simon et al, 1994; Doncheva et al, 2005; Poschenrieder et al, 2008; Amenós et al, 2009; Zhou et al, 2009). Furthermore, Al toxicity of plants grown in acid soils is generally considered to be closely related to phosphorus (P) nutrition. Phosphorus fixation in acid soils by precipitation of aluminum phosphate is recognized as an important factor for low P availability. P deficiency strongly limits plant biomass accumulation, reduces leaf area, decreases photosynthetic efficiency, and changes biochemical metabolic pathways (Tan and Keltjens, 1990; Chaudhary et al, 2008; Vieira et al, 2008).

However, some plant species and genotypes within a species have developed various strategies to cope with acid soil conditions. Immobilization Al at cell walls, increase pH in rhizosphere, compartmentalization Al in vacuoles, and evolution of Al-tolerant enzymes are the most important Al tolerant mechanisms for plants (Matsumoto, 2000; Monette et al, 2010; Garzón et al, 2011). Similarly, elongation of root hairs, exudation of organic acids and increase of root surface phosphatase are the main morphological and physiological mechanisms that plants adapt to low P conditions (Kamh et al, 2001; Wang et al, 2007).

Many studies on the effects of Al toxicity and P deficiency on plant growth, metabolism and the adaptive mechanism have been reported. However, little work has been done on the interaction of Al toxicity and P deficiency in plants in spite of its ecological significance. Meanwhile, rice is known as an Al-tolerant crop, although its tolerance is widely different among varieties. In our previous experiments, some genotypes with extreme Al tolerance were identified according to the evaluation of growth and yield performance. Therefore, the objectives of the present study are to investigate P deficiency resistance of rice genotypes differing in Al tolerance and the interaction of Al and P deficiency in rice seedlings.

MATERIALS AND METHODS

Rice materials, growth conditions and treatments

The experiment was conducted in 2010 at Shaoxing University, Shaoxing City, China. Two rice (Oryza sativa L.) genotypes Xiushui 132 and Yongyou 8 were used. Xiushui 132 is relatively Al resistant and Yongyou 8 is relatively Al sensitive. The seeds were surface sterilized in 2% H2O2 for 20 min, rinsed with distilled water and germinated in moist quartz sand in a culture room. When seedlings grew the second leaf, they were selected for uniformity and transplanted into a 5-L pot containing 4.5 L nutrient solution, which was covered with a polystyrol-plate with seven evenly spaced holes, and placed in a greenhouse with...
the photoperiod of 8 h light/16 h dark and the temperature of (25 ± 2) °C throughout the experiment. The composition of the basic nutrient solution (mg/L) was: (NH₄)₂SO₄ 48.2, MgSO₄ 65.9, K₂SO₄ 15.9, KNO₃ 18.5, Ca(NO₃)₂ 59.9, KH₂PO₄ 24.8, Fe-citrate 5.0, MnCl₂·4H₂O 0.9, ZnSO₄·7H₂O 0.11, CuSO₄·5H₂O 0.04, HBO₃ 2.9 and H₂MoO₄ 0.01. One week after transplanting to the basic solution culture, Al was added to the nutrient solution in the form of AlCl₃·6H₂O, further the solution pH was adjusted with HCl for following four treatments: CK (pH 6.5), Al addition (Al⁺, 1.5 mmol/L Al, pH 4.5), low P (P⁻, 1/30 of normal P supply), and Al⁺ and P⁻. The solution pH in each container was adjusted every other day with HCl or NaOH as required. The experiment was laid out in a completely randomized design with five replicates, and the nutrient solution in the growth container was renewed every 5 d.

Plant growth and chlorophyll content

On the 20th day after treatment, shoot height, root length and root volume (measured by the method of draining water) of rice plants were measured. Leaf chlorophyll content was determined according to the method of Sims and Gamon (2002) using fresh leaves collected from at least three different rice plants.

