# ALUMINUM EFFECTS ON CITRIC AND MALIC ACID EXCRETION IN ROOTS AND CALLI OF RICE CULTIVARS

**CRISTIANE ELIZABETH COSTA DE MACÊDO[1], JEAN MARIE KINET[2] AND STANLEY LUTTS[3]**

Laboratoire de Cytogenétique, Faculté de Science, Université Catholique de Lovain,- Louvain-La-Neuve, 1348, Belgique.

**ABSTRACT -** Citric and malic acid excretion in the medium and malic acid accumulation in seedling roots and embryo-derived calli as possible mechanisms of aluminum (Al) resistance and the effects of a 17-h Al stress period on root growth in *Oryza sativa* have been studied. Four-day-old seedlings and embryo-derived calli of Al-resistant (IRAT 112 and IR6023) and Al-sensitive (Aiwu and IKP) cultivars were treated with 250 and 500 µM $\{Al_2(S0_4)_3.18H_20\}$ of total aluminum or without Al for 36 hours. After 3 to 36 hours of stress, seedlings and calli were removed from the flasks and concentration of citric and malic acids was estimated in the Al and control solutions. Malic acid was also assayed in roots tips and in callus tissues. After 17-h of Al stress, inhibition of root growth was a typical effect of Al in rice and the extent of the inhibition depended on both cultivar and Al concentration. At 500 µM of Al, strong reduction of root elongation occurred in all cultivars while at 250 µM of Al, only IRAT was unaffected, when compared to their control. In the absence of Al, all varieties excreted comparable amounts of citric and malic acid. Al treatments, were without effect upon citrate excretion in both Al-resistant and Al-sensitive cultivars. Al treatment, for periods from 3 to 24h, slightly stimulated the excretion of malic acid from seedlings, in all cultivars. Malic acid concentrations in root apices, in the presence or absence of aluminum, were not correlated with aluminum resistance. No differences in malic excretion and internal concentrations were detected between Al-treated and untreated rice calli of the same four cultivars. It is therefore concluded that, in our experimental conditions, differences in Al resistance in our rice cultivars cannot be attributed to citric and malic acids. Further research needs to be carried out to examine other possible mechanisms of Al-resistance in rice and to determine whether organic acids such as succinic and oxalic acid are implicated.

**ADDITIONAL INDEX TERMS -** Malate, citrate, mechanisms Al resistance, Al detoxification, internal tolerance.

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Received: 10/6/2001 – Accepted: 2/4/2001

1. Bióloga PhD (Université Catholique de Louvain-Belgique) Professora Substituta, Departamento de Botânica, Ecologia e Zoologia, Universidade Federal do Rio Grande do Norte, Natal-RN, Corresponding author e-mail cristianemacedo.costa@bol.com.br ou criseli@cb.ufrn.br
2. Biólogo PhD, Professor Titular, Laboratoire de Cytogenétique, Faculté de Science, Université Catholique de Lovain,- Louvain-La-Neuve, 1348, Belgique.
3. Engenheiro Agrônomo e Biólogo PhD, Professor Titular, Laboratoire de Cytogenétique, Faculté de Science, Université Catholique de Lovain,- Louvain-La-Neuve, 1348, Belgique.

