STIMULATION OF ORGANIC ACID EXCRETION BY ROOTS OF ALUMINUM-TOLERANT AND ALUMINUM-SENSITIVE WHEAT VARIETIES UNDER ALUMINUM STRESS[1]

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ABSTRACT- With the objective to investigate the hypothesis that the excretion of organic acid is a mechanism of Al tolerance in wheat, four-day-old seedlings of Al-tolerant wheat varieties, Atlas 66 (AT) and Shirosanjyaku (SH), and Al-sensitive ones, Chikushikomugi (CK) and Saitama 27 (SA), were treated with 150μM $AlCl_3$ (+0.4 mM $CaCl_2$) or without Al (+0.4 mM $CaCl_2$) for 24 hours. After 8 hours, the solutions were renewed and then treated for further 16 hours. Organic acids (malic and citric acid) were assayed by enzymatic method in both samples. In other experiment, four-day-old seedlings of SH and CK were treated for 40, 80 and 120 minutes under the same conditions. The bathing solutions were colected and malic acid was analyzed. After the Al-treatment was imposed, the roots were treated with 0.5 mM K-citrate (pH 4.5) or with only deionized water for 30 minutes under ice-cold and aerated conditions. Afterwards, the roots were stained with hematoxylin. Al inhibited root growth of the Al-sensitive (CK and SA) varieties more severely than that of the Al-tolerant (AT and SH) ones. Presence of Al induced differential pH changes in the bathing solution. Al triggered malate excretion in all varieties. Nevertheless, the Al-tolerant varieties were able to excrete 3-5 fold more malate than the Al-sensitive ones. On the other hand, citric acid was not detected in the treatment solution. The ability of malic and citric acids to protect the root growth from Al stress was tested in four-day old seedlings of SH and SA. SH was treated with 250 M Al and CK with 100μM Al ($AlCl_3$ + 0.2 mM $CaCl_2$, pH 4.5) for 1, 2, 3, and 5 hours in presence of 0, 50, 100, and 400μM of malic or citric acid. In the presence of the organic acids, the roots of both Al-sensitive SA and Al-tolerant SH were slightly or not at all stained by hematoxylin. The Al absorbed by roots of Al-sensitive and Al-tolerant plants within 2-hour treatment was mainly apoplasmically associated and could be removed from this compartment by an Al chelator (citrate), as indicated by the lack of hematoxylin staining. During the period of the experiment, symplasmic Al was not visually detected even in the roots of the Al-sensitive variety. The sensitivity of the hematoxylin method to detect low levels of Al in the symplasm is questionable. The mechanisms involved in the differential tolerance among those varieties seem to be related to the greater excretion of malate from root apices of Al-tolerant varieties under Al-stress. Due to its high affinity for Al, malate might immobilize Al in the apoplasm and/or rhizosphere, reducing its harmful effects on important metabolic sites in the root. Thus, whether the Al-induced organic acid excretion requires an apoplasmic or symplasmic Al signal remains to be elucidated in future experiments.

Additional index terms: apoplasm, Al chelating, hematoxylin, malate.

EXCREÇÃO DE ÁCIDOS ORGÂNICOS POR VARIEDADES DE TRIGO TOLERANTES E SENSÍVEIS AO ALUMÍNIO, SOB CONDIÇÕES DE ESTRESSE DE ALUMÍNIO.

