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Assessment of tolerance to Aluminum toxicity in olive (*Olea europaea)* based on root growth and organic acid Al^{3+} exclusion mechanism

Tesfahun Alemu Setotaw[1*], Claudinéia Ferreira Nunes[1,6], Cristina Soares de Souza[1], Ana Paula Ribeiro[2], Gustavo de Faria Freitas[2], Daniel Angelucci de Amorim[5], Dalilhia Nazaré dos Santos[1], Moacir Pasqual[2], Juliano Lino Ferreira[3], Geraldo Magela de Almeida Cançado[4]

[1]Plant Biotechnology Laboratory, Agricultural Research Agency of Minas Gerais (EPAMIG), Av. Santa Cruz, 37780-000, Caldas, Brazil
[2]Federal University of Lavras (UFLA) - Department of Agriculture, Plant Tissue Culture Laboratory - Post Office Box 3037, 37200-000, Lavras, MG, Brazil
[3]Brazilian Agricultural Research Organization, Embrapa Pecuária Sul – CPPSUL, Bagé, RS – Brazil
[4]Embrapa GenClima, Center of Genetic Engineering and Molecular Biology, AC Unicamp University City, 13083970, Campinas, SP – Brazil
[5] Agricultural Research Agency of Minas Gerais (EPAMIG), Rua Afonso Rato, 1301, Mercês, 38060040 - Uberaba, MG – Brazil
[6]Instituto Capixaba de Pesquisa, Assistência Técnica e Extensão Rural (Incaper), Cx. Postal 62, CEP 29900-970, Linhares, ES, Brasil

***Corresponding author: setotaw2006@gmail.com**

Abstract

Aluminum toxicity is a major agricultural problem at low pH that inhibits the root growth and plant development. Therefore, selecting cultivars with Aluminum (Al) tolerance will be crucial step for the breeding programs. This work was done with the objective of evaluating the Al tolerance of six principal olive genotypes: 'MGSASC315'; 'Barnea'; 'Leccine'; 'CLO0025'; 'Coratina' and 'Mission' based on relative root growth, organic acid exudation, and root apex hematoxylin staining analysis. For root growth and hematoxylin staining, the experiment was laid on 4 x 6 factorial (4 doses of $AlCl_3$ (0, 250, 500 and 1000 µM), 6 genotypes of olive) in hydroponic solution under greenhouse condition. The root growth was measured for five consecutive weeks in a week interval. The organic acid exudation was evaluated after 24 and 48h exposure for Al in solution containing 0, 100, 200 and 400 µM of $AlCl_3$. The 1000 µM of $AlCl_3$ severely inhibited the root development of olive genotypes while 250 and 500 µM $AlCl_3$ produced small damage when compared to plantlets grown in the control solution. The analysis of organic acid exudation after 24 and 48h exposure to Al^{3+} showed citric acid involved on Al tolerance mechanism in olive, whereas malate and oxalic acid did not. Among the olive genotypes MGSASC315, Barnea and Leccine recorded high relative root growth and high citric acid exudation under Al stress that showed these genotypes are tolerant for Aluminum stress. The result also showed hematoxylin staining of the root apex of olive tree was not efficient in discriminating among control, 250 and 500 µM AlCl3 treatments within each genotype.

Keywords: abiotic stress, Al-tolerance, Olive tree, organic acid exudation, relative root growth.
Abbreviations: HPLC_High Pressure Liquid Chromatography; Al _Aluminum; EPAMIG_Empresa de Pesquisa Agropecuária de Minas Gerais; FAPEMIG_Fundação de Amparo à Pesquisa do estado de Minas Gerais; FINEP_Financiadora de Estudos e Projetos; CAPES_Coordenação de Aperfeiçoamento de Pessoal de Nível Superior; EMBRAPA_Empresa Brasileira de Pesquisa Agropecuária.

