

Evaluation of aluminium tolerance in grapevine rootstocks

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Summary

Aluminum (Al) toxicity is a major worldwide agricultural problem. At low pH, Al speciates into the soluble and phyto-toxic form Al³⁺, inhibiting the root growth and affecting plant development. In Brazil, agriculture in acidic soils with elevated concentration of Al has significantly increased in the last decades. Therefore, in order to achieve efficient agriculture practices, the selection of plant cultivars with improved Al resistance has become crucial in this type of soils. In this work we have evaluated the Al resistance of six genotypes of grapevine rootstocks. The grapevine hardwood cuttings were grown in nutrient solution in the absence and presence of 250 and 500 µM Al at pH 4.2. The phenotypic indexes of relative root growth, fresh and dry root weight, root area, hematoxylin staining profile, and Al content were evaluated for all six genotypes. These phenotypic indexes allowed us to identify the 'Kober 5BB', 'Gravesac', 'Paulsen 1103', and 'IAC 766' grapevine rootstocks genotypes as the ones with the highest resistance to Al. Likewise, 'IAC 572' and 'R110' genotypes were the most Al-sensitive cultivars. We evaluated the root organic acid exudation profile in the most Al-resistant ('Kober 5BB') and most Al-sensitive ('R110') in plantlets cultivated *in vitro* in the absence and presence of 100, 200, and 400 µM of Al. Among several compounds detected, citrate was the only organic acid related to the Al resistance phenotype observed in the 'Kober 5BB' genotype. The high constitutive citrate exudation observed in 'Kober 5BB' strongly suggests that exudation of this particular organic acid may impart Al-resistance/amelioration in grapevine.

Key words: *Vitis spp.*, abiotic stress, aluminum-tolerance, aluminum-toxicity, organic acid exudation, root growth.

Introduction

Acid soils found in large areas of the world pose a major limitation to agriculture. In soils with pH < 5.0, toxic forms of Al are released into the soil solution at levels leading to root growth inhibition, thus affecting the whole plant development (FOY *et al.* 1978, KOCHIAN 1995). Roots suffering from Al toxicity tend to become underdeveloped reducing nutrient uptake and increasing susceptibility to drought

(LUGANY *et al.* 1994, SASAKI *et al.* 1996). Although soluble Al can be temporarily removed from these soils by liming, this practice is not sustainable as it is time and economically unfeasible. Given the extensive inter- and intra-species genetic variability with respect to Al resistance in plants, screening of the genetic diversity in grapevine rootstocks is a more suitable approach to improve the performance of this crop in acidic soils. Evaluation of the physiological mechanism(s) that grant grapevines improved agricultural performance in this stress conditions will aid in future development of new genotypes with improved Al-resistance.

Although a number of possible physiological Al resistance/tolerance mechanisms have been proposed and studied over the years, the most compelling experimental evidence supports the existence of resistance mechanisms based on excluding Al from entering the root via exudation of organic acids such as citrate at the root surface (and into root apoplastic spaces) in response to Al stress (KOCHIAN *et al.* 2004, 2005). The organic acids released can immobilize Al³⁺ by forming stable, nontoxic complexes preventing it from entering the root. The cellular mechanism mediating organic acid release have recently being characterized (PIÑEROS *et al.* 2008 a and b, SASAKI *et al.* 2004). However, alternative Al-resistance mechanisms based on oxidative stress alleviation and gene expression regulation have also been reported (CANÇADO *et al.* 2005, 2008).

Given there is limited information regarding the behavior of grapevine rootstocks under Al stress, in the present study we evaluate phenotypic indexes associated with Al resistance in six genotypes of grapevine rootstocks.

