JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

J. Agric. Food Chem. **2006,** *54,* 10033–10039 **10033**

Aluminum Tolerance of Two Wheat Cultivars (*Brevor* and *Atlas66*) in Relation to Their Rhizosphere pH and Organic Acids Exuded from Roots

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Phytotoxicity of aluminum (Al) has become a serious problem in inhibiting plant growth on acid soils. Under Al stress, the changes of rhizosphere pH, root elongation, absorption of Al by wheat roots, organic acids exuded from roots, and some main factors related to Al-tolerant mechanisms have been studied using hydroponics, fluorescence spectrophotometry, and high performance liquid chromatography (HPLC). Two wheat cultivars, *Brevor* and *Atlas66*, differing in Al tolerance are chosen in the study. Accordingly, the rhizosphere pH has a positive effect on Al tolerance. *Atlas66* (Al-tolerant) has higher capability to maintain high rhizosphere pH than *Brevor* (Al-sensitive) does. High pH can reduce Al^{3+} activity and toxicity, and increase the efficiency of exuding organic acids from the roots. More inhibition of root elongation has been found in *Brevor* because of the exposure of roots to Al^{3+} solution at low pH. *Brevor* accumulate more Al in roots than *Atlas66* even at higher pH. Al-induced exudation of malic and citric acids has been found in *Atlas66* roots, while no Al-induced organic acids have been found in *Brevor*. These results indicate that the Al-induced secretion of organic acids from *Atlas66* roots has a positive correlation with Al tolerance. Comprehensive treatment of Al^{3+} and H^+ indicates that wheat is adversely influenced by excess Al^{3+}, rather than low pH.

KEYWORDS: Al stress; rhizosphere pH; organic acids; root exudates; wheat (*Triticum aestivum* L.)

INTRODUCTION

Aluminum (Al) is the most abundant metal in the earth's crust and occurs in a number of different species in the soil. Al toxicity restricts crop productivity in acid soils that cover almost 40% of the world's arable land (*1*, *2*). In neutral or alkaline soil, Al is found mainly to exist as oxide or silicate precipitates that are not toxic to plants. However, in acid soil (pH < 5.0), Al speciates to a soluble octahedral hexahydrate form, which is believed to be the primary phytotoxic Al species (*3*). In China, acid soil has been found in 15 provinces with a total area of 2.0×10^6 km^2 and covers up to 21% of the overall cultivable land (*4*). Active Al^{3+} has poisonous effects on plant root systems, inhibition of root growth is a well-known effect of Al toxicity, and root tips have been suggested as a primary site for Al-induced injury in plants (*5*, *6*). Thus, Al-tolerant mechanisms focusing on the complicated rhizosphere environment have mainly been investigated in various plants in order to improve productivity on acid soils (*7*, *8*). Recently, there have been many publications on the Al-tolerant mechanism in various plants. Many scientific hypotheses have been put forward *(9)*, including release of other Al-chelating ligands, changes in internal levels of Al-chelating compounds in the root, and Al translocation to the shoot (*10*). Foy et al. indicated that the elevation of rhizosphere pH was one of the Al-tolerant mechanisms. Elevation of rhizosphere pH could reduce the Al solubility and its potential toxicity, which was in favor of the formation of less-toxic Al species such as Al hydroxides and Al phosphates etc., and would also help the exudation of organic acids from roots (*11*). In studying the Al tolerance of wheat (*Triticum aestivum* L.), barley (*Hordeum vulgare* L.), pea (*Pisum sativum* L.), sorghum (*Sorghum bicolor* L. *Moench*), ryegrass (*Lolium perenne*), clover (*Trifolium resupinatum* L.), etc., it was found that there was also a positive correlation between transient increases in rhizosphere pH and Al tolerance (*12*−*17*). All of these studies were based on pH measurements of the rhizosphere solution, which reflected that the pH changes were only associated with the whole root rather than the root tip which was thought the primary part of Al toxicity (*18*).

Rhizosphere is a dynamic microenvironment, in which many new substances are released constantly and more secondary compounds will be produced under environmental stress (*19*). Rhizosphere can influence plant growth and crop productivity. Some organic acids are identified in root exudates of many plants (*20*, *21*). Ma et al. thought that the plant roots under Al

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10.1021/jf0611769 CCC: $33.50
Published on Web 12/20/2006

stress might exude various organic acids, including citric and malic acids. These organic acids can play a very important role in degrading the soil Al poisoning (*22*). Organic acids are known to be strong Al chelators. The chelated Al species is less phytotoxic than the ionic form Al^{3+} (*23*). The rhizosphere chelation of Al^{3+}, Fe^{3+}, and Ca^{2+} with organic acids can reduce rhizosphere pH and release less-soluble P and increase notably the concentration of available PO_4^{3-} for plant uptake in rhizosphere soil, which act in important adaptive processes in the rhizosphere, such as PO_4^{3-}, Fe^{3+}, and Ca^{2+} acquisition and Al tolerance. The organic acid metabolism and its potential manipulation may represent a way to understand fundamental aspects of plant physiology and lead to new strategies to obtain crop varieties better adapted to environmental stress, including Al stress (*24*).