# EFEITOS DO ALUMÍNIO NA EXCREÇÃO DOS ÁCIDOS CÍTRICO E MÁLICO NAS RAÍZES E CALOS DE CULTIVARES DE ARROZ

**RESUMO -** A excreção dos ácidos málico e cítrico no meio de cultura, assim com a acumulação do ácido málico nas raízes e em calos derivados de embriões, foram estudadas como um possível mecanismo de resistência ao alumínio em arroz. Plântulas de 4 dias e calos derivados de embriões das cultivares resistentes ao alumínio (IRAT 112 e IR6023) e das cultivares sensíveis (Aiwu e IKP) foram tratadas com 0, 250 e 500μM de alumínio {$Al_2(S0_4)_3.18H_20$ }. Em seguida, de 3 a 36 horas de estresse, as plântulas e os calos foram removidos dos frascos e as concentrações dos ácidos cítrico e málico, determinadas. A concentração do ácido málico foi também determinada nos ápices das raízes e nos calos. Após 17 horas de estresse, o crescimento radicular foi inibido, mostrando um efeito tipico do Al em arroz. Entretanto, a extensão da inibição depende da cultivar e da concentração em Al. Na presença de 500 μM de Al, ocorreu uma forte redução no alongamento radicular em todos as cultivares, ao passo que a 250 μM de Al, a cultivar IRAT não foi afetada. Na ausência de alumínio (solução-controle), todas as cultivares excretaram quantidades comparáveis de ácido cítrico e málico. Os diferentes tratamentos com alumínio não exerceram nenhum efeito na excreção do citrato nos dois grupos de cultivares (Al-resistentes e Al-sensiveis). Em todas as cultivares estudadas, e no intervalo de 3 a 24h, o Al estimulou ligeiramente a excreção do malato. As concentrações de ácido málico determinadas nos ápices das raízes, tanto em ausência como na presença de Al, não apresentaram nenhuma relação com a resistência ao alumínio, visto que nenhuma diferença foi detectada entre as cultivares. Nenhuma diferença foi detectada, tanto na excreção como nas concentrações internas de malato, entre calos tratados ou não com Al nas quatro cultivares estudadas. Portanto, conclui-se que, nas condições experimentais deste trabalho, diferenças com relação à resistência ao Al entre as cultivares de arroz estudadas não podem ser atribuídas aos ácidos málico e cítrico. Há necessidade de novos estudos, tanto para avaliar outros possíveis mecanismos de resistência do arroz ao alumínio, como, por exemplo, para participação de outros ácidos orgânicos.

**TERMOS PARA INDEXAÇÃO**: Ácido málico, ácido cítrico, mecanismos de resistência ao Al, detoxificação de Al, tolerância interna.

## INTRODUCTION

Al toxicity is a major factor limiting plant growth in strongly acid soils (Foy, 1984; Foy, 1988). The primary initial response to Al toxicity is inhibition of root elongation (Taylor, 1988; Kochian, 1995). However, the fundamental basis of aluminum rhizotoxicity and genotypic differences in sensitivity to aluminum are still poorly understood in higher plants (Kochian, 1995). It is not known whether the primary lesions are located in the apoplastic or in the symplastic (or both) compartments (Parker, 1995). Due to the many hypotheses trying to explain the mechanisms of aluminum toxicity, numerous associated resistance mechanisms have been proposed. They can be classified in two categories according to the site of aluminum detoxification or immobilization: exclusion of aluminum out of the symplasm and detoxification or immobilization of Al in the plant (Taylor, 1988, 1991).One of these resistance mechanisms is the chelation and detoxification of Al by organic acids, either within the plant (internal tolerance) or in the rhizosphere (exclusion). Plants are know to exude organic acids into the rhizosphere in response to mineral stress. Citrate, malate, malonate, acetate, aconitate, glycolate, oxalate and succinate have been found in the root exudates from a number of species (Vancura and Hovadik, 1965; Jayman and Sivasubramaniam, 1975; Smith, 1976; Gardner *et al.*, 1983; Christiansen-Weniger *et al.*,1992; Ma *et al*., 1997; Gallardo *et al*., 1999).

Root exudation of organic acids which can chelate and detoxify Al in the rhizosphere has been consistently reported in several studies. Citrate was found to be released by Al-resistant snapbean and maize cultivars (Miyasaka *et al.*, 1991; Pellet *et al.*, 1995; Jorge and Arruda, 1997) while malate was excreted by roots of Al-resistant wheat (Delhaize *et al.*, 1993b; Basu *et al.*, 1994; Ryan *et al.*, 1995 a,b; Andrade *et al*., 1997) and sorghum cultivars (Gonçalves and Cambraia, 1999). The existence of an internal detoxification of Al by organic acids is more debated. Cambraia *et al.* (1983) showed that the response of *Sorghum* roots to Al was an increase in t-aconitic and malic acids. This increase was higher in Al-tolerant than in sensitive cultivars. Similar results, showing that better Al tolerance was correlated with higher concentrations of citrate in roots of resistant plants exposed to aluminum were reported for pea (Klimashevskii and Chernysheva, 1980), for maize (Suhayda and Haug, 1986), and for barley (Foy and Lee, 1987). However, Scott *et al*. (1990) observed higher concentrations of citric and malic acids in the roots of Al-sensitive wheat cultivar 'Katepwa' and Pellet *et al.* (1995) reported that the increase in root malic acid content was similar in both Al-tolerant and Al-sensitive maize cultivars. Up to now, there is no available information concerning a possible mechanism of chelation and detoxification of Al by organic acids in rice. The aim of the present work was to determine whether organic acids are implicated in Al resistance mechanism in this species.