RESUMO- Com o objetivo de investigar a hipótese de que a excreção de ácidos orgânicos é um mecanismo de tolerância ao Al em trigo, plântulas de 4 dias de variedades tolerantes, Atlas 66 (AT) e Shirosanjyaku (SH), e variedades sensíveis, Chikushikomugi (CK) e Saitama 27 (SA), foram tratadas com 150 μM de Al ($AlCl_3$ + 0.4 mM $CaCl_2$) ou sem Al (0.4 mM $CaCl_2$) por 24 horas. Após 8 horas, as soluções foram renovadas e as plantas foram tratadas por mais 16 horas. Nas soluções, foram determinados os ácidos málico e cítrico, por método enzimático. Em outro experimento, plântulas de SH e CK, com 4 dias, foram tratadas com

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Al por 40, 80 e 120 minutos, sob as mesmas condições descritas anteriormente. Após o tratamento com Al, as soluções foram coletadas e o ácido málico foi analisado. Em seguida, as raízes foram tratadas com 0.5 mM de citrato-K (pH 4.5) ou com somente água deionizada, gelada, por 30 minutos, com aeração. Depois, as raízes foram coloridas com hematoxilina. Al inibiu o crescimento radicular das variedades sensíveis (CK e SA) mais severamente que das variedades tolerantes (SH e AT). Em presença de Al, as plantas induziram mudanças do pH das soluções de crescimento. Al estimulou a excreção de malato em todas as variedades. Entretanto, as variedades tolerantes foram capazes de excretar 3 a 5 vezes mais malato que as sensíveis. Por outro lado, o ácido cítrico não foi detectado nas soluções-tratamento. Quando quantidades crescentes dos ácidos málico e cítrico foram adicionadas às soluções contendo Al, as raízes de ambas as variedades (SH e SA) ficaram levemente coloridas ou não coloriram com hematoxilina. O Al absorvido pelas raízes das duas variedades no intervalo de tratamento de 120 minutos, estava associado ao apoplasma e não pode ser removido deste compartimento por um quelante de Al (citrato), como indicado pela ausência de coloração com hematoxilina. Durante o período do experimento, o Al localizado no simplasma não foi visualmente detectado, mesmo na variedade sensível. A sensibilidade do método de coloração de raízes com hematoxilina de detectar baixos níveis de Al no simplasma é questionável. Os mecanismos envolvidos na diferença em tolerância ao Al entre as variedades estudadas parecem estar relacionados com a maior excreção de malato pelas raízes das variedades tolerantes, sob condições de estresse de Al. Devido à sua alta afinidade por Al, o malato pode imobilizá-lo no apoplasma e/ou na rizosfera, reduzindo seus efeitos danosos em importantes sítios metabólicos nas raízes. Assim, se o estímulo a excreção de ácidos orgânicos pelas raízes, sob condições de estresse de Al, requer um sinal localizado no apoplasma ou simplasma, ainda precisa ser esclarecido em futuros experimentos.

Termos para indexação: ácidos orgânicos, apoplasma, hematoxilina, malato.

INTRODUCTION

In the studies about Al tolerance in plants many hypotheses have been proposed to explain why some species or varieties in the same species can grow normaly in the presence of Al and others are sensitive and develop symptoms of Al toxicity when grown in the same conditions. Taylor & Foy (1985a, b) suggested that one aspect for differential tolerance to Al among wheat varieties was related to their ability to change the pH in rhizosphere and consequently the Al solubility. Further, Taylor (1988a, b) demonstrated that the correlation among N nutrition, plant-induced pH changes and Al tolerance had not a causal relationship. He suggested that the tolerance was maybe related to the synthesis of organic acids and polyamines, since it is known that synthesis of organic acids increases in plants fed with NO_3-, and polyamines in $NH4^+$-fed plants. It has been suggested that the preference to N sources was not always related to Al-tolerance among wheat varieties (Andrade et al., 1996).

The potential role of organic acids in Al tolerance has been reported in recent publications. The data point out differences in organic acid concentration in tissues between Al-tolerant and Al-sensitive varieties and/or stimulation of excretion of organic acids by root apices under Al-stress conditions. Suhayda et al. (1986) hypothesized that citric and malic acid, because of their higher stability constants with Al, reduce the impact of toxic Al ions in maize root tissue, especially in the Al-tolerant lines. Miyasaka et al. (1991) showed that excretion of citric acid was one of the possible mechanism of Al tolerance in snapbeans. Christiansen-Weniger et al. (1992) found high concentrations of dicarboxylic acids, *i.e.* succinic, malic and oxalic acids, in the exudates of Al-tolerant wheat cultivars. Recently, Delhaize et al. (1993b) demonstrated that differential Al tolerance in near-isogenic wheat lines was based on the stimulation of malic acid excretion in Al-tolerant genotypes when they were exposed to Al.