In this paper, attempts are made to link the Al tolerance, rhizosphere pH, and organic acids exuded from wheat roots under Al stress. Two wheat (*Triticum aestivum* L.) cultivars, *Atlas66* (Al-tolerant) and *Brevor* (Al-sensitive), are selected to elucidate the correlation among the change in rhizosphere pH, organic acids in root exudates, and wheat Al tolerance by hydroponics and high performance liquid chromatography (HPLC). This study will provide the experimental results to reveal wheat Al tolerance scientifically.

MATERIALS AND METHODS

Plant Materials and Growth Conditions. Two wheat cultivars, *Brevor* and *Atlas66*, were chosen as the tested materials (*25, 26*). The uniform seeds were surface sterilized in 1% (v/v) sodium hypochlorite for 20 min. They were then washed, soaked for 1 day, and placed on a net tray in a lightless culture box (Changzhou GuoHua Instrument Co., China) at 25 °C for accelerating germination for 2 days. The tray was put on a plastic container filled with 0.5 mM $CaC1_2$ solution at pH 4.5. The solution was renewed every day. Those well-developed emergence seeds were placed on the nylon network, which was floated on a 1 L plastic pot (ten seedlings per pot) containing aerated Foy's nutrient solution after 4−5 days (*11*). In a 250 L culture box at 25 °C, the solution was adjusted to pH 4.5 with 1 M HC1 and renewed every other day. After 8 to 10 days of culture in the above-mentioned nutrient solution, the wheat seedlings were grown in a controlled culture box with a 14 h light/10 h dark cycle under 40 W m^{-2} light. The light/dark temperatures were set at 25/20 °C, and relative humidity was kept at 65%.

Preparation of Root Exudates. To obtain samples of root exudates, the procedure of Tyler and Ström was followed (*27*). To avoid interaction between $A1^{3+}$ and other nutrients such as PO_4^{3-}, a simple salt solution containing 0.5 mM $CaC1_2$ was used as the basal treatment. The pH of solutions with or without nutrients was adjusted with 10% NaOH and 10% HCl. The treated wheat roots need to be carefully washed numerous times after being placed in tap water for 0.5 h: 5 to 10 times starting with tap water, then another 3 to 5 times with distilled water, and finally a further 2 to 3 times with Milli-Q purified water. Subsequently, due to different study objectives, the specific procedure for collection of root exudates was as follows:

(1) To examine the influence of Al treatment on the secretion of organic acids, two-week-old wheat seedlings were grown in nutrient solution with no Al for 8 days, and root exudates were collected every other day in 0.5 mM $CaCl_2$ solution at pH 4.5 after 12 h. On 10, 12, 14 and 16 days, these roots were exposed to 80 mg·L^{-1} Al solution for 12 h (for **Figure 1**).

(2) To confirm the Al-induced secretion of organic acid, two-week-old wheat seedlings were cultivated in pH 4.5−0.5 mM $CaCl_2$ solution containing 0, 20, 40, 60, 80 mg·L^{-1} Al to further grow for 12 h (for **Figure 2**).

(3) To check the effects of Al stress and acid stress on secretion of organic acids, two-week-old seedlings were exposed to pH 4.5−0.5 mM $CaCl_2$ solutions without Al and containing 80 mg·L^{-1} Al, and to pH 6.8−0.5 mM $CaCl_2$ solution without Al for 12 h. For comparison, exudates of roots continuously exposed to 80 mg·L^{-1} or 0 mg·L^{-1} Al (pH 4.5 and pH 6.8) were also collected at the same interval (12 h) (for **Figure 4**).

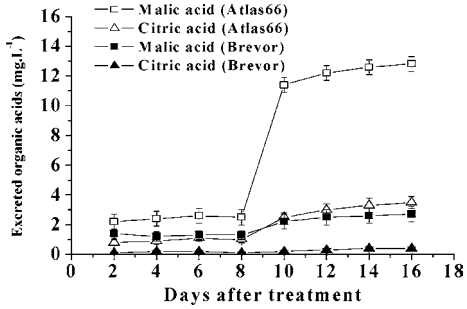

Figure 1. Effect of without Al and followed by Al treatment on the secretion of organic acids by wheat roots. Two-week-old wheat seedlings were grown in nutrient solution with no Al for 8 days, and root exudates were collected every other day in 0.5 mM $CaCl_2$ solution at pH 4.5 after 12 h. On days 10, 12, 14, and 16 after no Al, the roots were exposed to 80 mg·L^{-1} Al solution, and the root exudates were collected after 12 h. Error bars represent ± SD ($n = 9$).