## MATERIAL AND METHODS

### Plant Material

Four rice (*Oryza sativa* L.) cultivars were used in this work. Seeds of Al-resistant cultivars IRAT 112 (IRAT) and IR6023 (IR) were obtained from IRRI (International Rice Research Institute, Philippines) and seeds of Al-sensitive cultivars I Kong Pao (IKP) and Aiwu were obtained from WARDA (West Africa Rice Development Association, Senegal).

### Germination and root growth evaluation

Seeds were germinated on nylon wire nettings (with 2mm meshes) spread over plastic containers completely filled with 600 mL of deionized water, at the rate of 20 seeds per container. Germination and seedling growth were carried out in a phytotronic growth room under controlled temperature (28°C/23°C, day/night). Illumination was provided by Sylvania fluorescent tubes for 12 h $d^{-1}$ at a photon flux density (PAR) of ca. 300 µmol $m^{-2}$ $s^{-1}$. Relative humidity of the air was between 60 and 80% during the day. After 96 h, seedlings having a root 30 ± 1 mm long were selected and transferred to new containers. Aluminum was added as $Al_2(S0_4)_3.18H_20$ solutions up to reaching different concentrations: 0, 250 and 500 µM of total Al. The pH of the Al and control solutions were adjusted to 4 with $HN0_3$. The basal nutrient solution was the same composition as previously described by Delhaize *et al.*, (1993a). Exposure time to Al was 17 h. For each treatment (Al x cultivar), root length of 20 seedlings was measured before and after the 17-h stress period.

Root elongation rates at the end of the Al treatment (RERt) were estimated as follows: RERt = (LAT/LBT) x 100 where LBT = root length before the Al treatment, LAT = root length after the Al treatment.. For each cultivar, to account for genotypic differences in root elongation, RERs of seedlings that were treated with Al are expressed as percentages of RERs of seedlings grown without Al.

### Culture conditions and Al treatment of whole plants

Seeds were surface sterilised for 30 s in ethanol 95%, followed by treatments in formaldehyde 0.8% (v/v) for 40 min and calcium hypochlorite 5% (w/v) for 20 min. Seeds were then rinsed several times with sterile deionized water, placed in sterile Petri dishes containing filter paper moistened with 7 ml of sterile deionized water and placed for germination in a growth chamber under controlled temperature (28°/23°C, day/night).

Illumination was provided by Sylvania fluorescent tubes for 12h $d^{-1}$ at a photon flux density (PAR) of ca. 300 µmol $m^{-2}$ $s^{-1}$. Relative humidity of the air was between 60 and 80% during the day. Two-days seedlings were aseptically placed on perforated plastic top of flasks (15 seedlings per flask) completely filled with 50 ml of sterile deionized water in such a way that the primary root was dipped in the water. In order to prevent microbial infections, the flasks were placed into glass vessels covered with a plastic Petri dish, sealed with parafilm. The glass vessels were fixed on a rotary shaker (125rpm) to ensure seedling aeration and then maintained in the same environmental conditions as described above. After 2 days, the 4-days-old seedlings were rinsed three times with 50 ml of Al or control solutions for a total of 30 min in a sterile laminar-flow. The basal nutrient solution was the same composition as previously described by Delhaize *et al.*, (1993a). Aluminum was added as $Al_2(S0_4)_3.18H_20$ solutions up to reaching different concentrations: 0, 250 and 500 µM of total Al. The pH of the Al and control solutions were adjusted to 4 with $HN0_3$. Seedlings having a root 3.0 ± 1 cm long were selected and transferred to new flasks (15 per flask) containing 30 ml of Al or control solutions. These 15 seedlings were referred to as one sample. The flasks were maintained in the same conditions as described above. After 3 to 36h of stress, seedlings were removed from the flasks and concentration of citric and malic acids was estimated in the Al and control solutions. Malic acid was also assayed in root tip (5 mm long). For each cultivar and each duration of exposure to the Al and control solutions, the experiments were carried out three times and made in tripiclate (repetition), and for each repetition three samples were analysed.