Introduction

Acid soil is a chronic agricultural problem in the large part of the world that limits agricultural activities. It comprises 40 % of the arable land in the world (Foy et al., 1978) and this problem is more prominent in tropical areas where the weather promotes the soil acidification. Due to the localization, Brazil also suffers from acid soil problem where Aluminum (Al) toxicity is a bottleneck for agricultural activities. In the soil with pH<5.0 the toxic form of Al is released in the soil solution that lead to root growth inhibition thus affecting the whole plant development (Foy et al., 1978; Kochian et al., 1995). The Al toxicity in plants promotes severe reduction in root growth reduce water and nutrient uptake that caused susceptibility for drought (Llugang et al., 1994; Sasaki et al., 1996) and yield reduction. To overcome this constraint farmers used frequently the practice of liming to neutralize the toxic effect of Al^{3+}. However, its effects doesn't last longer and not considered as a sustainable practice to mitigate the problem because of its high cost, volume required and requirement of appropriate mechanization to incorporate it into the soil (Troeh and Thompson 2005; Fageria and Baligar 2008), besides this method is not affordable for the small scale farmers in the tropics and subtropics. This showed the importance of environmental friendly and economically viable solution to overcome the problem such as developing olive cultivars tolerant and well adapted to acid soil condition. Nowadays the cultivation of olive orchards is expanding in countries with acid soil/Al toxicity problem such as Brazil. That showed the importance of developing olive cultivars with improved tolerance for Al toxicity. The first step in developing cultivars tolerant to Al toxicity is studying the response of the olive cultivars for Al toxicity that represent

Table 1. ANOVA table of the relative root growth of six olive genotypes after (1^{st}, 2^{nd}, 3^{rd}, 4^{th}, 5^{th}) week exposure of $AlCl_3$ solution. The ANOVA was done using the PROCGLM of SAS.

Source of variation	DF	Mean squares				
		1^{st} week	2^{nd} week	3^{rd} week	4^{th} week	5^{th} week
Genotype	5	148.88**	290.9**	412.92*	773.91**	1446.28**
Treatment	3	432.47**	1226.94**	1720.87**	2598.05**	3081.09**
Genotype*Treatment	15	65.59*	136.39ns	184.25ns	229.76ns	314.83ns

the gene pool of olive cultivars grown in Brazil (Val et al. 2012). Until now, there is no report about the behavior of olive trees in Brazil in the presence of Al that showed the importance of developing the research activity in this direction. Different authors used physiological parameters such as relative root growth, organic acid exudation and hematoxylin staining of the root apex to evaluate Al tolerance in various crops such as maize (Cançado et al. 1999), Vittis (Cançado et al. 2009), common bean (Rangel et al. 2005; 2007) and proven efficient in identifying genotypes tolerant for Al toxicity. The tolerance mechanism that prevent entrance the toxic form of Al in the root system via organic acid exudation such as citrate acid in the presence of Al^{3+} was also reported in different crops (Kochian et al., 2004;2005; Cançado et al. 2009). To our knowledge, we did not come across a research reports about Al tolerance in olive species tree that showed the importance of developing research activities to address this problem. In addition, due to the increasing importance of this crop in Brazil and its expansion in tropics and subtropics region where the acidification is a prevalent agricultural problem producing information in this direction will benefit the future breeding program of olive and its production in the region. Therefore, the work was done with the objective of studying Al tolerance in olive cultivars with diverse genetic architecture (Val et al., 2012) based on relative root growth reduction, hematoxylin staining, and organic acid exudation activities.

Results

Relative root growth analysis

The analysis of variance of the relative root growth among the olive genotypes showed significant difference ($p<0.05$) among genotypes along the period of measurement (Table 1). The highest relative root growth was recorded by genotype MGSASC315, while the minimum by Mission at all the treatments (control, 250, 500, and 1000 µM of $AlCl_3$) along the measurement time followed by Barnea (Fig 1 and Table 2). Fig. 1 showed the response of olive genotypes at different level of $AlCl_3$ at each week of measurement that indicated genotypes MGSASC 315, Barnea and CLO0025 are tolerant and Mission is the most susceptible. The Scott-knott mean separation test ($p<0.05$) among genotypes also confirmed similar results (Table 2).

Organic acid exudation

The analysis of aluminum induced organic acid exudation with the presence of $AlCl_3$ showed significant difference ($p<0.05$) among olive genotypes for oxalic acid and citric acid (Figure 2 &3). The result also confirmed during this process only oxalic acid and citric acid were released by the genotypes of olive (Figure 2 & 3). Among the six genotypes of olive Leccine and CLO0025 started exudation of citrate acid within 24h exposure for $AlCl_3$ solution (Figure 2). In contrast olive cultivars MGSASC0315 and Barnea started citrate acid exudation after 24h exposure for $AlCl_3$ solution (Fig 3). In addition, Barnea and CLO0025 exudate citrate acid after 24 and 48h of exposure for $AlCl_3$ (Fig 2 and Fig 3). The absence of oxalic acid exudation after 48h exposure showed this organic acid is not involved in Al tolerant mechanism in olive genotypes evaluated in this experiment. Besides the absence of malate acid and succinic acid exudation during the evaluation period showed these acids are not involved in Al tolerance mechanism in olive tree. The relationship among organic acid exudation and relative root growth (Fig 4) show the strong relation between the citric acid exudation and relative root growth under the Al treatment. The graph also showed with the increase of citric acid exudation the relative root growth increase (Fig 4A) in contrast the increase in the malic acid exudation is not accompanied with the increase in relative root growth (Fig 4B). This evidence showed the importance of citric acid on Al tolerance mechanism in olive tree.