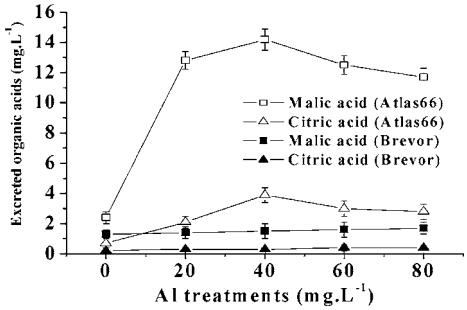

Figure 2. Effect of Al contents on the secretion of organic acid by wheat roots. Two-week-old wheat seedlings were exposed to pH 4.5 0.5 mM $CaCl_2$ solution containing 0, 20, 40, 60, 80 mg·L^{-1} Al. After 12 h the root exudates were collected and organic acids were analyzed. Error bars represent ± SD ($n = 9$).

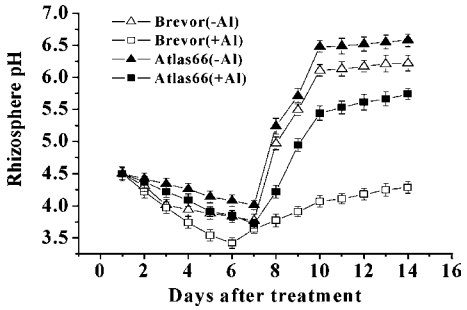

Figure 3. Rhizosphere solution pH with culture time under normal conditions or Al stress. Two-week-old wheat seedlings were exposed to pH 4.5 nutrient solutions without Al as well as containing 40 mg·L^{-1} Al for the collection of rhizosphere solution every day. Error bars represent ± SD ($n = 9$).

(4) To reveal the correlation of rhizosphere pH and organic acid secretion, two-week-old wheat seedlings were grown in pH 4.5, 5.5, and 6.5 0.5 mM $CaCl_2$ solution without Al for 12 h, respectively (for **Table 3**).

The corresponding root exudates were filtrated and then concentrated to a certain volume in a rotatory evaporator, respectively. They were analyzed immediately or stored in a refrigerator at −20 °C for future analysis.

Al Accumulation, Root Elongation, Organic Acid, and Rhizosphere pH Measurements. Two-week-old *Brevor* and *Atlas66* seedlings were exposed to pH 4.5 0.5 mM $CaCl_2$ solutions with 0, 20, 40,

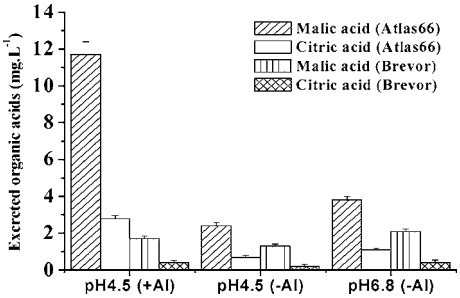

Figure 4. Effects of Al stress and acid stress on secretion of organic acids. Two-week-old seedlings were exposed to pH 4.5 0.5 mM $CaCl_2$ solutions without Al and containing 80 mg·L^{-1} Al, and to pH 6.8 0.5 mM $CaCl_2$ solution without Al for 12 h. For comparison, exudates of roots continuously exposed to 80 mg·L^{-1} or 0 mg·L^{-1} Al (pH 4.5 and pH 6.8) were also collected at the same interval. Error bars represent ± SD (n = 9).

Table 1. Aluminum Accumulation in *Atlas66* and *Brevor* under Different Al Treatments[a]

	Al accumulation (mg·kg^{-1}·fresh·wt)	
Al (mg·L^{-1})	*Atlas66*	*Brevor*
0	0.2 ± 0.03[b]	0.8 ± 0.05
20	0.5 ± 0.04	1.5 ± 0.1
40	0.9 ± 0.05	2.7 ± 0.2
60	1.1 ± 0.06	3.0 ± 0.2
80	1.2 ± 0.06	3.2 ± 0.2

[a] Two-week-old wheat seedlings were exposed to pH 4.5 0.5 mM $CaCl_2$ solutions with 0, 20, 40, 60, 80 mg·L^{-1} Al respectively, and the wheat roots were collected for 24 h. [b] Data are means ± SD (n = 9) .