**Culture conditions and Al treatment of calli**

Seeds were dehusked and surface sterilised for 30 s in ethanol 95%, followed by treatments in formaldehyde 0.8% (v/v) for 40 min and calcium hypochlorite 5% (w/v) for 20 min. Seeds were then rinsed several times with sterile deionized water and incubated in the dark for 24h at 25°C. Aseptically dissected embryos were transferred to Petri dishes containing 25 ml of a callogenesis medium. The basal callogenesis medium was the same composition as previously described by Van Sint Jan *et al.*, (1997). Five embryos (100 embryos per cultivar and per repetition) were placed in each Petri dish and maintained in the dark at 28°C. After six weeks (with 1 transfer onto fresh callogenesis medium) and under sterile conditions, the calli were weighed, and the calli with approximately 100 mg of fresh weight were selected and transferred in Erlenmeyer flasks containing 30 ml of Al or control solutions. The basal nutrient solution was the same composition as previously described by Delhaize *et al.*, (1993a). Aluminum was added as $Al_2(S0_4)_3.18H_20$ solutions up to reaching different concentrations: 0, 250 and 500 µM of total Al. The pH of the Al and control solutions were adjusted to 4 with $HN0_3$.The flasks were wrapped in an aluminum foil and incubated on a rotary shaker (125 rpm) at 28-23°C. After 3, 6, 12 and 24h of stress, the samples were filtered (sterile Whatman filter paper ), the calli were collected and the malic acid concentration in the Al and control solutions and in callus tissues was then assayed. For each cultivar and each duration of exposure to the Al and control solutions, the experiments were carried three times, and two calli were analysed in each of these repetitions.

**Citric and malic acids assays**

Citric and malic acids concentration in Al and control solutions, and malic acid concentration in roots and callus tissues were assayed according to Delhaize *et al.*, (1993b). For citric acid determination, a 2.52 ml aliquot of solution (Al and control solutions) was preincubated with 0.24 ml of buffer (1 M tris-Cl, pH 7.8), 30 µl of 10 mM NADH and 10 µl of a lactate dehydrogenase/malate dehydrogenase mixture for 30 min at room temperature to obtain a stable $A_{340}$ reading before the addition of 10 µl of citrate lyase. The decrease in $A_{340}$ due to oxidation of NADH was measured and is directly proportional to the amount of citric acid in the sample. Al at the

concentrations of 250 and 500 µM added to the citric acid solutions ranging from 0.6 to 20 µM citric acid (the range of citric acid concentrations assayed and used in the standard curve) did not interfere with the assay. For malic acid determination, a 1.35 ml aliquot of solution (Al and control solutions), was incubated with 1.5 ml of buffer (0,5 M glycine; 0,4 M hydrazine pH 9.0) and 0.1 ml of 40 mM NAD. The reaction mixture was preincubated for 30 min at room temperature to obtain a stable $A_{340}$ reading before the addition of 5µl of malate dehydrogenase. The increase in $A_{340}$ due to the production of NADH was measured after 30 min and is directly proportional to the amount of malic acid in the sample. Al at the concentration of 250 µM added to malic acid solutions ranging from 2 to 80 µM malic acid (the range of malic acid concentrations assayed and used in the standard curve) did not interfere with the assay.

For malic acid determination in root apices, 15 root tips 5mm long were collected, pooled, weighed (approximately 20 mg per sample) and immediately ground using a mortar and pestle in 1 ml of 0.6 N perchloric acid. The sample was centrifuged at 15.000g for 5 min, and 0.9 ml of supernatant solution was collected and neutralised with 80 µl of $K_2CO_3$ (5M). The neutralised solution was centrifuged at 15.000g for 5 min, and 0.5 ml of supernatant was assayed for malic acid as described above after adding 0.85 ml of sterile deionized water to make up the volume (1.35 ml). To analyse malic acid in calli, 20 mg of tissue per callus were taken and the conditions of extraction and assay were the same as described above for root apices.

The results of citric and malic acids concentration in Al and control solutions, and malic acid concentration in roots and callus tissues were expressed respectively in µmoles/flask and in µmoles/mg of fresh weight according to Delhaize *et al.*, (1993b). Since the results of the three experiments (at plant and at callus level) were similar, pooled data are presented. Statistical analyses (ANOVA) of data were conducted with absolute values, using aluminum concentration and the cultivar as variables.

## RESULTS

### Root growth

At the end of the Al treatment, all cultivars showed root growth inhibition that increased with Al concentration (Figure 1) at the lower Al dose (250 µM), however, the two resistant cultivars were less affected. This was especially the case for IRAT in which root elongation at 250 µM was significantly higher than in the other three cultivars and not different from that of the controls kept in control solution without Al. Genotypic differences were undetectable at higher Al levels (500 µM).