The relationship between the organic acid synthesis, which is located in the symplasm, and the Al trigger is not described yet. Because Al absorbed during short period treatments is accumulated in the apoplasm (Zhang & Taylor, 1990, 1991; Ownby and Popham, 1989; Tice et al., 1992), it is supposed that the regulation of the organic acid excretion across the plasma membrane could be signaled by an apoplasmic Al.

The objective of the present study was to investigate the hypothesis that the Al-induced excretion of organic acid by roots of Al-tolerant varieties is a mechanism of Al tolerance in wheat. A second objective was to better understand the nature of the early Al uptake (apoplasmic or symplasmic) using the hematoxylin stain method for the detectation of Al localization in the roots.

MATERIALS AND METHODS

a) Growth conditions and Al treatment:

Seedlings of Al-tolerant wheat varieties, Atlas 66 (AT) and Shirosanjyaku (SH), and Al-sensitive ones, Chikushikomugi (CK) and Saitama 27 (SA) were grown aseptically. The seeds were disinfected by soaking in 1.5 % NaClO for 90 minutes, rinsed many times with sterile deionized water, and then

germinated on moistened paper in sterile Petri dishes in the dark for about 48 hours. Twenty seedlings were transferred onto stainless steel screen in tests tubes (20 cm in length 4 cm in diameter) over 50 mL of sterile nutrient solution (pH 4.5) containing macronutrients (mM): 0.50 $NH4^+$; 1.00 $NO3^-$; 0.50 Ca^{2+}; 0.50 K^+; 0.30 Mg^{2+}; 0.40 Cl^-; 0.40 $SO4^{2-}$; 0.30 Na^+; 0.0025 H_2PO_4-; and micronutrients (μM): 0.91 Mn^{2+}; 0.03 Cu^{2+}; 4.63 H_3BO_3; 0.08 Zn^{2+}; 3.2 Fe as $FeNa_2EDTA$. The tubes were placed on a rotary shaker (120 rpm) for 4 days in an environmentally controlled chamber, at 20°C, with a 12-hour photo-period, 120 mol m^{-2} s^{-1} (PAR). On the fifth day, after the solutions were aspirated from the tubes the seedlings were treated with 150 M Al ($AlCl_3$ + 0.4 mM $CaCl_2$) and without Al (0.4 mM $CaCl_2$) at pH 4.5, for 24 hours. Each tube contained 40 mL treatment solution. Al was added after Al solution was sterilized using 0.45μm membrane filter. Samples were collected after 8 hours to analyze excreted organic acids. Subsequently, the solutions were renewed, and the plants were treated for further 16 hours. Root length and solution pH were measured at the end of the experiment.

Malic and citric acids in the solution were assayed by the enzymatic methods according to Delhaize et al. (1993b).

b) Alleviation of Al toxicity by addition of organic acids to the bathing solution:

The ability of malic and citric acids to protect the root growth from Al stress was tested in seedlings of SH and SA. The seedlings were grown during four days in a solution containing 0.2 mM $CaCl_2$, pH 4.5 under aerated conditions. On the fifth day, SH was treated with 250 M Al and CK with 100μM Al ($AlCl_3$ + 0.2 mM $CaCl_2$, pH 4.5) for 1, 2, 3, and 5 hours in the presence of 0, 50, 100, and 400μM of these organic acids. After treatments, the roots were rinsed many times with deionized water and stained with hematoxylin (2.0 g L^{-1} hematoxylin, Nacalai Tesque, 0.2 g L^{-1} $NaIO_3$).