Root staining analysis

Hematoxylin staining analysis in olive tree roots with and absence of $AlCl_3$ showed that this tool is important to discriminate genotypes for Al tolerance in maximum dose. However, it is not possible to see the coloring intensity with different level of $AlCl_3$ especially for 250 and 500 µM $AlCl_3$ except 1000µM (Fig 5). Among the genotypes CLO0025 and Mission showed high root apex damage at 1000 µM AlCl3 (Fig 5). The study showed that hematoxylin analysis is not efficient method to differentiate among treatments of Al within each cultivar.

Discussion

The data from this research showed olive genotypes MGS ASC0315 and Barnea are tolerant for Al stress, while Mission is the most susceptible one. In addition relative root growth and citrate acid exudation are proved efficient methods to select olive genotypes resistant to Al stress condition. Our result showed that the presence of differential response among olive genotypes evaluated for relative root growth and organic acid exudation. Differential relative root growth, Al induced organic acid exudation, and hematoxylin staining of the root apex are frequently used by researchers to evaluate Al tolerance in different crops such as maize (Cançado et al. 1999; Llungany et al. 1994), Vittis (Cançado et al. 2009), common bean (Rangel et al. 2005) and proved efficient in olive tree. Our result also showed high relative root growth is accompanied with citrate acid exudation under Al stress condition that showed this organic acid is involved in Al exclusion mechanism in olive tree. In our study olive genotypes MGS315 and Barnea showed significant relative root growth accompanied with the high citric acid exudation that indicated these cultivars use citrate acid exudation as a mechanism to neutralize the toxic effect of Al in the nutrient solution. Similarly research reports are presented in maize (Llugany et al. 1994), wheat (Delhaize et al. 1993), common bean (Rangel et al. 2005) and *Vittis* (Cançado et al. 2009). According to Cançado et al. (1999) Al induced root growth inhibition alone cannot be used as the only criteria for screening of Al tolerance due to the complex nature of Al