Material and Methods

Plant genotypes and growth conditions: The six grapevine rootstock genotypes characterized in this study were: 'IAC 766'; 'IAC 572'; 'Gravesac'; 'R110'; 'Paulsen 1103'; and 'Kober 5BB', supplied by the germplasm of EPAMIG (Caldas, Brazil). Rootstock hardwood cuttings were propagated in a sand bed under fog irrigation. After 20 d, hardwood cuttings with vigorous and healthy root development were transferred to opaque plastic box containing 25 L of nutrient solution of the following composition (mg·L⁻¹): 100 NH₄NO₃; 1,000 KNO₃; 150 MgSO₄·H₂O; 50 KH₂PO₄; 200 Ca(NO₃)₂·4H₂O; 1.2 MnSO₄·4H₂O; 1 H₃BO₃; 1 ZnSO₄·7H₂O; 0.025 CuSO₄·5H₂O; 0.025 CoCl₂·6H₂O; 1 KI; 1 Na₂MoO₄·2H₂O; 27.5 FeSO₄·7H₂O;

37.5 Na₂EDTA, pH 5.5. The nutrient solution was continuously aerated and the hardwood cuttings were grown during 14 d for previous adaptation.

Aluminum treatment: Following the adaptation period, the initial root length of tagged roots was measured (time zero) and the nutrient solution was replaced by an identical solution supplemented with 0, 250 or 500 µM of AlCl₃ (corresponding to 0, 44, and 80 µM of Al³⁺ activity, respectively). The nutrient solutions were continuously aerated, and their pH was monitored daily and adjusted to 4.2. The experiment was carried out in 12 plastic boxes (experimental units) with 6 replicates per treatment.

Root growth: To evaluate the root growth, two roots from each hardwood cutting were tagged with plastic rings. The length of each root was measured at 24 h intervals during 4 consecutive days. The net root length for each 24 h period was calculated after 24, 48, 72 and 96 h. The relative growth rate was calculated as the percentage of root growth length grown in the presence of Al (250 and 500 µM) in relation to the root growth length in control solution (CANÇADO *et al.* 2005).

Root area: Roots of six hardwood cuttings grown in nutrient solution (0, 250, 500 µM of Al) were collected after 15 d of treatment and individually scanned. The images were analyzed using software SIARCS 3.0 (Embrapa, Brazil). Root area was calculated in pixels.

Fresh and dry weight of roots: The complete roots of each hardwood cutting with 15 d of treatment were washed in water and dried in paper towel. Fresh weights were determined using an analytical balance. Root samples were dried at 50 °C until a constant weight was reached prior to recording the dry weights. Six replicates were evaluated for each treatment.

Aluminum content: Dried tissue samples of the complete root after 15 d of treatment were pulverized, homogenized, and 1 g of the powdered root was digested in nitric-perchloric acid solution. Al content was determined from the mineral extract as described in WANG and WOOD (1973). Six replicates were evaluated for each treatment.

Hematoxylin staining: The staining was carried out as described by CANÇADO *et al.* (1999). Five roots tips of 10 cm in length from each genotype grown in nutrient solution lacking or containing 250 µM of Al for 24 h were tested. Excised roots were rinsed in distilled water during 20 minutes and then placed in a solution consisting of 1 % hematoxylin (Sigma-Aldrich, USA) and 0.1 % potassium iodine during 2 min. Then rinsed and washed in distilled water during 1 hour. Stained roots tips were photographed under a stereoscope.

Tissue culture of grapevine rootstocks: In order to produce samples for organic acid evaluation the genotypes 'R110' and 'Kober 5BB' were cultivated *in vitro* in sterile condition in liquid MS medium (MURASHIGE and SKOOG 1962) at pH 5.5. After two months the medium was replaced with filter-sterilized solution consisting of 500 µM of CaCl₂ at pH 4.2 during 48 h for adaptation prior to Al-treatment. Subsequently, the medium was exchanged by solution with 0, 100, 200, or 400 µM of AlCl₃ (pH 4.2), corresponding to 60, 130, and 250 µM

of Al³⁺, respectively. The plantlets were cultivated in the treatment solution for 24 h and 48 h and samples of 10 ml of the nutrient solution containing the exudates were collected.

Organic acid evaluation: The samples were lyophilized and concentrated in 100 µl of water HPLC grade. The soluble extracts were purified in nitrocellulose filters (0.45 µm Millipore) and 30 µl of each sample were injected in a HPLC (Hewlett-Packard, model 1100) equipped with a pre-column SupelcoGuard C610H (50 mm x 4.6 mm) and a column SupelcoGel C610H (300 mm x 7.8 mm) warmed-up to 30 °C. The liquid phase consisted of a 0.5% H₃PO₄ solution injected in a flux rate of 0.4 ml·min⁻¹ and a running time of 40 min. The organic acids absorbance was monitored at 210 nm. Citrate, oxalate, malate, tartaric (Sigma-aldrich), succinate (Supelco), and trans-aconitate (Fluka) standards were used for the identification and quantification of organic acids present in the samples. Ten replicates for each treatment were evaluated.