Free proline content

The upper second fully expanded leaves were sampled for assay of proline content on the 20th day after treatment using the procedure described by Demiral and Turkan (2005). Fresh leaf samples (0.5 g) were homogenized in 3% sulfosalicylic acid and centrifuged at 10 000 × g for 15 min. One milliliter aliquot of supernatant was mixed with 1 mL of acid ninhydrin and glacial acetic acid at the ratio of 1:1. The resulting mixture was heated at 100 °C for 1 h in a water bath and cooled quickly on an ice bath to stop the reaction. Toluene (5 mL) was added to each tube and the absorbance was read at 520 nm. Free proline content was determined using a calibration curve and expressed as µmol/g FW.

Anti-oxidative enzymes activities and malondialdehyde (MDA) content

The samples were washed with distilled water and ground with a mortar and a pestle under the chilled condition in homogenization buffer specific for each enzyme. The homogenate was filtered through four layers of muslin cloth and centrifuged at 10 000 × g for 20 min at 4 °C, and the supernatant was used for enzyme and MDA assays. The activities of superoxide dismutase (SOD), peroxidase (POD) and the accumulation of MDA content were simultaneously determined according to Zhang (1992).

SOD activity was assayed by using the photochemical nitro-blue tetrazolium (NBT) method. The samples (0.5 g) were homogenized in 5 mL extraction buffer consisting of 50 mmol/L phosphate, pH 7.8, 0.1% bovine serum albumin (BSA), 0.1% ascorbate, 0.05% β-mercaptoethanol. The assay mixture (3 mL) contained 50 mmol/L phosphate buffer, pH 7.8, 9.9 mmol/L L-methionine, 57 mmol/L NBT, 0.025% Triton X-100 and 0.0044% riboflavin. The photo-reduction of NBT (formation of purple formazan) was measured at 560 nm and an inhibition curve was made against different volumes of extract. One unit of SOD is defined as being present in the volume of extract that causes inhibition of the photo-reduction of NBT by 50%.

POD activity was measured with guaiacol as the substrate in a total volume of 3 mL. The reaction mixture consisted of 50 mmol/L potassium phosphate buffer (pH 6.1), 1% guaiacol, 0.4% H₂O₂ and enzyme extract. Increase in the absorbance due to oxidation of guaiacol [\( E = 25.5 \text{ mmol/(L·cm)} \)] was measured at 470 nm. Enzyme activity was calculated in terms of µmol of guaiacol oxidized per gram fresh weight in 1 min at (25 ± 2) °C.

The level of lipid peroxidation was expressed as MDA content and was determined as 2-thiobarbituric acid (TBA) reactive metabolites. Plant fresh tissues (0.2 g) were homogenized and extracted in 10 mL of 0.25% TBA in 10% trichloroacetic acid (TCA). Extract was heated at 95 °C for 30 min and then quickly cooled on ice. After centrifugation at 10 000 × g for 10 min, the absorbance of the supernatant was measured at 532 nm. Correction of non-specific turbidity was made by subtracting the absorbance value taken at 600 nm. The level of lipid peroxidation was expressed as mmol/g fresh weight by using an extinction coefficient of 155 mmol/(L·cm).

Statistic analysis

The measurements were done in triplicate and all data presented are the mean values. Statistical analysis was performed using the software SPSS 13.0 (SPSS Inc., 1989–2004). The results were subjected to a one-way ANOVA, and the Tukey test was used to determine significant differences between the means.

RESULTS

Growth response and chlorophyll content

Changes in plant growth expressed as shoot height,
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Root length and root volume of the two rice genotypes after 20 d in solution culture treatment are shown in Table 1. The most obvious response of rice plants to Al addition was characterized by a significant reduction in root growth, while root growth was favored at the expense of shoot growth as evidenced by a higher root/shoot ratio (data not shown) in the P individual stressed plants. In comparison with the control, root length of stressed plants under Al toxicity and P deficiency was decreased by 24.8% and 15.6%, respectively. P deficiency further enhanced Al toxicity in terms of growth inhibition. Moreover, both stress treatments led to a more severe growth inhibition in the Al sensitive genotype than in the Al-tolerant genotype, especially for root growth.