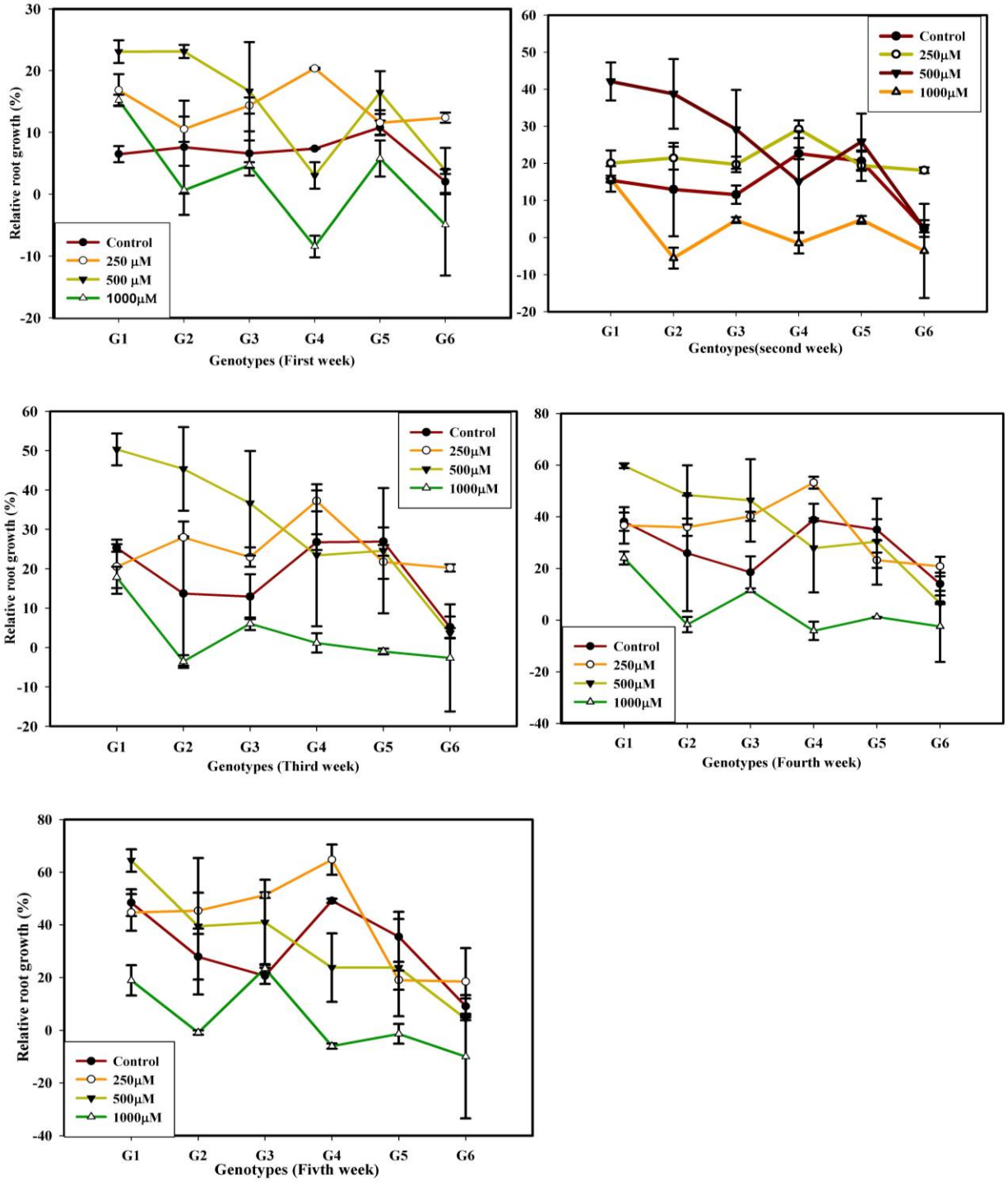

Fig 1. The relative root growth at 1st, 2nd, 3rd, 4th and 5th weeks of measurement at different level of Al treatment. All the data showed significant difference among genotypes and treatment at 5% level of probability. (G1) 'MGSASC 315'; (G2) 'Barnea'; (G3) 'Leccine'; (G4) 'CLO0025'; (G5) 'Coratina' and (G6) 'Mission').

stress response in plants there for it is recommended the use of different Al tolerance screening techniques at the same time. Ma and Furukawa (2003), Ma et al. (2001), and Kochian et al. (2005) reported the importance of the citrate acid exudation to neutralize the toxic effect of Al in different crops by forming a stable chelate that prevent the movement of Al^{3+} to other part of the plant. Our result showed the activation of the gene responsible for citrate acid exudation in Leccine and CLO0025 seems constitutive that did not required some time to activate since it started the citrate acid exudation immediately after exposed for Al^{3+} solution (Fig 2), as reported in some species by Ma et al. (2001) and Kochian et al. (2005). In contrast, the organic acid exudation for genotypes MGSASC315 and Barnea needs stress of Al to activate the gene responsible for citrate acid exudation that can only observed after 48h exposure for $AlCl_3$ (Fig 3). The study also showed oxalic acid was not responsible for Al tolerance in olive tree since the exudation of this organic acid was not accompanied with relative root growth and was not consistent during 24h and 48h of exposure (Fig 2 & 3). Since this is the first report about Al tolerance mechanism in this species, it can contribute much for the breeding program of

Table 2. The mean comparison test of the relative root growth among the six olive genotypes at different level of $AlCl_3$ concentration along the time of measurement using Scott-Knott.