60, 80 mg·L^{-1} Al solution respectively, and the wheat roots were collected for 24 h (For **Table 1**). The 8-hydroxyquinoline-fluorescence spectrophotometry was used to determine Al absorption of their roots (*28*, *29*). These treated wheat roots needed to be carefully washed numerous times. The sample surface was then dried by filter paper. After being cut to small pieces, treated wheat roots (2 g), ground with 5 mL of deionized water in a grinding mill, were transferred to a centrifuge tube. The grinding mill was cleaned with 5 mL of deionized water and the rinsing of the sample solution was poured into the centrifuge tube. Then, by centrifugation (ca. at 12*g*), for 15 min the supernatant was passed through anion- and cation-exchange columns respectively, and the upper sample solution was eluted with a suitable eluent and then filtrated and concentrated to a certain volume in a rotatory evaporator. The total Al in the sample reacted with added 8-hydroxyquinoline to form a fluorescent complex in the $NH_4Ac-NH_3\cdot H_2O$ buffer system. The complex was extracted with $CHCl_3$. The fluorescence intensity of the extract was measured at emission wavelength 530 nm in a 1 cm quartz cell by a VerdFluor fluorophotometer [Bole (B10-RAB), USA], keeping the excitation wavelength 365 nm, and the blank solution was measured simultaneously. This established fluorescence spectrophotometry, which uses standard quinine sulfate as the reference for fluorescence strength, is suitable for the determination of Al content from 0.002 to 0.24 mg·L^{-1}.

To observe Al- and proton-induced effects on root growth of *Atlas66* and *Brevor*, two-week-old wheat seedlings were exposed to pH 4.5 and 5.0 0.5 mM $CaCl_2$ solutions with or without 80 mg·L^{-1} Al^{3+} for 16 h (For **Table 2**). Root lengths were measured with a ruler before and after treatments. The inhibition rate was calculated by dividing the growth of roots without Al exposure by the difference of that with and without Al stress (*9*).

The organic acid analysis was modified from the methods of Shen et al. (*30*). The root exudates and root extracts were passed through a cation-exchange column (16 × 14 mm) filled with 5 g of Amberlite IR-120B resin (H^+ form, Chemical Co., Shanghai, China), followed by an anion-exchange column (16 × 14 mm) filled with 2 g of Dowex 1 × 8 resin (100−200 mesh, format form) at room temperature. The

Table 2. Aluminum- and Proton-Induced Effects on Root Growth of *Atlas66* and *Brevor*[a]

		root elongation (cm·16 h^{-1})	
Al (mg·L^{-1})	rhizosphere pH	*Atlas66*	*Brevor*
0	4.5	1.4 ± 0.1[b]	1.2 ± 0.1
80	4.5	1.3 ± 0.1	0.8 ± 0.1
0	5.0	1.9 ± 0.2	1.3 ± 0.1
80	5.0	1.8 ± 0.1	1.1 ± 0.1

[a] Two-week-old wheat seedlings were exposed to pH 4.5 and 5.0 0.5 mM $CaCl_2$ solutions with or without 80 mg·L^{-1} Al for 16 h. [b] Data are means ± SD (n = 30).

Table 3. Correlation of Rhizosphere pH and Organic Acid (mg L^{-1}) Contents in Root Secretion[a]

	Altas66		*Brevor*	
rhizosphere pH	malic acid	citric acid	malic acid	citric acid
4.5	2.4 ± 0.2[b]	0.7 ± 0.1	1.3 ± 0.1	0.2 ± 0.05
5.5	3.4 ± 0.2	1.0 ± 0.1	1.7 ± 0.1	0.2 ± 0.05
6.5	3.7 ± 0.2	1.2 ± 0.1	1.9 ± 0.1	0.3 ± 0.05

[a] Two-week-old wheat seedlings were exposed to pH 4.5, 5.5, and 6.5 0.5 mM $CaCl_2$ solution without Al. After 12 h the root exudates were collected. [b] Data are means ± SD (n = 9).

organic acids retained on anion-exchange resin were eluted by 1 M HCl, and the eluate was concentrated to dryness by a rotary evaporator (40 °C). After that, the residue was redissolved with dilute $HClO_4$ solution at pH 2.1. The malic and citric acids were analyzed by Waters 600 HPLC with Waters 486 ultraviolet detector; 25 μL of the filtered solution was injected onto a Biorad HPX-87H column (*31*−*33*). The column was placed in an oven set at 55 °C. The mobile phase, 5 mM H_2SO_4 solution, was flowed at 0.6 mL·min^{-1}. The detection wavelength was 210 nm. Qualitative analysis was based upon comparing data with regard to the retention time, while quantitative analysis was conducted using the peak height external standard method. At the same time, malic and citric acids standards (3−20 μM) were added to the root exudates to confirm the analytical results (*34*).

To test whether the rhizosphere pH for both *Brevor* and *Atlas66* changed significantly under normal growth conditions or Al stress (40 mg·L^{-1} Al), two-week-old wheat seedlings were exposed to pH 4.5 ± Al nutrient mediums for the collection of rhizosphere solution every day (for **Figure 3**), and the nutrient solution pH was determined by a PHS-25 digital display acidimeter (Shanghai Precision Instruments Co., China) at the same time daily.