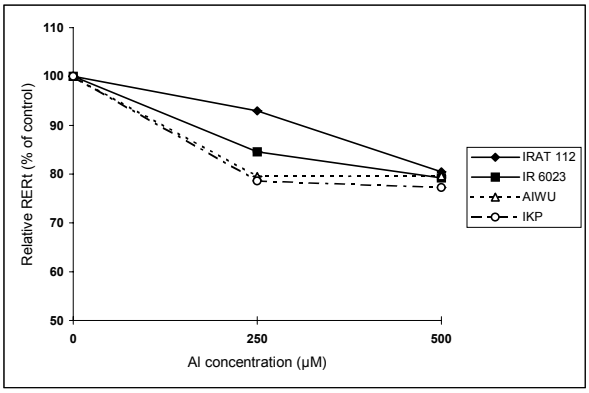


**FIGURE 1** - Relative root elogation rates (% of control without Al) after 17 h of Al-stress (RERt), for four rice cultivars differing in Al-sensitivity (Aiwu and IKP are Al-sensitive and IRAT and IR are Al-resistant.

### Excretion of citric acid by seedling roots

In all solutions and irrespective of the duration of exposure, the roots of all cultivars tested excreted approximately the same amount of citric acid. Al treatments were without effect upon citrate excretion in both Al-resistant and Al-sensitive cultivars (Figure 2a-d).

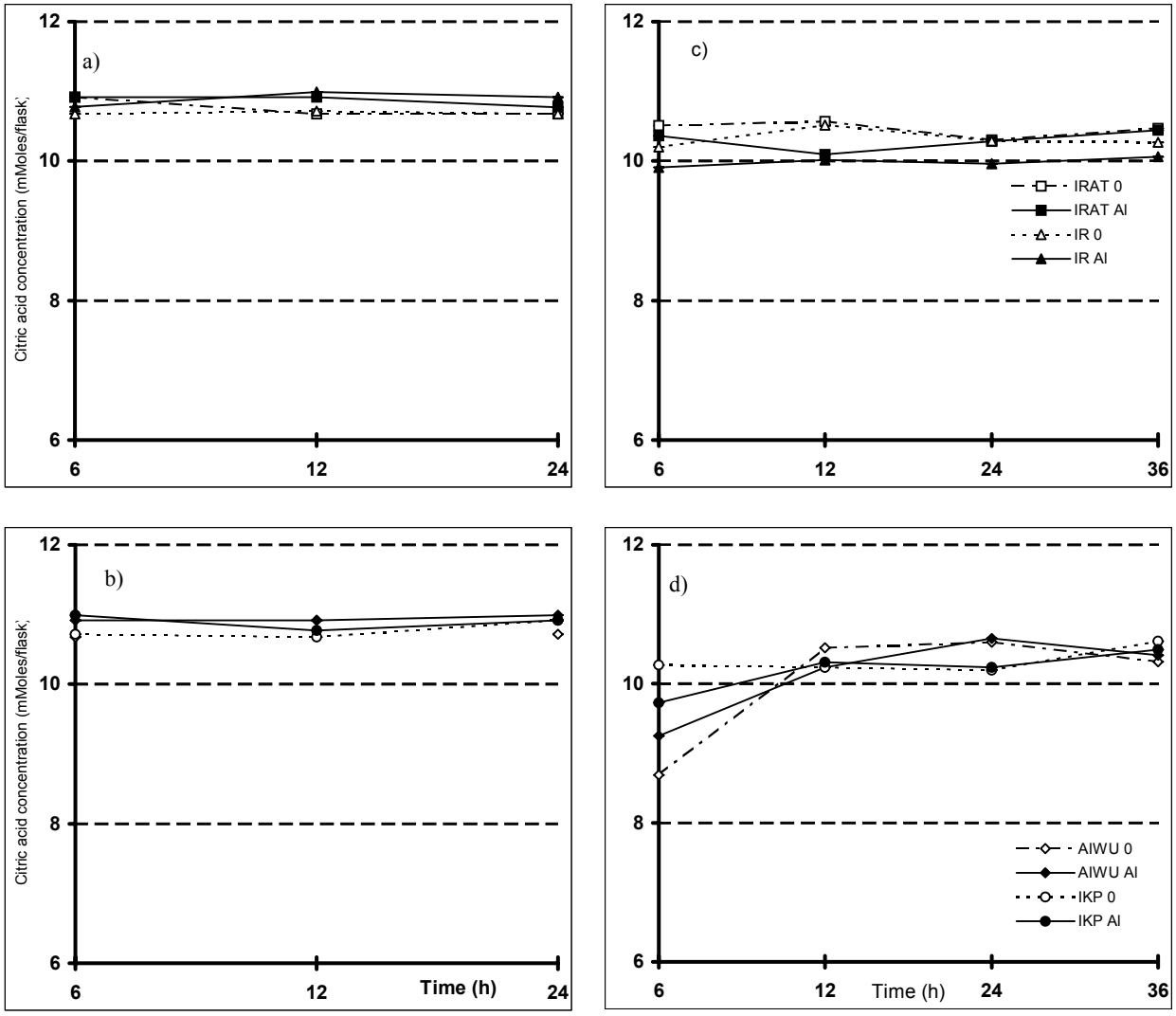


**FIGURE 2** - Excretion of citric acid by root apices of Al-resistant (IRAT and IR) and Al-sensitive (Aiwu and IKP) rice cultivar seedlings as a function of exposure to solutions containing Aluminium (Al) 250µM (a, b) and 500µM (c, d) and control (zero Al). The coeficient of variation ranged from 2 to 7%.