Genotypes	Treatments	First week	Second week	Third week	Fourth week	Fifth week
MGSASC315 (G1)	Control	6.48 c	15.42 b	25.26b	38.10b	48.45a
MGSASC315 (G1)	250 µM	16.84b	20.08 b	20.50b	36.69b	44.73a
MGSASC315 (G1)	500 µM	23.06a	42.12 a	50.31a	59.80a	64.44a
MGSASC315 (G1)	1000 µM	15.23b	15.79b	17.80b	24.04c	18.92b
Barnea (G2)	Control	7.60 c	12.96b	13.67c	25.89b	27.89b
Barnea (G2)	250 µM	10.52b	21.41b	27.98 b	35.97b	45.42a
Barnea (G2)	500 µM	23.10 a	38.76a	45.34a	48.49a	39.49a
Barnea (G2)	1000 µM	0.63 c	-5.53c	-3.56c	-1.71d	-0.87c
Leccine (G3)	Control	6.6c	11.55c	12.90c	18.50c	20.66b
Leccine (G3)	250 µM	14.35b	19.72b	22.95b	40.20b	51.31a
Leccine (G3)	500 µM	16.67b	29.17 a	36.62a	46.37a	40.97a
Leccine (G3)	1000 µM	4.70c	4.73c	6.04c	11.61c	23.45b
CLO0025 (G4)	Control	7.38c	22.66b	26.75b	38.81 b	49.15a
CLO0025 (G4)	250 µM	20.35a	29.23a	37.23a	53.25a	64.78a
CLO0025 (G4)	500 µM	3.02c	15.09b	23.42b	27.90 b	23.80b
CLO0025 (G4)	1000 µM	-8.44d	-1.51c	1.15c	-4.11d	-6.01c
Coratina (G5)	Control	10.77b	20.57b	26.88b	34.99b	35.45a
Coratina (G5)	250 µM	11.57 b	19.38b	21.72b	23.20c	19.05b
Coratina (G5)	500 µM	16.46b	25.85b	24.58b	30.41b	23.76b
Coratina (G5)	1000 µM	5.76c	4.81c	-1.01c	1.22 d	-1.39c
Mission (G6)	Control	2.01c	2.47c	5.15 c	14.01c	9.13c
Mission (G6)	250 µM	12.38b	18.14b	20.20b	20.79c	18.44b
Mission (G6)	500 µM	3.86c	2.45c	3.62c	6.54d	4.33c
Mission (G6)	1000 µM	-4.94 d	-3.62c	-2.66c	-2.41d	-10.05c

NB- treatment mean along the column with different letter showed significant difference at 5% probability.