As shown in Table 2, Al addition and P starvation single and combination all significantly decreased chlorophyll (Chl) content in the both rice genotypes. It was noted that there was more seriously decrease in the Chl a content than in the Chl b content, as shown by the value of Chl a/Chl b. P starvation in the presence of Al induced the further decrease in Chl content, especially for Al-sensitive genotype Yongyou 8.

Free proline content

P starvation and Al toxicity both resulted in significant increase in proline content in rice leaves (Fig. 1). P deficiency aggravated this increase in the plants exposed to Al solution. Further, a great genotypic difference in proline accumulation in response to stress condition was observed, and more obvious increase was detected in Yongyou 8.

MDA content, and SOD and POD activities

On the 20 d after treatment, necrotic lesions and yellow dots could be observed on the rice leaves of the plants exposed to Al toxicity and P deficiency. These toxicity symptoms were consistent with 1–2 folds increase of MDA content in both roots and leaves. More significant increase of MDA content was detected in Yongyou 8 seedlings under the combined solution of Al addition and P deficiency than other treatments, which was about 2-fold of the control in roots (Table 3).

Al toxicity and P deficiency induced the similar changes of SOD and POD activities, resulting in the decrease in leaves and increase in roots for the two

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**Table 1. Growth of rice seedlings exposed to Al toxicity and P deficiency.**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Shoot height (cm)</th>
<th>Root length (cm)</th>
<th>Root volume (mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Xiushui 132</td>
<td>Yongyou 8</td>
<td>Xiushui 132</td>
</tr>
<tr>
<td>CK</td>
<td>39.5 ± 1.6 a</td>
<td>41.6 ± 0.9 a</td>
<td>26.2 ± 1.0 a</td>
</tr>
<tr>
<td>Al+</td>
<td>33.5 ± 1.2 b</td>
<td>32.3 ± 1.1 c</td>
<td>20.8 ± 0.6 c</td>
</tr>
<tr>
<td>P-</td>
<td>35.3 ± 1.1 b</td>
<td>35.2 ± 0.9 b</td>
<td>23.0 ± 0.4 b</td>
</tr>
<tr>
<td>Al+ and P-</td>
<td>30.8 ± 0.5 c</td>
<td>29.3 ± 1.4 d</td>
<td>18.0 ± 0.6 c</td>
</tr>
</tbody>
</table>

**Table 2. Chlorophyll content in rice leaves exposed to Al toxicity and P deficiency.**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Treatment</th>
<th>Chl a</th>
<th>Chl b</th>
<th>Total Chl</th>
<th>Chl a/Chl b</th>
</tr>
</thead>
<tbody>
<tr>
<td>Xiushui 132</td>
<td>CK</td>
<td>2.82 ± 0.21 a</td>
<td>1.27 ± 0.22 a</td>
<td>4.10 ± 0.16 a</td>
<td>2.21 ± 0.19 a</td>
</tr>
<tr>
<td>Al+</td>
<td>1.95 ± 0.11 c</td>
<td>0.93 ± 0.05 b</td>
<td>2.88 ± 0.14 c</td>
<td>2.08 ± 0.27 b</td>
<td></td>
</tr>
<tr>
<td>P-</td>
<td>2.18 ± 0.05 b</td>
<td>1.04 ± 0.11 b</td>
<td>3.22 ± 0.09 b</td>
<td>2.09 ± 0.33 b</td>
<td></td>
</tr>
<tr>
<td>Al+ and P-</td>
<td>1.54 ± 0.09 c</td>
<td>0.73 ± 0.08 c</td>
<td>2.27 ± 0.08 c</td>
<td>2.10 ± 0.34 b</td>
<td></td>
</tr>
<tr>
<td>Yongyou 8</td>
<td>CK</td>
<td>2.99 ± 0.26 a</td>
<td>1.40 ± 0.10 a</td>
<td>4.38 ± 0.24 a</td>
<td>2.13 ± 0.12 a</td>
</tr>
<tr>
<td>Al+</td>
<td>1.52 ± 0.26 c</td>
<td>0.85 ± 0.07 b</td>
<td>2.37 ± 0.25 b</td>
<td>1.78 ± 0.21 c</td>
<td></td>
</tr>
<tr>
<td>P-</td>
<td>1.89 ± 0.18 b</td>
<td>0.91 ± 0.05 b</td>
<td>2.80 ± 0.12 b</td>
<td>2.07 ± 0.32 b</td>
<td></td>
</tr>
<tr>
<td>Al+ and P-</td>
<td>1.15 ± 0.16 d</td>
<td>0.58 ± 0.06 c</td>
<td>1.73 ± 0.18 c</td>
<td>1.99 ± 0.06 bc</td>
<td></td>
</tr>
</tbody>
</table>
anti-oxidative enzymes activities. Moreover, more changes were observed in Yongyou 8 than in Xiushui 132. Further, in P deficiency and Al toxicity individual solution, POD activity was enhanced in roots but reduced in leaves, whereas the combined solution decreased SOD activity both in roots and leaves, compared with the single stress treatments and CK (Table 3).