Statistical analysis: All treatments were organized in a completely randomized design. The data were examined through a Variance Analysis (F test) and Tukey test for multiple average comparisons, at a 5 % significance level.

Results

Relative root growth: Among all six genotypes studied, 'Kober 5BB' was the rootstock genotype which showed the least root growth inhibition under Al stress (Fig. 1). The root growth rates calculated for this genotype over 24, 48, 72 and 96 h of exposure to 250 µM Al were not significantly different to those observed in roots grown in control solution (*i.e.* lacking Al) over the same time periods. However, a slight root growth inhibition was observed after 72 and 96 h (relative to the control) when the Al concentration in the nutrient solution was doubled (*i.e.* 500 µM). The genotype 'Gravesac' showed a similar response to that described above for 'Kober 5BB'; however, the onset of growth inhibition in the presence of 500 µM Al was apparent at an earlier time point (*i.e.* 48 h). The Al-induced root growth inhibition recorded in genotypes 'IAC 766' and Paulsen resembled that described for the 'Gravesac' genotype. Genotypes 'IAC 572' and 'R110' were noticeably the most sensitive to the Al treatments, with the root growth rates being reduced as much as 40 % (*e.g.* genotype 'R110' exposed to 250 µM Al during 96 h).

Fresh and dry root matter: The genotypes 'Gravesac' and 'Paulsen 1103' had the largest fresh and dry root weight after 15 d of growth in the 250 and 500 µM Al treatments (Fig. 2). The genotype 'Kober 5BB' had intermediate fresh and dry root weights, followed by the genotypes 'IAC 572' and 'IAC 766' respectively. The of 'IAC 572' and 'IAC 766' showed the smallest fresh and dry root weights in both Al treatments, as well as in control conditions (Fig. 2).

Root area: Root areas were calculated from scanned images taken after 15 d old plants grown in nutri-

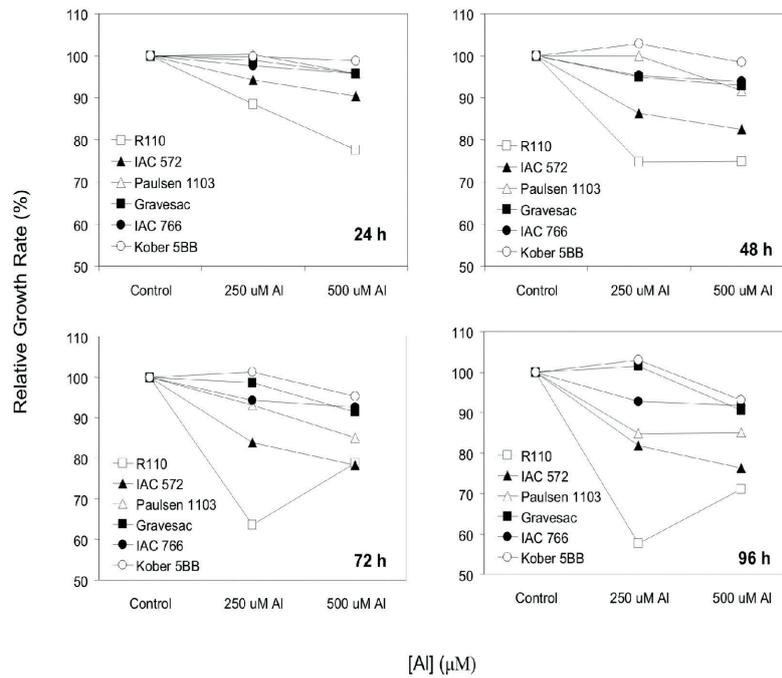


Fig. 1: Relative growth rate (%) of grapevine rootstocks genotypes: 'R110' (white square); 'IAC 572' (black triangle); 'Paulsen 1103' (white triangle); 'Gravesac' (black square); 'IAC 766' (black circle); and 'Kober 5BB' (white circle). Measurements were performed in plants treated with 0 (control), 250 μM and or 500 μM of Al. The Al-exposure times were 24, 48, 72 and 96 h.