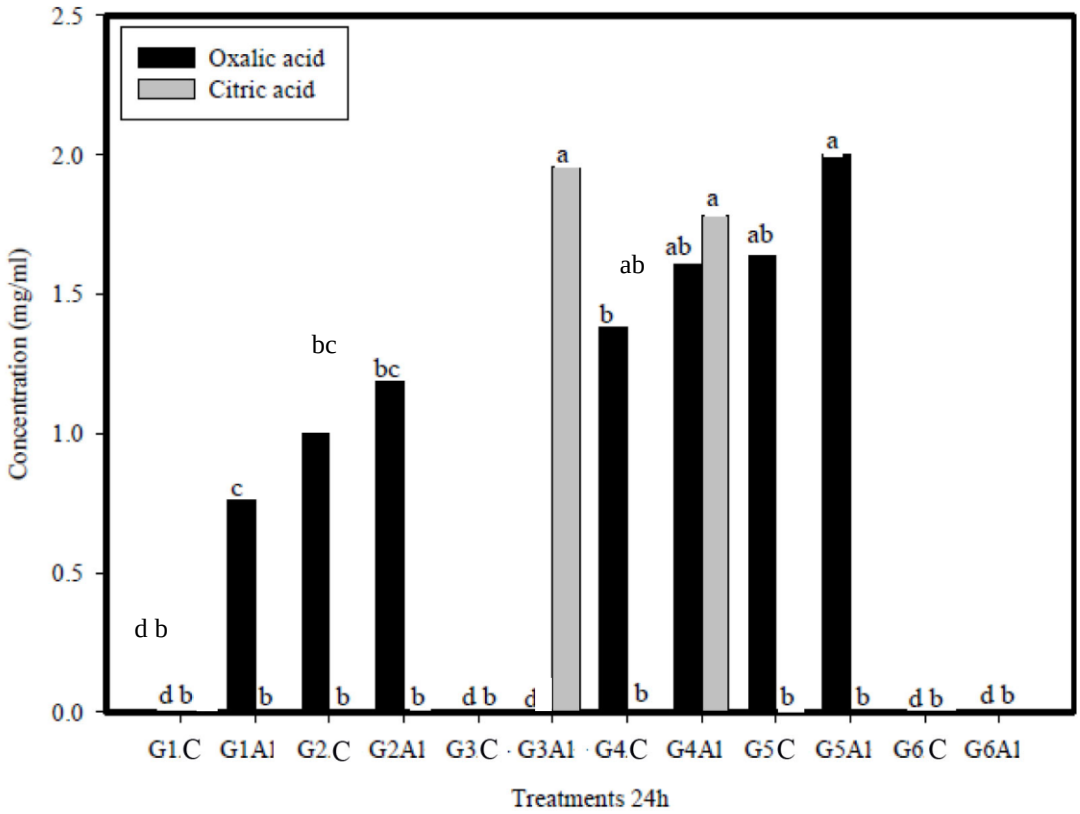

Fig 2. The organic acid exudation of olive genotypes [(G1) 'MGSASC 315'; (G2) 'Barnea'; (G3) 'Leccine'; (G4) 'CLO0025'; (G5) 'Coratina' and (G6) 'Mission'] with the presence (Al) and absence AlCl3[Control (C)] after 24h exposure. The treatments with different letter are significant at 5% probability using Tukey's test.

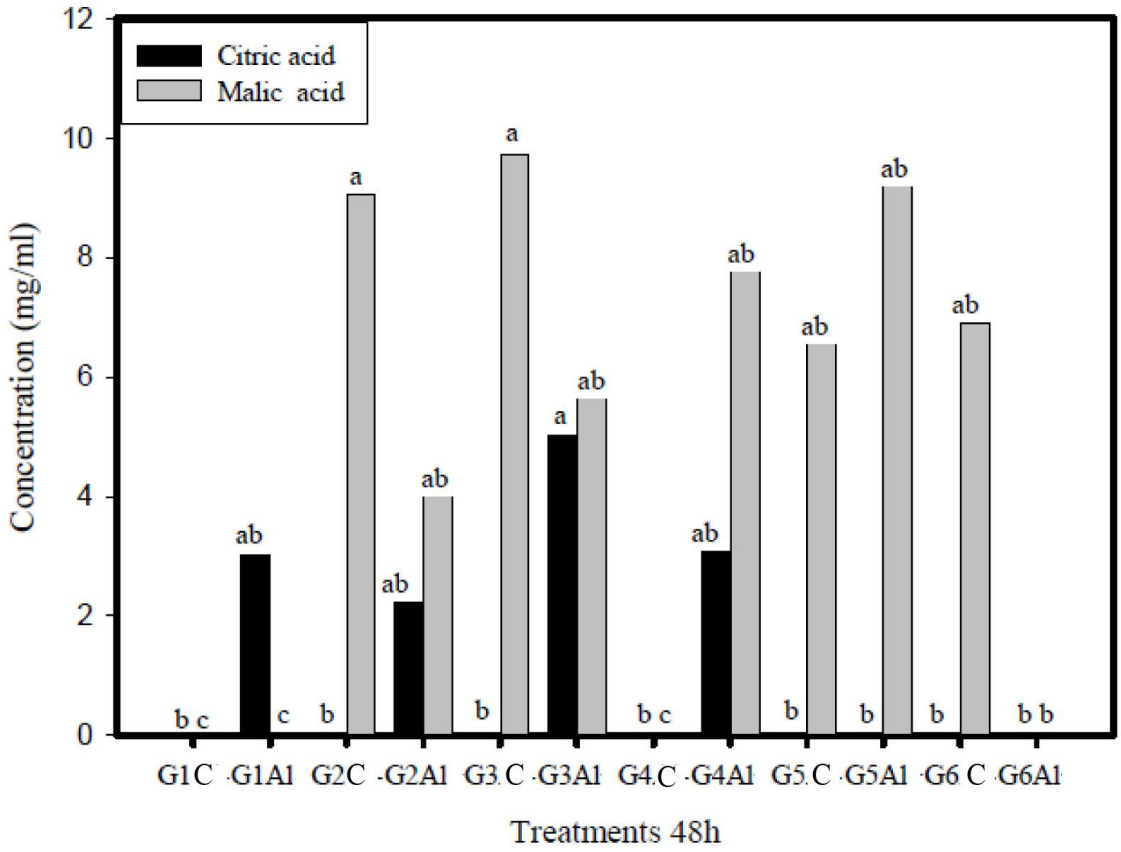

Fig 3. The organic acid exudation of olive genotypes [(G1) 'MGSASC 315'; (G2) 'Barnea'; (G3) 'Leccine'; (G4) 'CLO0025'; (G5) 'Coratina' and (G6) 'Mission') in the presence and absence of $AlCl_3$ (Control (C)] after 48h exposure. The treatments with different letter are significant at 5% probability using Tukey test.