Quality Control. The analytical method validation was confirmed with fortification and recovery studies. Nine replicates were made for each treatment. The overall precisions of the HPLC and fluorescence spectrophotometry were <15% for all the real samples. To characterize the distribution of the data, all data present here are expressed as arithmetic means of nine observations ± SD. All chemicals and standards were labeled as AR or GR levels, and Milli-Q purified water (Millipore, Eschborn, Germany) was used throughout the study period.

RESULTS

Aluminum Accumulation and Al Toxicity. The absorption of Al content by *Brevor* and *Atlas66* roots increased slightly as the external Al levels increased. After the two-week-old wheat seedlings were cultivated in pH 4.5 0.5 mM $CaCl_2$ solutions with 0, 20, 40, 60, 80 mg·L^{-1} Al for 24 h, respectively, the Al concentrations accumulated in *Brevor* and *Atlas66* roots increased. Compared with *Brevor*, Al-tolerant cultiver *Atlas66* absorbed less Al at the same Al concentration, as shown in **Table 1**.

The toxicity of Al^{3+} to plant is characterized by rapid inhibition of root elongation. To confirm Al resistance of wheat,

the effect of Al on root elongation is compared between *Atlas 66* and *Brevor*. After exposure to 80 mg·L^{-1} Al for 16 h (pH 4.5), the root elongations of *Atlas66* and *Brevor* significantly reduce with an average inhibition of 7% and 33% compared with the control **(Table 2)**. The reduced growth of *Brevor* root elongation seem to be due to higher Al^{3+} accumulation in the control (0.8 mg kg^{-1}·fresh·wt, **Table 1**) than in *Atlas66* (0.2 mg kg^{-1}·fresh·wt, **Table 1**), and also in the presence of 80 mg·L^{-1} Al^{3+}, *Brevor* accumulated more Al^{3+} (3.2 mg·kg^{-1}·fresh·wt) than *Atlas66* (1.2 mg·kg^{-1}·fresh·wt, Al^{3+}), which accumulated 2.0 mg·kg^{-1}·fresh·wt less Al^{3+} **(Table 1)**. These results indicated that *Atlas66* is more resistant for Al^{3+} inhibition at pH 4.5 due to genetic capacity to accumulate less Al^{3+}. When the rhizosphere pH is increased from 4.5 to 5.0, in the presence of A1, the root growth rates increase 38.5% and 37.5% for *Atlas66* and *Brevor*, respectively. Therefore, an increase in rhizosphere pH of 0.5 unit caused a significant increase in A1 resistance. The results indicate that when the pH is increased to the alkaline side, *Atlas66* develops a protection mechanism for Al toxicity to root elongation.

Al-Induced Organic Acid Exudation. The specificity of Al-induced secretion of organic acids was investigated by comparing it with the root's responses to Al deficiency. Organic acids in root exudates were monitored every other day after the initiation of Al-deficiency treatment, and it was not significantly secreted up to 8 days **(Figure 1)**. When Al was added to the Al-deficient roots on day 10, a significant amount of organic acids was secreted. The malic and citric acids secreted by *Atlas66* were significantly higher than those by *Brevor* at any time. Malic acid was the major component and was greatly affected by Al treatment. A marked difference was found in the presence or absence of Al for *Atlas66*. However, no marked difference was found for *Brevor* **(Figure 1)**. Under control experiment without Al in pH 4.5 nutrient solution for 8 days, the both cultivars did not induce significant secretion of malic and citric acids. After 2 days of treatment with 80 mg·L^{-1} Al, significant amounts of malic and citric acids were secreted, with a slight extent in *Brevor* but more extensively in *Atlas66*. Compared with unstressed control, on day 10, Al treatment let to 1.7−4.6-fold enhanced exudation of organic acids in the two cultivars, respectively **(Figure 1)**.

The Al-induced secretion of organic acids for *Brevor* and *Atlas66*, both pretreated with $AlCl_3$ solution for 12 h, is shown in **Figure 2**. For the Al-tolerant cultivar *Atlas66*, over the external Al concentration ranging from 0 to 40 mg·L^{-1}, the organic acids exuded from roots were increased with added Al. The highest concentrations of malic and citric acids were nearly 14.2 and 3.9 mg·L^{-1} after *Atlas66* roots were exposed to 40 mg·L^{-1} Al solution. When the external Al concentration was over 40 mg·L^{-1}, the exudation of organic acids decreased slightly with increasing Al concentration, and then gradually decreased to the control level (−Al). In contrast, malic and citric acids exuded from *Brevor* roots slightly increased with raising A1 concentrations (from 0 to 80 mg·L^{-1}). Malic acid exudation increased from 1.3 to 1.7 mg·L^{-1}, and citric acid was from 0.05 to 0.2 mg·L^{-1}, respectively. Their increase was so small that graphically the line for malic and citric acids exuded from *Brevor* roots was almost horizontal with a slightly upward trend. *Atlas66* exuded more malic and citric acids than *Brevor* at all Al concentrations **(Figure 2)**.