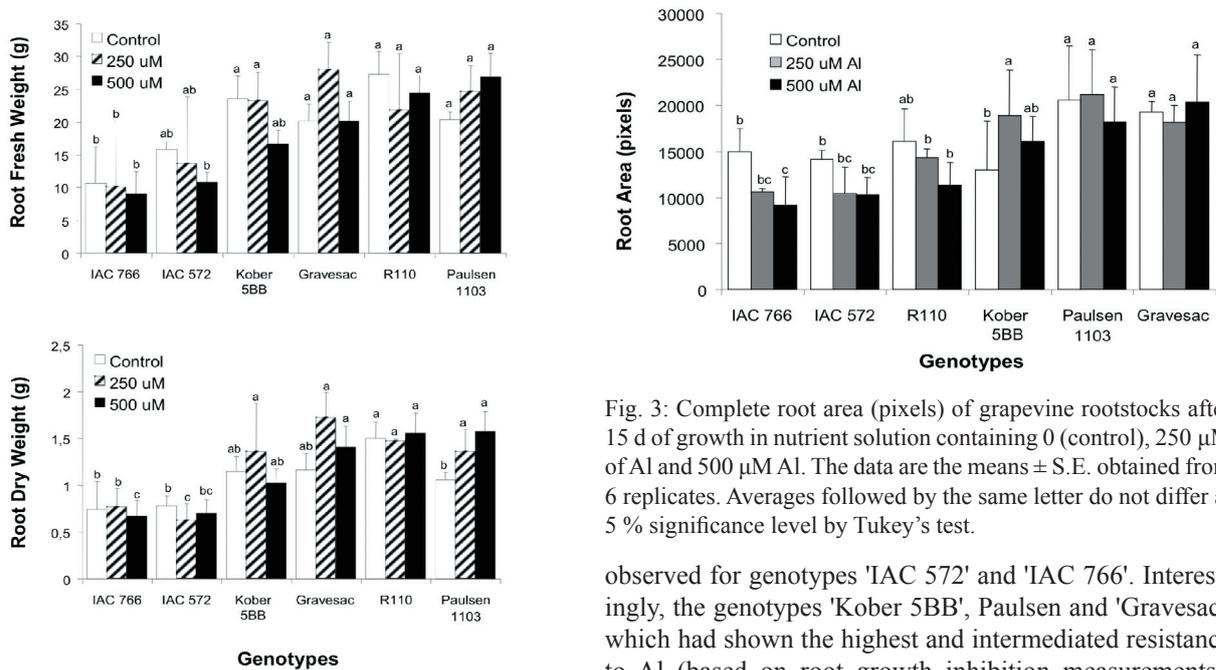


Fig. 2: Root fresh weight and root dry weight of grapevine rootstocks genotypes 'IAC 766', 'IAC 572', 'Kober 5BB', 'Gravesac', 'R110', and 'Paulsen 1103' after 15 d of cultivation in nutrient solution containing 0 (control), 250 μM of Al and 500 μM Al. The data are the means ± S.E. obtained from 6 replicates. Averages followed by the same letter do not differ at 5 % significance level by Tukey's test.

ent solutions lacking or containing Al (Fig. 3). The genotypes 'Gravesac' and 'Paulsen 1103' showed the largest total root area, regardless of the treatment, with the genotype 'Kober 5BB' ranking at an intermediate value. Likewise, the genotype 'R110' showed a larger root area than that

Fig. 3: Complete root area (pixels) of grapevine rootstocks after 15 d of growth in nutrient solution containing 0 (control), 250 μM of Al and 500 μM Al. The data are the means ± S.E. obtained from 6 replicates. Averages followed by the same letter do not differ at 5 % significance level by Tukey's test.

observed for genotypes 'IAC 572' and 'IAC 766'. Interestingly, the genotypes 'Kober 5BB', Paulsen and 'Gravesac', which had shown the highest and intermediated resistance to Al (based on root growth inhibition measurements), showed no significant reduction in root area in the presence of Al (relative to their control). Likewise, genotypes 'R110' and 'IAC 572', previously ranked as Al-sensitive show a significant reduction in root area (relative to the control) in the presence of Al.