**Excretion of malic acid by seedling roots and callus culture**

In the absence of Al and irrespective of the duration of exposure, the roots of all cultivars tested excreted approximately the same amount of malic acid (Figure 3a-b). In the presence of Al and whatever the duration of exposure, malic acid concentration in the incubation medium slightly increased (figure 3a-b). This increase was significant in all cultivars, after 6h of aluminum exposure. There was no further increase in malic acid excretion with longer exposures and all cultivars behaved similarly (Figure 3a-b).

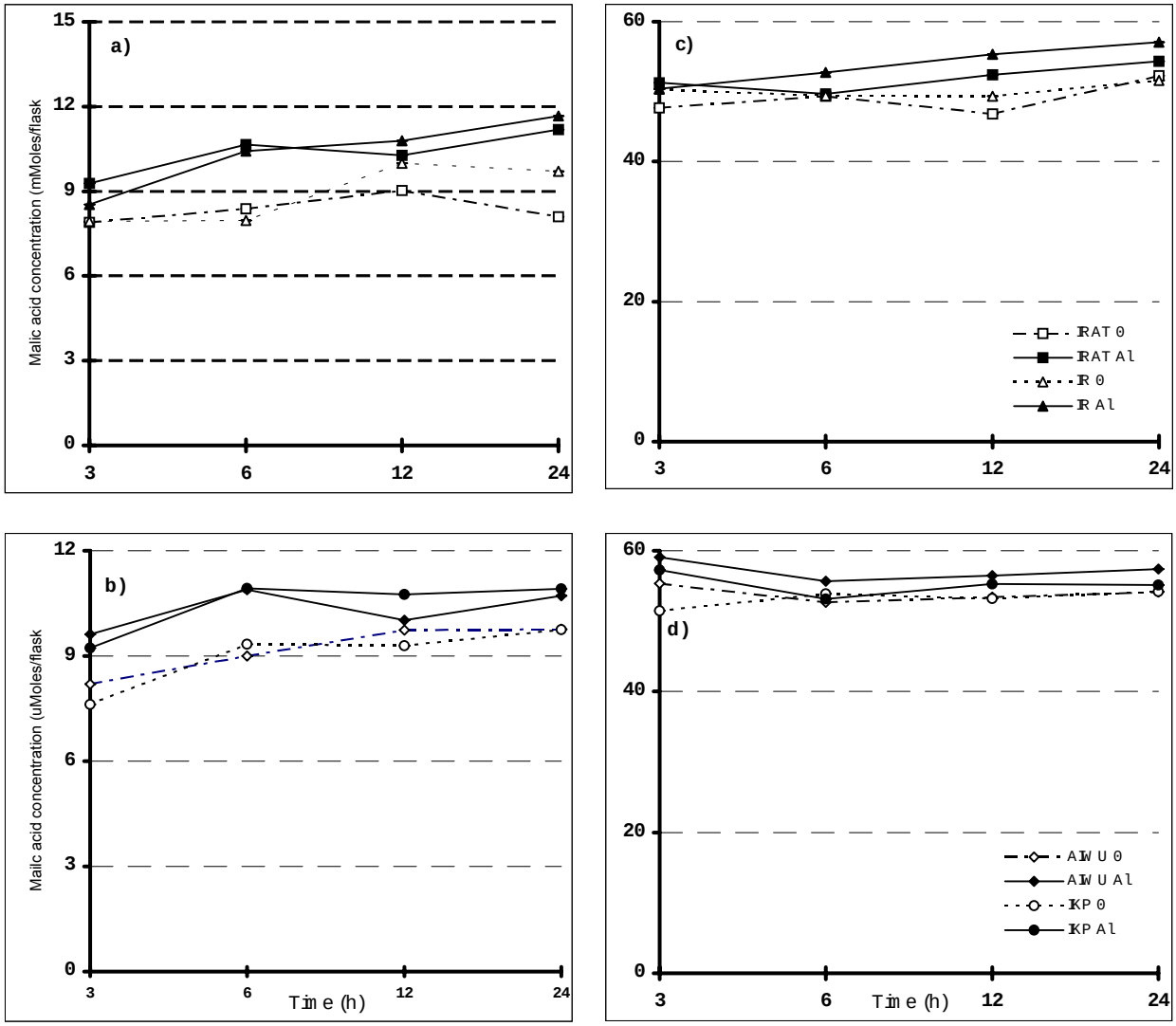


**FIGURE 3** - Excretion of malic acid by root apices of Al-resistant (IRAT and IR) and Al-sensitive (Aiwu and IKP) rice cultivar seedlings (a, b) and by rice mature embryo-derived calli (c, d) as a function of exposure to solutions containing Aluminium (Al) 250µM and control (zero Al). The coeficient of variation ranged from 8 to 12%.

Similar results were obtained when rice calli were exposed to aluminum, i. e., there was a slight, but non-significant, increase in the amount of excreted malic acid in Al-treated versus untreated calli, and in the presence of aluminum, o significant differences between cultivars and durations of exposure were detected (Figure 3c-d).

**Accumulation of malic acid in root apices and callus tissues**

In the absence of Al, malic acid concentration in root apices was similar in all cultivars, except after 24h of exposure, when IRAT accumulated more malate than the other three cultivars, but this accumulation was not significant (Figure 4a-b).