c) Al desorption from apoplasm:

Seedlings of SH and CK (15 seedlings/tube) were grown in 50 mL of sterile 0.4 mM $CaCl_2$, pH 4.5. On the fifth day, the seedlings were treated with 30 mL of sterile 150 M $AlCl_3$ solution containing 0.4 mM CaCl2, pH 4.5 for 40, 80 and 120 minutes. The loosely bound Al in the apoplasm that could react with hematoxylin was desorbed by washing the seedlings with 0.5 mM K-citrate (pH 4.5) or with deionized water for 30 minutes under ice-cold and aerated conditions. Then, the roots were rinsed briefly with deionized water and stained with hematoxylin for 20 minutes.

The bathing solutions collected at the end of the treatments were dried under low pressure conditions and redissolved in water. Afterwards, the malic acid was analyzed by the enzymatic procedure.

RESULTS

Root growth of the control plants was greater in Al-sensitive than in Al-tolerant cultivars. Al affected root growth of all varieties (Fig. 1). Al-tolerant SH had the lowest inhibition (19 %), while the Al-sensitive SA had the highest (32 %).

The solution pH measured after the 16-hour treatment with Al decreased by 0.5 pH unit only in SH, while the other varieties tended to induce little pH changes (increment of 0.1 unit) (Fig. 2). In the absence of Al, the pH of the treatment solutions increased by more than 1.0 unit for all varieties.

Even when plants were not treated with Al, Al-tolerant varieties, especially AT, excreted more malic acid than Al-sensitive ones (Fig. 3), but the amounts of malic acid were not so large. Exposure to Al caused increased excretion of malic acid in all varieties. Tolerant varieties were able to excrete malic acid 3- to 5-fold more than sensitive ones. SH excreted more malic acid than AT. The rates of excretion of malic acid were greater during the first period (8 hours) than the second period (16 hours). The seedlings of both Al-tolerant and Al-sensitive varieties excreted very low amounts of citric acid into the solution (not detectable in most of the cases) and it was not stimulated by Al (data not shown).

In vitro citric and malic acids were able to compete with hematoxylin for Al binding (data not shown). Citric acid was found to be stronger Al-chelator than malic acid at the same molar concentration. Addition of malic and citric acids to the bathing treatment solutions did not induce symptoms of Al toxicity in both Al-sensitive (SA) and Al-tolerant (SH) roots. A lack or attenuation of the intensity of hematoxylin staining was observed with increasing concentrations of these organic acids, even in extending time of exposure to Al (Table 1).

Al desorption from apoplasm experiment: During the short exposure to Al, i.e. 120 minute-experiment, differential staining between Al-tolerant and Al-sensitive root tips were not observed, therefore not photographed. After 40 minutes, staining was not observed in the citrate-washed roots of both varieties and only slight staining was observed when washed in water (not shown). Staining intensities slightly increased following the 80 and 120-minute Al treatment, mainly in more basal regions. However, that staining pattern seemed to be related with Al localized on the root surface. The water-washed roots were stained more intensely than the citrate-washed ones, but Al staining in cytoplasm and nuclei was not apparent. The attenuation in the staining intensity when the roots tips were washed with citrate suggests