Hematoxylin staining: Accumulation or exclusion of Al at the actively growing root region (*i.e.* root apex) was evaluated using hematoxylin stain after 24 h of treatment. This dye has the property of turning blue when it forms a complex with Al, such that the penetration and retention of this ion in the roots can be assessed, allowing for a direct and quantitative measure of Al sensitivity based on

the color intensity of stained root apices grown in nutrient solution (POLLE *et al.* 1978, DELHAIZE *et al.* 1993). In the present study, only roots from the treatments containing Al showed blue coloration after staying with hematoxylin, with the distinct staining profiles among the genotypes studied. The genotypes 'Kober 5BB' and 'Paulsen 1103' showed no staining in the 5 mm apical root region (Fig. 4). The genotype 'IAC 572' showed no staining in a smaller region (2 mm from the apex), while the genotypes 'IAC 766' and 'R110' showed a dark blue staining through out the root except for a small (1 mm length) root tip region. It is worth noticing that the root cap of the latter genotype was also strongly stained. Finally, genotype 'Gravesac' showed a dark blue staining through the root, including both the root tip and the root cap.

Aluminum content: The Table shows the Al content measured in the complete root system of all genotypes after 15 d of Al treatment. As expected, the amount of

Al found in the root tissue of plants cultivated in presence of 500 μM Al were bigger than in the root tissues of plants cultivated in presence of 250 μM Al. Only trace amounts of Al were found in the root tissues of plants cultivated in the control treatment. However, no major differences in Al root content were observed among the six genotypes.

Organic acid exudation: Given their significant differences in Al-sensitivity (Fig. 1) and Al-exclusion patterns (Fig. 4) the genotypes 'Kober 5BB' and 'R110' were selected as representative contrasting cultivars to evaluate potential differences in the nature of root exudates. Exudates samples were collected after 24 and 48 h of exposure to 0, 100, 200 or 400 μM Al. Fig. 5 shows representative HPLC chromatograms of the contrasting root exudates profiles from 'Kober 5BB' and 'R110' roots after 48 h of Al treatment. Citrate, malate, succinate and trans-aconitate peaks were regularly identified in the samples. Among these, citrate was the only organic acid that showed a clear relation between grapevine rootstock genotype (Fig. 6). Root citrate exudation from the 'Kober 5BB' genotype was significantly higher than that observed for the genotype 'R110'. Citrate exudation from 'Kober 5BB' roots was constitutive, with the exudation rates being unaffected by the presence of Al in the growth media.

Discussion

Several methodologies have been developed for evaluating the potential physiological processes underlying the Al tolerance trait in plants (CANÇADO *et al.* 1999, ZHANG *et al.* 1994, MOUSTAKAS *et al.* 1993). Root growth assessments in nutrient solutions is possibly one of the most attractive ones, as they allow control over the solution chemistry and speciation, providing adequate forms of Al stress, and thereby allowing preliminary screening of a large number of genotypes (MAGNAVACA *et al.* 1987). Using this methodology we established a differential degree of root growth inhibition among the six grapevine rootstock genotypes examined when grown under Al stress conditions (Fig. 1). For example, the rootstock genotype 'Kober 5BB' had the least root growth Al-induced inhibition, followed by the rootstock 'Gravesac' which showed an intermediate Al-sensitivity. In contrast, the rootstocks 'R110' and 'IAC 572' showed higher Al-induced root growth inhibition.