**DISCUSSION**

Crop production is greatly reduced when crops were grown in acid soils where P deficiency and Al toxicity are two major factors limiting plant growth (Kochian et al, 2004). Although liming of acid soils can ameliorate soil acidity, this is neither an economic option for poor farmers nor an effective strategy for alleviating subsoil acidity. In fact, plants grown under such conditions may have developed numerous adaptive strategies to cope with multiple stress such as P deficiency and Al toxicity.

In the present study, Al toxicity and P deficiency both reduced plant growth and chlorophyll content (Tables 1 and 2), which coincided with the report by Tan and Keltjens (1990), who observed similar reduction in shoot and root growth by 0.4 mg/L Al treatment and suboptimal P treatment in sorghum plants. Xiushui 132 showed higher tolerance to both Al toxicity and P deficiency, suggesting the potential of obtaining dual-tolerant genotypes from rice germplasm. Moreover, Al exposure under P starvation produced severe growth inhibition, indicating that there is synergistic effect of P deficiency on Al toxicity to rice seedlings growth.

Proline accumulation in response to environmental stress has been intensively reported (Watanabe et al, 2000; Ashraf and Harris, 2004; Mansour et al, 2005), and is expected to play a role in cellular osmotic adjustment. In this study, proline content significantly increased in rice seedlings exposed to both Al toxicity and P deficiency solution, which was similar to previous findings. These results showed Al toxicity and P deficiency induced proline accumulation in mungbean (Yang and Chen, 2001), carrot and radish (Ismail, 2005), and lentil seedlings (Sarker and Karmoker, 2011). The accumulation of proline might be primarily due to increased synthesis under stress conditions (Madan et al, 1995), and the increased content, in addition to its role as solute that protects macromolecules against denaturation (Schober and Tschesche, 1978), and reduces oxidative stress caused by the free radicals produced in response to stress conditions (Hare et al, 1998). P deficiency also aggravated the increase of proline accumulation in the plants exposed to Al solution. Moreover, higher increase of proline content was observed in Al-sensitive genotype, indicating that the difference between the varieties in response to P starvation and Al toxicity could be assessed by proline content.