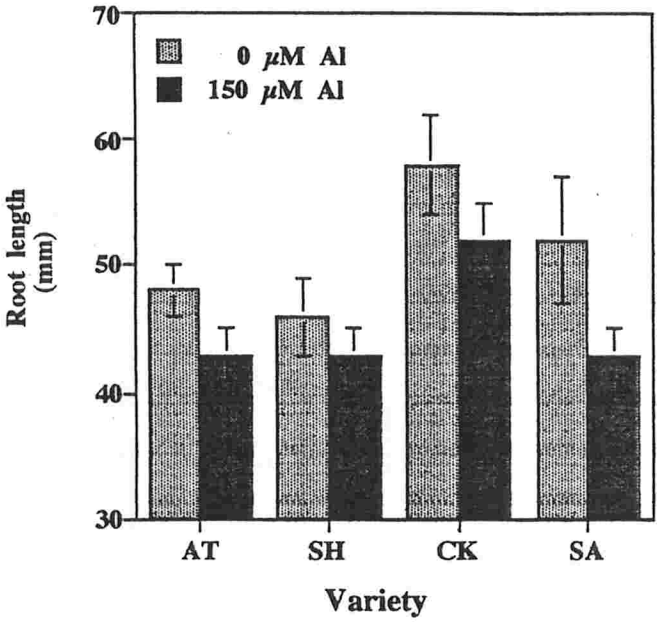

FIGURE 1- Effect of Al on root growth of 5-day-old seedlings of wheat cultivars differing in Al-tolerance. Root length was measured after treatment for 24 hours. (values = mean±SD of 20 seedling, n=20X5).

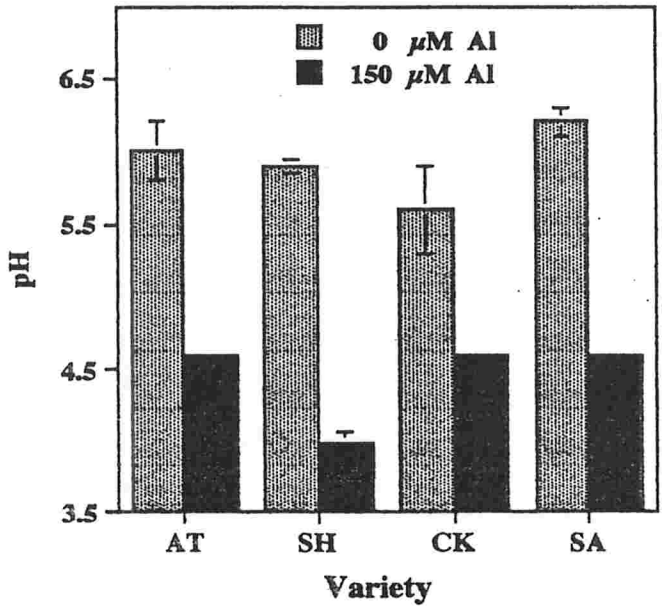

FIGURE 2- Effect of Al on solution pH changes in 5-day old seedlings of wheat varieties differing in Al-tolerance. The pH was 4.5. Vertical bars signify SD of the mean (n=5) where it is large enough to be represented.

that apoplastic Al might be at least partly responsible for the differential staining. Nevertheless, the short duration (within 2 hours) of the Al-treatment period might have been insufficient to distinguish differences in Al tolerance using staining pattern of root tips.

TABLE 1- Suppression of hematoxylin staining by organic acid in roots of 4-day-old seedlings of Al-sensitive Saitama (SA) and Al-tolerant Shirosanjyaku (SH) wheat varieties under Al-stress.

Organic Acid	Malic acid				Citric acid			
	Time of exposure (hours)							
μM	1	2	3	5	1	2	3	5
SA*								
0	+	+	+++	+++	+	+	+++	+++
50	-	-	+	+	+	-	-	-
100	-	-	±	±	-	-	-	-
400	-	-	-	-	-	-	-	-
SH**								
0	±	±	±	+	-	-	-	-
50	±	±	±	±	-	-	-	-
100	-	-	-	-	-	-	-	-
400	-	-	-	-	-	-	-	-

Treatment solutn : * 0.2 mM $CaCl_2$ + 100 μM $AlCl_3$
** 0.2 mM $CaCl_2$ + 250 μM $AlCl_3$

+++ large and deep staining, ++ small but deep staining, + slight staining, ± little staining, – no staining.