Al-Induced Variance of Rhizosphere pH and Effect of This Variance on Organic Acid Exudation. Figure 3 presents the changes of rhizosphere pH with culture time without or with Al (40 mg·L^{-1} Al) for *Brevor* and *Atlas66*. Initially, the pH value reduced slowly for both *Brevor* and *Atlas66*, dropping to a minimum at 6 or 7 days. However, after 7 days it increased rapidly under either normal conditions or Al stress. Even though the changes in rhizosphere pH under the two growth conditions had the same trend, there was a significant difference in magnitudes. Under Al treatment, the rhizosphere pH was lower than that under normal growth conditions. Note that pH in *Atlas66* was always higher than that in *Brevor*. Thus, in the presence of Al, *Atlas66* could alkalinize the rhizosphere at the root surface, the small increase in rhizosphere pH perhaps related to its increased Al resistance.

Increasing rhizosphere pH without Al stress could induce wheat roots to exude more organic acids **(Table 3)**. The Al-tolerant *Atlas66* exuded more organic acids (malic and citric acids) to its rhizosplere than the Al-sensitive *Brevor* in response to Al. The malic acid level exuded by the two cultivars was higher than the citric acid level.

Under Al treatment, *Brevor* and *Atlas66* exuded certain amounts of organic acids from their roots. But *Atlas66* exuded higher amounts of citric and malic acids than *Brevor* **(Figure 4)**. Under rhizosphere pH 4.5 without Al, the two cultivars exuded slightly higher amounts of organic acids than those under rhizosphere pH 6.8 without Al. Organic acid exudation at pH 4.5 was significantly lower than that at pH 6.8. The rate of citric acid excretion at pH 4.5 was about 62% that at pH 6.8. When they suffered from Al stress and acid stress simultaneously, the exuded organic acids induced by Al stress were obviously more than those by acid stress.

DISCUSSION

Aluminum Accumulation and Al Toxicity. Free Al^{3+} ions in nutrient solution can be absorbed by plant roots. A high concentration of $A1^{3+}$ will become toxic to the plant (*35*). At pH 5 and below, Al^{3+} may accumulate to toxic levels that prevent root growth and plant functions. How can Al^{3+} be tolerated by plants? Our results are probably best interpreted as follows: free Al^{3+} ions are generally considered to be the most phytoxic. It might be postulated that the OH^- and H^+ are transported across the plasma membrane in the root tips of wheat to maintain higher rhizosphere pH, and to alter rooting Al species by changing the plasma membrane charge positively and reject external Al (*36*). Simultaneously, root exudates play a fundamental role in the prevention of the accumulation of phytotoxic Al species (*37*). Exclusion of Al from root tips by exudation of organic ligands with high affinity for Al^{3+} is considered a major factor for Al resistance (*38*). In addition, the Al-tolerant cultiver, *Altas66*, has a strong capability to exclude Al^{3+} from its rhizosphere, where a series of processes are also involved, mainly including Al chelation by root exudates, Al exclusion, Al transport and Al uptake across plasma membranes, and Al accumulation in root (*23*, *39*, *40*).

In this paper, inhibition of root growth is the best measure for Al toxicity symptoms, especially for the Al-sensitive cultivar *Brevor* (*5*, *9*). Barceló and Poschenrieder reported that Al-induced effect on root growth is more closely related to Al-tolerant genotypes of wheat and P deficiency. Under Al stress, phosphate is probably precipitated as insoluble Al-phosphate in the culture solution. Phosphate deficiency restricts plant root growth (*37*). However, our results indicate that Al-induced root growth inhibition is also associated with the rhizosphere pH of wheat. Elevated rhizosphere pH can reduce the Al solubility and its potential toxicity (*11*), and will also help the wheat root growth. How is the rhizosphere pH formed? As published by Song et al., the rhizosphere pH is formed from (1) the absorption

of cation and anion being out of balance; (2) CO_2 being generated by rhizosphere breath; and (3) the secretion of organic acids, H^+, and other chemical components from the roots. H^+ transport is required from the rhizosphere to inside the cell or OH^- export to the rhizosphere is involved. Ca^{2+} participates in the rhizosphere pH formation (*41*). Al-induced root elongation, as found in *Atlas66*, has frequently been observed and can be attributed to amelioration of H^+ toxicity by Al^{3+} and/or the activation of defense mechanisms (*42*), whereas the rhizosphere pH and the Al species are thought to be more important (*43*).