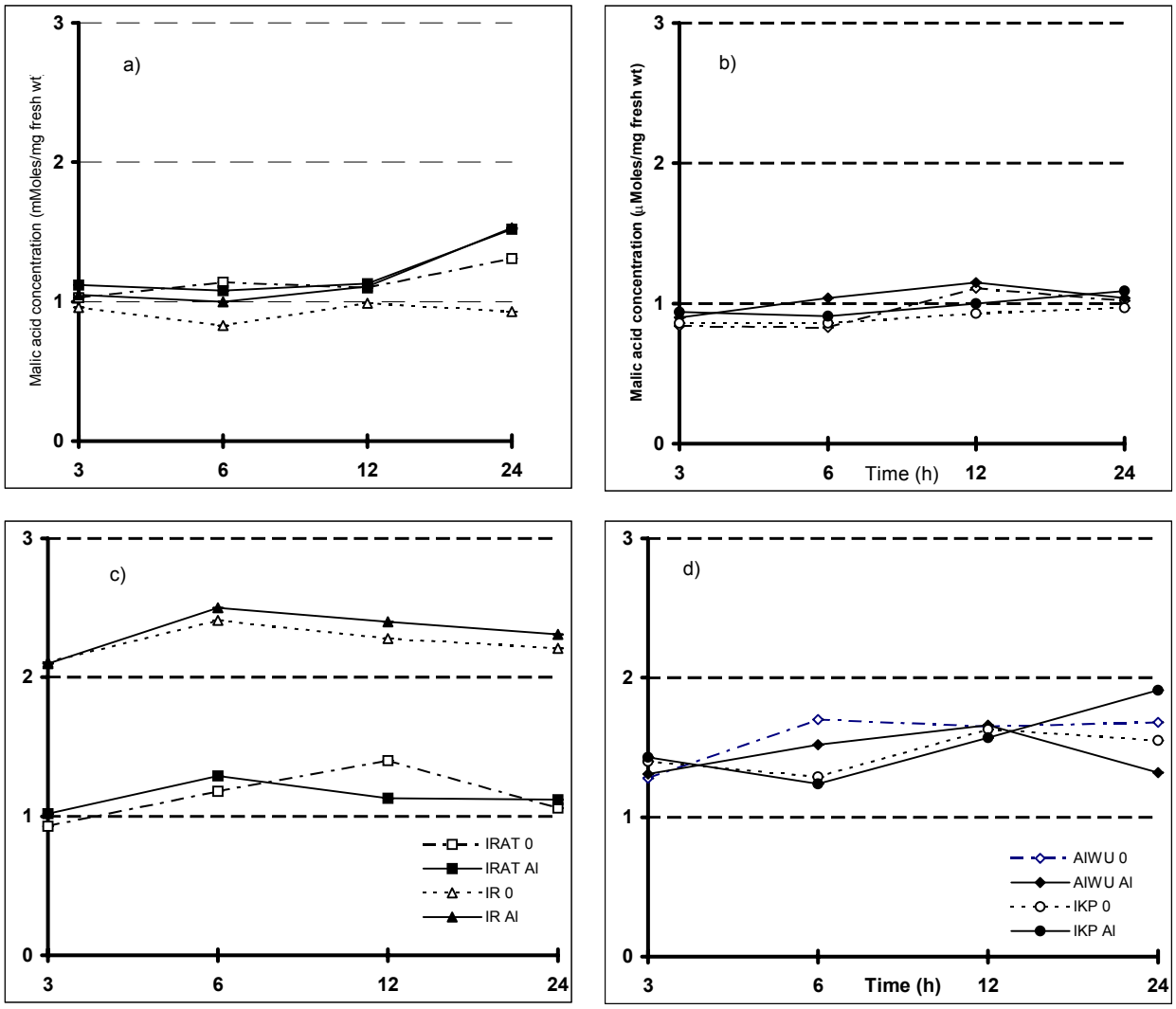


**FIGURE 4** – Concentration of malic acid in root apices of Al-resistant (IRAT and IR) and Al-sensitive (Aiwu and IKP) rice cultivar seedlings (a, b) and in rice mature embryo-derived calli (c, d) as a function of exposure to solutions containing aluminum (Al) 250 µM and control (zero Al). The coefficient of variation ranged from 5 to 9 %.

In the presence of 250 µM of aluminum, all cultivars showed no differences in malic acid concentration in root apices when compared to the untreated controls, irrespective of the duration of exposure to Al (Figure 4a-b).In callus tissues, the highest amount of malic acid was found in IR cultivar. In all cultivars, Al treatment had no effect upon malate concentration (Figure 4c-d).

## DISCUSSION

Inhibition of root growth is a typical effect of Al in rice that has already been reported in other species (Ryan *et al.*, 1993; Kochian, 1995). Our results clearly indicate that the extent of this inhibition depends on the cultivar and on the Al concentration applied during the stress period. At high Al levels (500 µM), all cultivars

that we have investigated behaved similarly, exhibiting strong reduction of root elongation. In contrast, IRAT was not significantly affected at low levels (250 μM).

The possibility that differences in amounts of citric or malic acids excreted by seedlings roots could be responsible for differences in Al resistance observed among 4 rice cultivars was investigated.

Our results indicate that neither the release of citric acid nor the release of malic acid is responsible for differential aluminum resistance observed in studied cultivars. This conclusion is valid for the two experimental systems we used, i.e. the whole plants and the callus cultures. In contrast, Miyasaka *et al.* (1991) reported that in snapbean the Al resistant 'Dade' cultivar excreted more citric acid than the sensitive cultivar 'Romano'. Similar results, in which higher aluminum resistance was correlated with higher excretion of citrate were found with maize (Pellet *et al.*, 1995) while aluminum resistance in wheat has been correlated with higher excretion of malate by seedling roots (Delhaize *et al.