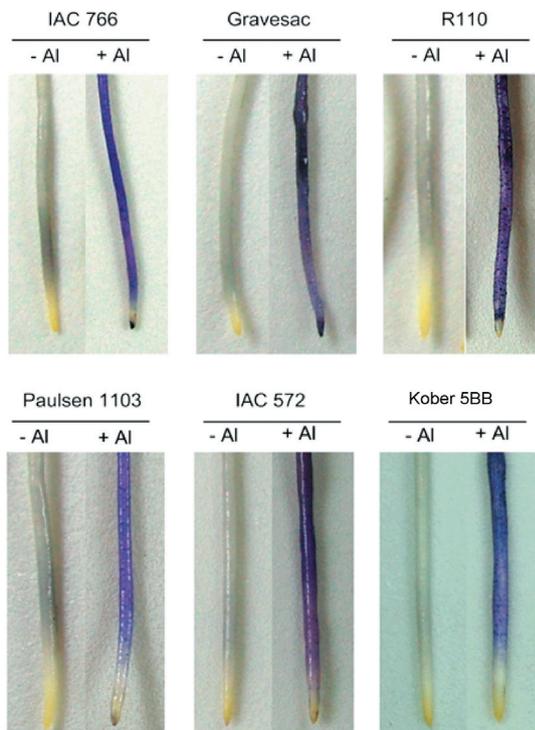


Fig. 4: Hematoxylin staining profile of grapevine rootstocks root tips cultivated in absence of Al (-Al) and presence of 250 μM of Al (+Al) during 24 h.

Table

Aluminum content ($\mu\text{g}\cdot\text{g}^{-1}$ of dry weight) in roots of grapevine rootstocks genotypes after 15 d of treatment in nutrient solution containing 0, 250 μM , or 500 μM of Al. The data are the means \pm S.E. obtained from 6 replicates. Averages followed by the same capital letter in the line and small letters in the column do not differ at 5% significance level by Tukey's test

Genotypes	Treatment +Al (μM)		
	0	250	500
IAC 766	0.023 \pm 0.076 Cb	7.158 \pm 3.754 Ba	29.980 \pm 9.870 Aa
Gravesac	0.075 \pm 0.003 Ca	7.122 \pm 1.079 Ba	33.551 \pm 11.803 Aa
R110	0.119 \pm 0.104 Ca	5.784 \pm 0.092 Bab	28.117 \pm 8.947 Aa
Paulsen 1103	0.082 \pm 0.010 Cb	4.740 \pm 1.250 Bb	31.470 \pm 6.211 Aa
IAC 572	0.091 \pm 0.007 Cb	6.315 \pm 0.562 Ba	22.123 \pm 3.420 Ab
Kober 5BB	0.055 \pm 0.021 Cbc	5.742 \pm 1.037 Bab	33.338 \pm 9.961 Aa