Although differential staining of the roots was not observed between the two varieties, the presence of Al for only 40 minutes effectively stimulated malic acid excretion in the Al-tolerant variety (Fig. 4).

DISCUSSION

One of the primary symptoms of Al toxicity is inhibition of root growth, which can be observed within 1~3 hours (Ownby & Popham, 1989). In the condition of the present experiments where the concentrations of Al was high (150μM), Al affected the root growth of all varieties. However, the inhibition was more severe in the sensitive varieties than in the tolerant ones (Fig. 1).

Differential nutrient uptake by roots causes changes in the solution pH. Excess anion over cation uptake will result in an increase of rhizosphere pH whereas excess cation uptake will induce a compensatory proton release that will acidify the root surroundings (Keltjens & Ulden, 1987). In regard to this work, the solution of the control treatment was composed by a simple salt solution ($CaCl_2$). The increase in the solution pH (Fig. 2) suggests that the uptake of the anion (Cl^-) was much more rapid than that of the cation (Ca^{2+}). In this uptake system, cytoplasmic electroneutrality might be reached by extrusion of OH^- into the solution.

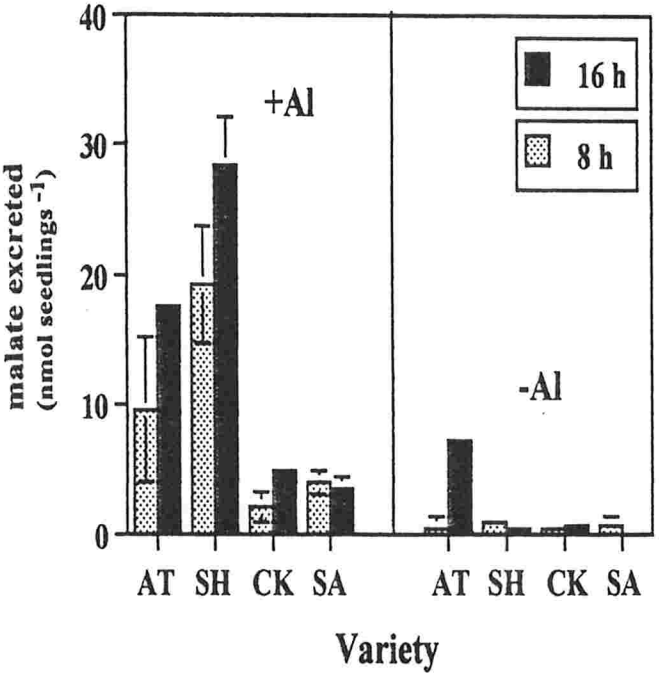

FIGURE 3- Malic acid excreted into nutrient solution by roots of 5-day-old seedlings of wheat varieties differing in Al-tolerance. Twenty seedlings were treated with 150μM Al ($AlCl_3$ +0.4mM $CaCl_2$) or without Al (0.4mM $CaCl_2$), pH 4.5, 40mL/tube, for 8 hours and subsequent 16 hours. Vertical bars signify SD of the mean (n=5 or 4) where it is large enough to be represented.

Al in the root medium changed the net anion uptake to net H^+ release or increased H^+ release. SH decreased the pH by 0.5 units, while the other varieties tended to induce little changes in solution pH (increment of 0.1 unit) (Fig. 2). On the basis of the ionic composition of the treatment solution, Al and Ca were assumed to be the nutrients responsible for the solution acidification. Even though the fluxes of Ca2+ were not measured in the present experiments, some evidence indicates that Al is likely to inhibit Ca^{2+} influx,