*, 1993b; Basu *et al.*, 1994; Ryan *et al.*, 1995 a,b). The discrepancies between the above results and our findings could reflect differences between species or in experimental techniques. The question as to whether a difference in organic acid excretion could be detected over the short time exposure used in our experiments remains open. In several other studies, however, enhanced exudation of malate (Delhaize *et al.*, 1993b; Ryan *et al.*, 1995a,b) or citrate (Pellet *et al.*, 1995) by seedling roots of resistant cultivars were observed after 30h or less. The observations of Basu *et al*., (1994) and Miyasaka *et al.*, (1991) were done after 48h or more of Al stress, but these authors did not report whether differences were already detectable earlier or not.

In root, there was usually no increase in malic acid in response to Al treatment. Similarly, in callus, no correlations could be established between differential malic contents and aluminum tolerance. Thus these results suggest that internal Al-detoxification in roots by malic acid cannot account for Al-resistance in our rice cultivars. This conclusion is in agreement with the views of Scott *et al.* (1990) and Pellet *et al.* (1995) who consider that differential aluminum tolerance in wheat and maize is not related to changes in root organic acids concentrations. Of course, we cannot exclude that differences in organic acids excretion or accumulation could be detected for other durations of aluminum exposure than those investigated in this work. Organic acids other than malate or citrate could be involved in Al-resistance in our rice cultivars. Further research needs to be carried out to examine other possible mechanisms of Al-resistance in rice and to determine whether other organic acids are implicated.

## ACKNOWLEDGMENTS

This work was suported by grants from F.N.R.S. of Belgium and the CAPES of Brazil to Cristiane Elizabeth Costa de Macêdo. The authors thank J. Bar for excellent technical assistance and Dr. P. Bertin for valuable comments and discussions.

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