olive tree to develop Al tolerant cultivars for commercial production, especially due to the expansion of its production in the tropical regions like Brazil that are affected by acid soil problem. Hematoxylin has a special property of turning blue when it form a complex with Al (Cançado et al., 1999) and the method allow the direct quantitative measure of Al susceptibility based on the coloring intensity of the root apices (Polle et al., 1978, Delhaize et al., 1993). Our result showed hematoxylin staining was efficient to differentiate the control and maximum concentration of $AlCl_3$ (1000 µM) for all genotypes and among genotypes (Fig 4) but not efficient technique in determining Al tolerance in the olive genotypes at different level of $AlCl_3$ solutions. This may be due to the root structure of olive that is perennial species. In our case hematoxylin staining work well at the maximum dose (1000µM $AlCl_3$) and due to this reason the technique cannot be used as sole screening technique for Al tolerance in olive tree genotypes. Therefore, for an efficient screening of Al tolerance in olive, the relative root growth, Al induced organic acid exudation and hematoxylin staining should be used together.

Materials and Methods

Plant genotypes and growth conditions

The six genotypes characterized in this study were: G1) 'MGSASC 315'; G2) 'Barnea'; G3) 'Leccine'; G4) 'CLO0025'; G5) 'Coratina' and G6) 'Mission', obtained from the Olive germplasm bank of EPAMIG located at the Experimental Farm of Maria da Fé, Minas Gerais, Brazil. The six genotypes were selected randomly from each cluster groups formed based on the genetic diversity study analysis of olive genotypes of Brazil using SSR molecular marker (Val et al. 2012). The hardwood cuttings were propagated in a sand bed under fog irrigation. After 40 days, the plantlets 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 NH_4NO_3; 1,000 KNO_3; 150 $MgSO_4.H_2O$; 50 KH_2PO_4; 200 $Ca(NO_3).4H_2O$; 1.2 $MnSO_4.4H_2O$; 1 H_3BO_3; 1 $ZnSO_4.7H_2O$; 0.025 $CuSO_4.5H_2O$; 0.025 $CoCl_2.6H_2O$; 1 KI; 1 $Na_2MoO_4.2H_2O$; 27.5 $FeSO_4.7H_2O$; 37.5 Na_2.EDTA, pH 4.2 (Magnavaca et al. 1987). The nutrient solution was continuously aerated and the plantlets were grown during 14 d under the solution for subsequent adaptation.

Aluminum treatment

Following the adaptation period, from selected roots, the initial root length measurement (Time zero) was done and the root tagged with plastic rings for subsequent measurements. After the initial measurement, the nutrient solution was replaced by an identical solution supplemented with 0, 250, 500 or 1000 µM of $AlCl_3$. 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 four replicates per treatment.

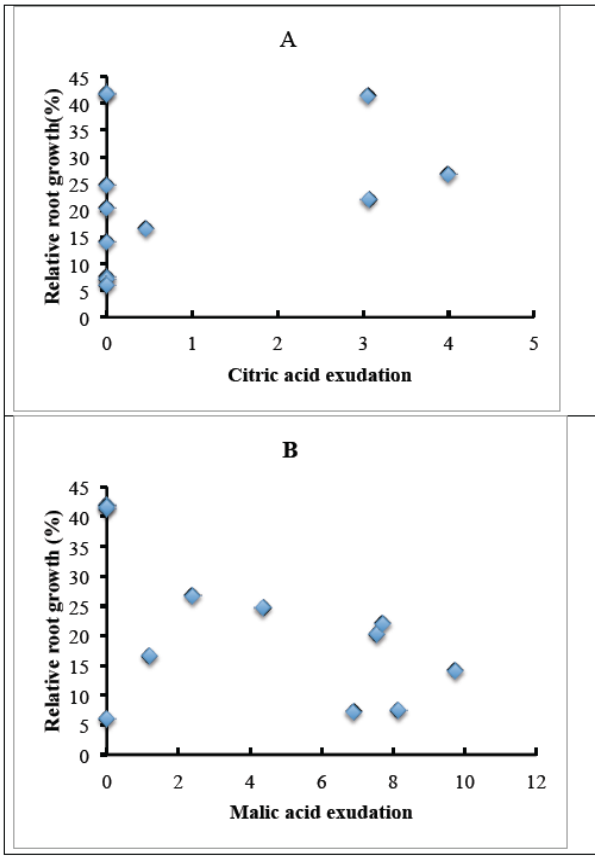

Fig 4. The scatter graph showing the relationship between organic acids (citric acid (A) and malic acid (B)(mg/ml)) and relative root growth in olive genotypes with the presence and absence of $AlCl_3$.