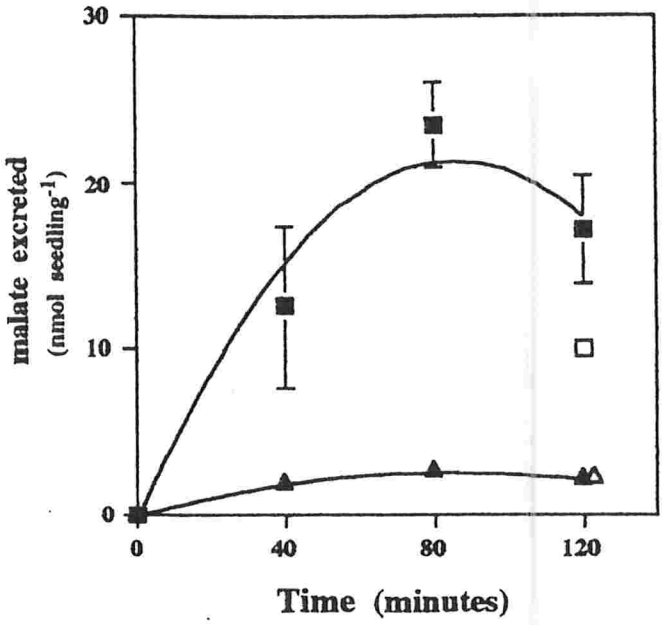

FIGURE 4- Malate excreted to solution by Al-tolerant (SH = o), and (CK = Δ) wheat varieties treated with 150μM (closed symbol) and without Al (open symbol). Vertical bars signify SD of the mean (n=2) where it exceeds symbol size.

at least in Al-sensitive plants; moreover, Al-tolerant one is likely to be able to resist Al inhibition of Ca uptake and translocation of Ca to shoots (Ryan et al., 1992; Ryan & Kochian, 1993; Huang et al., 1995).

Many theories are trying to explain the mechanisms involved in the synthesis and release of malate by Al-tolerant roots. A possible explanation could be related to the stimulation of malate synthesis in response to an excess of cation uptake in the cytoplasm. With the increasing in cation absorption, the cytosolic pH starts to increase and hence the synthesis of organic acids is stimulated (Kolek & Holobroda, 1992). In order to keep the cytosolic neutral pH, the anion produced in excess ($malate^{2-}$) would be driven out to the apoplasm. This exuded malate might be accompanied by a correspondent proton exclusion (Hoffland et al., 1989; 1992; Luttge & Ball, 1974), hence a decreasing in the rhizosphere pH. The operation of a H^+-ATPase may be considered to be involved in this process. In fact, the function of H^+-ATPase, with a optimum pH of 6.6 (Michelet & Boutry, 1995) is activated when protons start accumulation in the cytoplasm, resulting in the efflux of the excess of H^+ from the cell. The lowest pH induced by SH roots, which excreted the highest amount of malate (Fig. 3), might be explained under the light of those speculations. In regard to the Al-tolerant AT, this variety excreted 3-fold more malic acid than Al-sensitive varieties but caused little changes in the solution pH. Although not investigated

in this experiment, consistent evidence also shows K+ excretion as an accompanied cation in malate excretion (Ryan et al., 1995a) and this might explain the unchanged solution pH induced by AT.

In case of the Al-sensitive varieties, it seems that cytoplasmic pH regulation was disturbed in the presence of Al. The relative stability of the intracellular pH is the result of the buffering capacity of the cytoplasm, of rapid reaction in organic acid synthesis or of the production of salts of these acids which is connected with the release of H^+ or OH^- (Kolek and Holobrada, 1992). Distinct from the Al-tolerant varieties, the presence of Al on the bathing solution did not cause higher rates of organic acids excretion or changes in solution pH in Al-sensitive CK or SA (Fig. 2 and 3). Thus, it is possible that in the presence of Al not only the biophysical (H^+-ATPase) but also the biochemical (synthesis of organic acids) pH-stat system failed to control cytoplasmic pH.