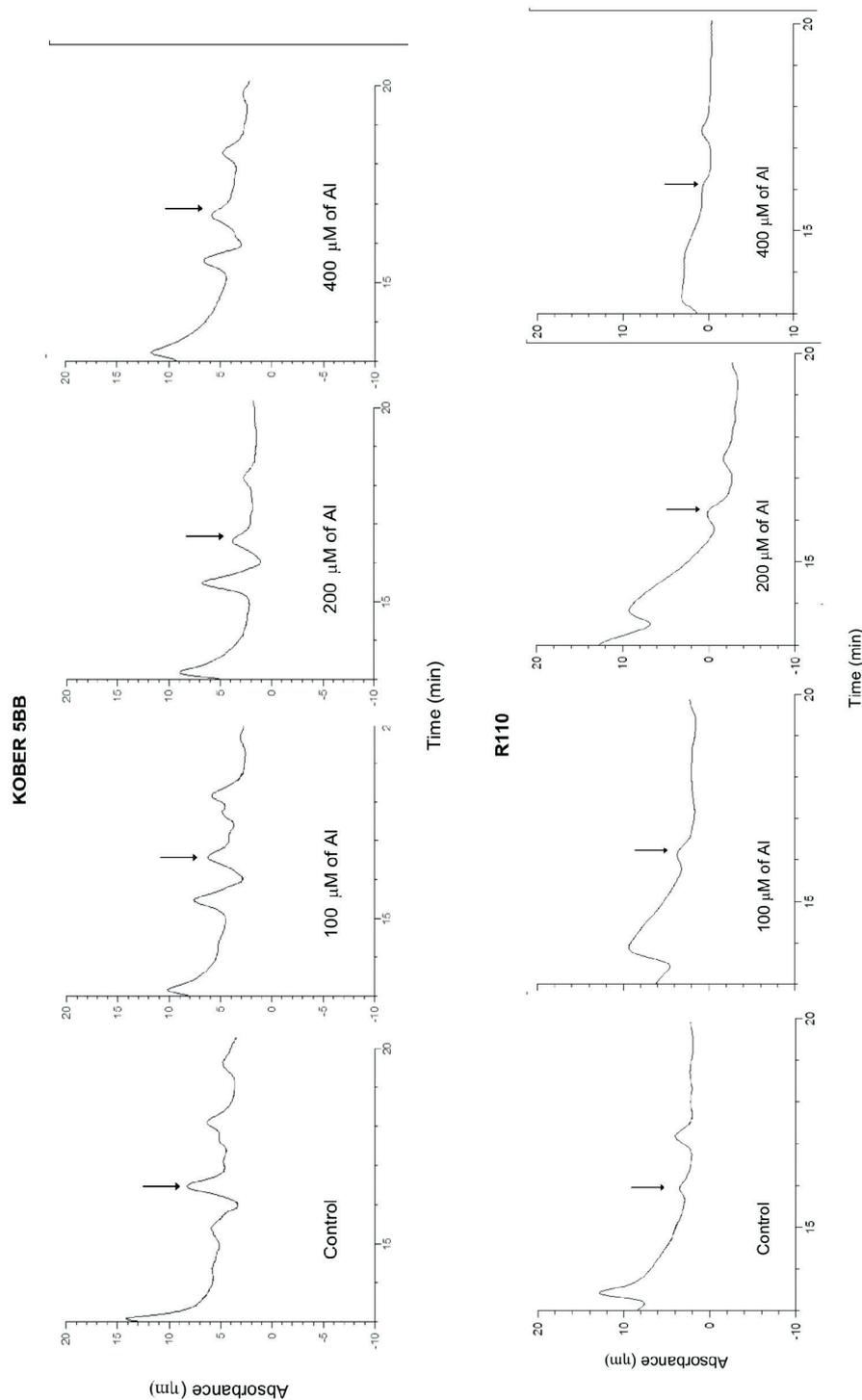


Fig. 5: Representative HPLC chromatograms of root exudates from 'Kober 5BB' and 'R110' after exposition to 0 (control), 100, 200 and 400 μM of Al for 48 h. Arrows indicate the citrate peak.

These results indicate the existence of genetic diversity on the Al-resistance trait among the grapevine rootstock evaluated in the present study. However, although root length has been frequently used as a suitable phenotypic index for Al tolerance in plants cultivated in nutrient solution (for example see CANÇADO *et al.* 1999), due to the complexity of the Al-stress responses, it should not be used as the single criterion to score Al-resistance.

The evaluation of alternative parameters such as fresh and dry root weights yield a contrasting scoring profile

(Fig. 2). The genotypes 'Paulsen 1103', 'R110' and 'Gravesac' had the highest root weights, while 'Kober 5BB' occupied only an intermediate position. Genotypes 'IAC 766' and 'IAC 572' showed the smallest root weights when grown both in the presence and absence of Al. These observations indicate that differences in root tissue mass are a product of the different genetic background among the genotypes, rather than a product of differential responses to Al stress. Alternatively, the difference in the root growth and root weight profiles could be biased by morphological

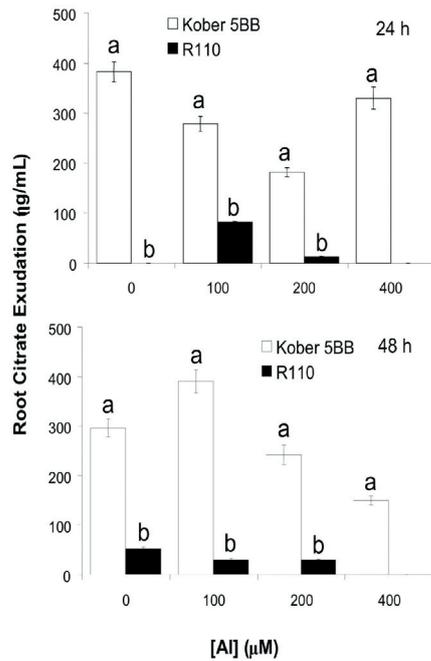


Fig. 6: Citrate root exudation from 'Kober 5BB' and 'R110' cultivated in sterile medium without Al (control) and 100, 200 and 400 μM of Al at pH 4.2 for 24 and 48 h. The data are the means \pm S.E. obtained from 10 replicates. Averages followed by the same letter do not differ at 5 % significance level by Tukey's test.

changes due to Al-induced injuries, such as the severe root thickening and the production of larger number of secondary and capillary roots observed in the 'R110' genotype.