Al-Induced Exudation of Organic Acids. Accumulating evidence has shown that the secretion of organic acids plays an important role in plant Al resistance. One of the mechanisms responsible for this high A1 resistance is the secretion of organic acids from the roots that occur within 30 min after exposure to $A1^{3+}$ and increase with rhizosphere Al concentration (*26*, *44*). Under Al stress, Al may enhance the secreting rate of organic acid from the roots of *Atlas66* and *Brevor*. According to our results, for the same Al concentrations, the Al-induced exudation of malic and citric acids can be observed clearly in *Atlas66*, but only slightly in *Brevor*. Our followup experimental data indicate that, at the 3.0 ± 0.4 mg·L^{-1} level, $A1^{3+}$ activates the production of malic acid. The secretion of malic acid is high within a certain level of external Al^{3+} (3.0−120 mg·L^{-1}). When *Atlas66* was exposed to 120 mg·L^{-1} $A1^{3+}$, the secretion of malic acid decreased to the control level (−Al). **Figure 1** presents that the cultivar *Atlas66* exudes 11.4 ± 0.5 mg·L^{-1} of malic acid in 2 days exposed with 80 mg·L^{-1} A1. **Figure 2** shows that increasing Al concentration at 40 mg·L^{-1} can exude the maximum concentration of malic acid at 14.2 ± 0.7 mg·L^{-1}. Here, at 40 mg·L^{-1}, Al can trigger the cultivar *Atlas66* to exude 11.7 ± 0.6 mg·L^{-1} of malic acid. Higher activities of $A1^{3+}$ may be attributed to the higher secretion of malic acid, especially for *Altas66*. Higher secretion of malic acid seems to be related to Al-tolerant genotypes of wheat. According to our results, at equal effect concentrations Al-induced exudation of malic and citric acids observed in *Atlas66*, and *Brevor* accession also exhibited slight induction on organic acid exudation. In the rhizosphere, malic acid can form a complex with Al. One molecule of malic acid per Al is needed. At a 1:1 ratio the Al−ligand complexes also have little toxic effects in Al-sensitive wheat, and the complex prevented Al accumulation in the root tip. Our results are consistent with the results of Ma et al. (*45*). Al-induced organic acid exudation can alleviate Al toxicity, which has gained wide acceptance for plants. But there is little or no conclusive evidence for whether these acids play a protective role and the levels of organic acids are sufficient to provide direct protection against $A1^{3+}$ in the rhizosphere. It is still unknown whether the amount of organic acids secreted is sufficient to detoxify Al. This should be investigated in more detail.

Al-Induced Variance of Rhizosphere pH and Effect of This Variance on Organic Acid Exudation. The possibility that *Atlas66* altered rhizosphere pH was investigated by contrast tests. This pH-mediated Al-resistant mechanism had often been hypothesized in the literature, but had not been conclusively shown to exist in terrestrial plants (*46*). In previous studies, rhizosphere pH differences of similar magnitude were found along root tips of differential genotypic tolerance of wheat roots exposed to nutrient solution with Al (*47*, *48*). Results showed that in the Al-tolerant cultivar *Atlas66*, the small Al-induced increases in rhizosphere pH (0.1−0.5 unit) were sufficient to account for the observed Al resistance. The increased Al resistance of *Atlas66* appeared to be caused by an Al-induced alkalinization of the rhizosphere. The increased alkalinization was localized to wheat root tip, which was the site of A1 toxicity (*23*, *46*). Under our experimental conditions, Al-induced alkalinization of rhizosphere pH can be found in the cultivar *Atlas66*. The possible explanation is that Al-induced organic acid anions exude to the rhizosphere, which makes wheat root tips positively charged. Then the root tips can exclude Al^{3+} and have an attraction for OH^-, and thus tolerate Al. On the contrary, the cultivar *Brevor* does not. This difference may relate to their genotypic tolerance to Al.

The correlation between rhizosphere pH and exuded organic acids indicated that, if the wheat roots could maintain high rhizosphere pH, the exuded organic acid concentrations could be elevated and Al toxicity to wheat would be reduced. In this paper, high rhizosphere pH was found in Al-tolerant *Atlas66*, and more organic acids exuded. But Al-sensitive *Brevor* has no capacity to increase rhizosphere pH, or to exude more organic acids. Plant roots are responsible for changes of up to 1−2 units of rhizosphere pH (*48*, *49*). The small Al-induced increase in rhizosphere pH for *Atlas66* can alkalinize the rhizosphere at the root surface and affect rhizosphere Al speciation, and therefore increase Al resistance. Our results were probably best interpreted as follows: with rhizosphere pH increasing, wheat root tips would adsorb more OH^- and at the same time also release more malic and citric anions to maintain their charge balance. Aluminum-tolerant *Atlas66* generally maintained high rhizosphere pH, which might elevate its Al tolerance. In contrast, secreted organic acids could reduce rhizosphere pH. The increased organic acid reactivity and elevated rhizosphere pH under Al treatment were associated with the organic acid chelated with Al. When treated by external Al^{3+} and H^+ simultaneously, wheat is influenced by Al stress significantly, rather than acid stress. The Al-tolerant genotype *Atlas66* has the capability to exude more organic acids and to maintain higher rhizosphere pH than the Al-sensitive genotype *Brevor*, which is consistent with their Al tolerance and is a specific response to Al.