Exudation of organic acids has recently been connected to differential Al-tolerance among species and among varieties in the same species. Under Al stress conditions, citric acid was reported to be exuded by Al-tolerant snapbeans (Miyasaka et al., 1991) and maize roots apices (Pellet et al., 1995), and malic acid from Al-tolerant wheat varieties (Delhaize et al., 1993b; Ryan et al.; 1995a, b; Basu et al., 1994). The *in vitro* affinity of malic and citric acid to chelate Al was investigated to know a correlation between excretion of organic acids by roots and imobilization of Al in the rhizosphere. The data (not shown) confirmed that both acids have strong affinity for Al. Citric acid was a more potent chelator of Al than malate. These results indicated the potential of those acids to chelate Al in the rhizosphere. Moreover, when malic and citric acids were added to the bathing solution containing Al, the lack or attenuation of the hematoxylin staining of the roots fo both Al-tolerant SH and Al-sensitive SA varieties, despite of the increase in the Al-treatment duration, indicates that those organic acids chelated Al in such a way that Al could not enter and make a complex with hematoxylin (Table 1). Similar results were obtained by Delhaize et al. (1993a, b), who demonstrated that citric and malic acids, with high affinity for Al binding, were able to protect roots of Al-sensitive wheat lines from Al injury.

In this study the rate of malate excretion decreased when plants were treated with Al for longer time. Delhaize et al. (1993b) showed malate excretion continued at an almost constant rate within 24 hours in Al-tolerant wheat lines which were treated with 50μM $AlCl_3$. In this experiment 150μM $AlCl_3$ was used. Hence it can be assumed that Al concentration was too high to maintain a constant rate of malate excretion over 24 hours, probably because Al gave damage to excretion function involving plasma membrane or metabolism.

Attempts to understand the basis of the mechanisms for Al toxicity have been guiding to investigate the localization of Al in the cell compartments, as apoplasm and symplasm. Strong evidence indicates that Al in apoplasm is responsible for rapid inhibition of root growth and alteration of certain physiological processes within minutes (*e.g.* Ca^{2+} influx) (Kochian, 1995). Zhang & Taylor (1990) examined the Al uptake kinetics in wheat and observed an initial 30-minute rapid uptake that represents apoplastic binding and a linear phase that corresponds to transport across the plasma membrane. Our results clearly showed increased malate excretion into the apoplasm / rhizosphere by SH roots after exposure to Al for only 40 minutes (Fig. 4). Loosely bound Al was effectively desorbed by citrate or water from the roots of the Al-tolerant as well as the Al-sensitive variety, as indicated by the lack of Al staining in the apoplasm and cell wall. This result indicates that the signal for Al-induced excretion of organic acid appears before the entry of Al into the symplast. Delhaize et al. (1993a) showed that Al trigger malate excretion within only 15 minutes, which also suggestes an action of Al in the apoplasm. Recently, the use of more sensitive analytical techniques, e. g. secondary ion mass spectrometry, enabled to demonstrate that Al could enter into the cell even within 30 minutes (Lazof et al., 1994). This rapid uptake indicates that direct effects of Al on cell function can appear within this time frame. In the condition of the present work (Al-treatment for 40 minutes) the presence of symplastic Al may not be ruled out if the Al could penetrate in the cytoplasm and it was associated with intracellular ligands, which would limit combination (and thus visible staining) with hematoxylin.

CONCLUSIONS

The present results suggest that when excreted by roots to the apoplasm and/or rhizosphere, malate and citrate can immobilize Al and might protect the plasma membrane of wheat roots from the Al damage. However, the lower concentration of citric acid found in the nutrient solution fails to indicate that this acid is related to the Al-tolerance in wheat varieties. On the other hand, the relatively high affinity of malate for Al binding *in vitro* and the alleviation of Al toxicity in solution strongly support the hypothesis that the excretion of this Al chelator might be an important mechanism for the amelioration or the prevention of Al toxicity in the Al-tolerant AT and SH. Thus, whether the Al-induced organic acid excretion requires an apoplasmic or symplasmic Al signal remains to be elucidated in future experiments.

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