Furthermore, the root area and root weight measurements are absolute values, while the root growth rate is a relative value calculated between treatment and control. Thus, given the poor correlation between these parameters and Al-resistance, we conclude that at least for grapevine rootstocks, neither root weight nor root area can be used as an accurate parameter to categorize Al resistance in these plant species.

Grapevine genotypes were also scored based on the hematoxylin dye staining profiles, a precocious and non-destructive methodology commonly used in cytogenetic studies evaluating Al-exclusion in plant species (POLLE *et al.* 1978, RINCÓN and GONZALES 1992, DELHAIZE *et al.* 1993; CANÇADO *et al.* 1999;). This technique has proved conducive in identifying resistant and sensitive genotypes after a very short exposure time of plants to Al, well before differences in the root length become detectable (DELHAIZE *et al.* 1993). In the present study, the genotypes 'Kober 5BB' and 'Paulsen 1103' grown in the presence of Al showed no staining in the actively growing root regions (*i.e.* root tips). In contrast, the root tips of the 'IAC 766' and 'IAC 572' genotypes showed a light blue staining in the tips. The remaining two genotypes ('R110' and 'Gravesac') showed a strong blue staining throughout the root, including the root tip region. As with all other parameters measured, hematoxylin staining can not be used as an absolute criterion to score Al-resistance as it could potentially be misleading in plant genotypes that achieve Al-resistance by accumulating and sequestering high amounts of Al in the aerial part of the plant rather than excluding it from penetrating into

the root system (FOY and PETERSON 1994, MOUSTAKAS *et al.* 1993). Nonetheless, in the present study Al-exclusion as inferred from the staining profiles correlate with the differential Al-sensitivity scored from the root growth measurements. Thus, in the case of grapevine rootstock, relative root growth parameter can be associated with hematoxylin staining, and could be used simultaneously to phenotype Al resistant genotypes in a non destructive way (CANÇADO *et al.* 1999). Additionally, the strong hematoxylin staining observed at the root tip of the Al-sensitive grapevine rootstocks strongly suggests that the root tip of these plants might be the first target of Al toxicity. Thus, any Al-induced or constitutive Al resistant mechanism(s) ameliorating Al phyto-toxic effects is likely to operate primarily in this actively growing region.

The Al accumulation measured in the complete root tissue showed no correlation with the Al sensitivity scored by the root growth and hematoxylin staining parameters. This unexpected lack of correlation might be due to substantial Al accumulation in the mature regions of the root, masking the localized genotypic differences in Al-accumulation (as indicated by the hematoxylin staining) in the relatively small biomass of the growing root tip. Thus, analysis of Al content from exclusively the root apical region, rather than the entire whole root, might prove more adequate.

Based on the results of relative root growth rate and hematoxylin staining we chose 'Kober 5BB' and 'R110' as representative Al-resistant and Al-sensitive genotypes to examine their root exudation profiles under Al stress. Interestingly, the roots of the Al-resistant 'Kober 5BB' genotype showed a constitutive citrate exudation which was at least three times higher than that observed for the Al-sensitive 'R110' genotype (Figs 5 and 6). The fact that citrate exudation in 'Kober 5BB' was independent from the presence or absence of Al indicates that the exudation of this organic acid in this genotype is a constitutive mechanism, rather than an Al-triggered plant response. Nonetheless, as in other plant species, citric acid exudation may provide some amelioration to Al stress (KOCHIAN *et al.* 2004).

To our knowledge, the present study constitutes the first work addressing potential Al-resistance in grapevine rootstocks. The hematoxylin staining results indicate that as in many other plant species, the primary site and target of Al stress in grapevine rootstocks is confined to the actively growing root tip, and as such early Al stress symptoms manifest as a severe inhibition of root growth. Our observations demonstrate that in regards to the Al resistance trait, there is enough genetic diversity in grapevine rootstocks. These observations open the possibility to develop breeding programs focused at the increase of Al resistance, particularly in acid soil of tropical regions where Al-toxicity is a major agronomical constrain.

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