Ma strongly suggests that the secretion of organic acids plays an important role in the external detoxification of Al (*26*). According to Ma, there are two patterns of Al-induced organic acid anion secretion and different mechanisms are involved: In pattern I, there is no discernible delay between the addition of Al and the onset of the release of organic acids. Activation of the anion channel seems to be involved in this pattern. In pattern II, there is a marked lag phase between the addition of Al and the onset of organic acid release. The action of genes related to the metabolism and secretion of organic acids seems to be involved in this pattern. Internal detoxification of Al in Al-accumulating plants is achieved by the formation of an Al−organic acid complex (*26*). The characteristics of our finding drawn from this study are as follows: for cultivar Atlas66, when Al is added, the production and secretion of malic acid increase within 30 min, and the production and secretion of citric acid within 2−4 h. Thus, in our study, the production and secretion of malic and citric acids may be explained by the above two-mentioned patterns of Al-induced secretion of organic acids. Thus, if we only think of the experimental time between the addition of Al and the onset of the release of organic acids, the exudation of malic and citric acids is in line with pattern I and pattern II, respectively.

Some Further Considerations on the Mechanism Underlying Al Tolerance. Possible explanations to our understanding of the mechanisms underlying Al tolerance and toxicity for wheat can be drawn from this study:

(1) Inhibition of root growth is a well-known effect of Al toxicity, and root tips have been suggested as a primary site for Al-induced injury in plants (*4*). The two different genotype cultivars, *Atlas66* and *Brevor*, living in the same growth conditions behaved differently in root secretion, root growth inhibition, and rooting Al accumulation, mainly due to their different genotypes (*37*, *50*). Al resistance is related to Al-tolerant genotypes of wheat and is characterized by Al exclusion from the root tip, changes in rhizosphere pH, and increased release of organic acids (*26*, *43*, *46*).

(2) Under Al stress, different genotype wheat emits different rhizosphere electric signal in response to Al exposure (*51*). Around wheat roots, many exudates released from wheat roots (such as electrolyte, H^+, sugar, organic acids, amino acid endogenous hormones, enzymes and other secondary metabolites) react or balance with external Al ions to reach high Al tolerance (*49*). The composition of root exudates is related to the wheat genotype (*24*). The Al-tolerant genotypic wheat has strong capacity to exude more organic acids (*23*).

(3) Al stimulates the efflux of malic acid by activation of anion channels (*52*). Activation of anion efflux channels facilitating the efflux of malic acid seems responsible for Al resistance in wheat (*38*). The Al-induced organic acid anions are transported through the specific anion channel across the plasma membrane into wheat rhizosphere, which makes the plasma membrane positively charged and rejects external Al^{3+} (*36*) as well as H^+. The organic acid stained inside root tip may react with smaller amounts of Al^{3+}. These complexes are not toxic to plant cells (*3*). Outside wheat roots, the secreted organic acids react or balance with external $A1^{3+}$ to obtain high Al tolerance, which is also related to wheat genotype (*51*). Al-tolerant genotypes excrete more malic acid than Al-sensitive genotypes (*13*). The stable Al−organic acid complexes do not cross rooting plasma membrane (*8*).

(4) Finally, under Al stress, the Al-tolerant cultivar *Atlas66* can maintain higher rhizosphere pH in itself. The increased rhizosphere pH around the root apex should drive the A1 speciation toward less-toxic Al species, which would reduce Al toxicity (*10*, *11*, *48*). In addition, the Al-tolerant cultivar *Atlas66* also exhibits higher Al resistance at the limited Al level in this study.

ACKNOWLEDGMENT

We thank Shi Wang and Qiuyan Ding for carrying out the chemical analyses. Many thanks to the three anonymous referees for their comments on the manuscript.

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Received for review April 26, 2006. Revised manuscript received October 6, 2006. Accepted October 10, 2006. This work was supported by the Funding of Nanjing Forestry University (No. 063709), Research Funding of MOE for Young Teachers at Key Universities of China, and Visiting Fellowship of State Key Laboratory of Pollution Control & Resource Reuse of China.

JF0611769