The lipid peroxidation is the most prominent symptom of oxidative stress. MDA, an oxidized product of membrane lipids, can indicate the extent of oxidative stress (Chaoui et al, 1997). In the present study, the response pattern was similar in leaves and roots, with a significant MDA content increase from control plants to those grown in the stress solution. The observed increase in MDA content indicated that lipid peroxidation occurred as a result of ROS formation induced by high level of Al toxicity and P deficiency. This is in agreement with previous reports that MDA content in rice plants was increased under Al toxicity and P starvation stress (Kuo and Kao, 2003; Tewari et al, 2004). More increase of MDA content under the combined stress treatment, especially in roots, indicated the positive effect of P deficiency on Al toxicity in inducing oxidative stress. Simultaneously, to cope with the harmful effects of ROS, plants cells are equipped with a well-developed antioxidant defense system comprising enzymes, such as SOD, POD, catalase

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Treatment</th>
<th>MDA content (nmol/g)</th>
<th>SOD activity (U/g)</th>
<th>POD activity [μmol/(min·g)]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Leaf</td>
<td>Root</td>
<td>Leaf</td>
</tr>
<tr>
<td>Xiushui 132</td>
<td>CK</td>
<td>11.2 ± 1.3 d</td>
<td>11.8 ± 1.4 c</td>
<td>51.1 ± 4.3 a</td>
</tr>
<tr>
<td></td>
<td>Al+</td>
<td>14.9 ± 2.1 b</td>
<td>14.2 ± 1.6 b</td>
<td>40.8 ± 2.6 b</td>
</tr>
<tr>
<td></td>
<td>P-</td>
<td>12.7 ± 2.3 c</td>
<td>12.9 ± 2.0 bc</td>
<td>49.8 ± 4.9 a</td>
</tr>
<tr>
<td></td>
<td>Al+ and P-</td>
<td>17.0 ± 1.3 a</td>
<td>18.1 ± 2.2 a</td>
<td>35.6 ± 3.9 c</td>
</tr>
<tr>
<td>Yongyou 8</td>
<td>CK</td>
<td>10.9 ± 0.9 c</td>
<td>10.1 ± 1.2 d</td>
<td>56.8 ± 2.8 a</td>
</tr>
<tr>
<td></td>
<td>Al+</td>
<td>14.2 ± 2.6 b</td>
<td>16.3 ± 2.2 c</td>
<td>42.8 ± 3.7 b</td>
</tr>
<tr>
<td></td>
<td>P-</td>
<td>13.6 ± 1.7 b</td>
<td>13.7 ± 1.6 b</td>
<td>51.4 ± 5.1 c</td>
</tr>
<tr>
<td></td>
<td>Al+ and P-</td>
<td>18.3 ± 1.2 a</td>
<td>20.3 ± 2.0 a</td>
<td>35.7 ± 3.1 d</td>
</tr>
</tbody>
</table>

CK, Control; Al+, Al addition (1.5 mmol/L Al); P-, Low P level (1/30 of normal P supply).

The same lowercase letters after numbers within a column for the same genotype indicate no significant differences between treatments ($P > 0.05$).
(CAT), glutathione peroxidase (GPX), ascorbate peroxidase (APX) and glutathione reductase (GR) (Yamamoto et al., 2003). SOD, which is the most effective anti-oxidative enzyme in preventing cellular damage, catalyzes the conversion of the superoxide anion to $\text{H}_2\text{O}_2$, while POD utilizes $\text{H}_2\text{O}_2$ in the oxidation of various inorganic and organic substrates. In this study, both Al toxicity and P deficiency individual treatments increased SOD and POD activities in roots, but decreased them in leaves. However, it is reported that Al toxicity and P deficiency both increased SOD and POD activities in maize and rice plants (Tewari et al., 2004; Sharma and Dubey, 2007). Both genotypes showed low SOD and POD activities in leaves with severe inhibition of root growth in this study, suggesting that stress-damaged rice could not continuously elevate anti-oxidative enzymes activities. P deficiency in Al added solution produced positive effect on POD activity in roots. Whereas, the combined solution decreased POD activity both in roots and leaves, and POD activity in leaves, compared with the single stress condition, revealing a close association between the acid soil sensitivity and POD activity increase in roots of rice seedlings.

ACKNOWLEDGEMENTS

This work was supported by the Natural Science Fund of Zhejiang Province, China (Grant No. Y3100450) and the Educative Department of Zhejiang Province, China (Grant No. Z201018672).

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