Fig 5. The hematoxylin staining of the root tips of six olive genotypes evaluated under different level of $AlCl_3$ (control (0), 250, 500, and 1000 µM of $AlCl_3$) after 24h exposure in the nutrient solution.

Root growth measurement

To evaluate the root growth, tagged roots from each cutting were measured at weekly intervals during five consecutive weeks. The net root length for each week period was calculated after 1st, 2nd, 3rd, 4thand 5th week. The relative growth rate was calculated as the percentage of root growth length based on the difference between the measurement before the treatment applied to each week reading for each treatment (0, 250, 500 and 1000 µM $AlCl_3$). The experiment was laid with 6 x 4 factorial where six olive cultivars and four levels of $AlCl_3$ with 4 replications using completely randomized design (CRD).

Hematoxylin staining

The hematoxylin root staining was carried out as described by Cançado et al. (1999) using the five root tips of 10cm taken from each treatment (0, 250, 500 and 1000 µM of $AlCl_3$) after 24h of application. The excised roots were rinsed in distilled water during 20 min and then placed in a solution consisting of 1 % hematoxylin (Sigma-Aldrich Chemical Co., USA) and 0.1 % potassium iodine during 2 min. Then rinsed and washed in distilled water during 1 h. Stained roots tips were evaluated and photographed under a stereomicroscopy with magnification of 10x.

Organic acid extraction and HPLC analysis

For organic acid evaluation, olive plantlets were transplanted to filter-sterilized solution consisting of 500 µM of $CaCl_2$ at pH 4.2 during 48 h for adaptation prior to Al-treatment. Subsequently, the medium was replaced with a solution containing 0, 100, 200, or 400 µM of $AlCl_3$ (pH 4.2), corresponding to 60, 130, and 250 µM of Al^{3+} activity, respectively. The plantlets were nurtured in the treatment solution for 24 and 48 h, thereafter samples of 10 mL of the nutrient solution containing the exudates were collected. For organic acid evaluation, the collected samples were concentrated 10 times using a speed vacuum centrifuge (Eppendorf, Germany). Then the concentrated samples were purified in nitrocellulose filters with mesh of 0.45 µm (Millipore, USA). The 100µL of each sample were injected in a HPLC (PerkinElmer, USA) equipped with a guard-column ODS-C18 and two analytical columns Brownlee (PerkinElmer) ODS-C18 (250 mm x 4.6 mm) mounted in series and warmed-up to 30 °C. The liquid phase consisted of a 0.5 % H_3PO_4 solution injected in a flow rate of 0.5 mL min^{-1} for 50 min. The organic acids absorbance was monitored at 210 ηm using PerkinElmer HPLC equipped with UV/VIS reflectance. Pure standards of oxalate, malate, citrate, succinate and *t*-acconitate (Sigma-Aldrich Chemical Co., USA) were used for the identification and quantification of organic acids present in the samples. Four replicates for each treatment were analyzed.

Statistical analysis

The data of relative root growth and organic acid exudation was subjected for analysis of variance and mean separation. The ANOVA and Mean separation analysis was done using ProcGLM model of SAS software (SAS Institute, 2002). The mean separation test was done using Tukey'sand Scott-Knott statistical test at $p<0.05$ using the same software. The following model was used for analysis of variance

$Y_{ij} = \mu + G_i + T_i + G_iT_i + \varepsilon_{ij}$

Where, μ- grand mean, G_i- genotype effect, T_i- Treatment effect (Al), G_iT_i- Genotype x Treatment effect, and ε_{ij}– Residual error (Tamane 2009).
For all graphic analysis the Sigma plot software program was used.

Conclusion

This study showed that olive cultivars MGSASC315, Barnea, and Leccine are tolerant olive genotypes for Al toxicity whereas Mission the most susceptible genotype. Al induced relative root growth, and citric acid exudations are efficient techniques for screening Al tolerance genotypes in olive tree. In addition, citrate acid exudation is one of the principal organic acids used to neutralize the effect of Al in acid soil in olive tree. The study also proved the importance of using different Al tolerance screening techniques to select Al tolerance genotypes in olive tree. Hematoxylin staining technique is not an efficient screening technique for Al tolerance in olive. The information produced in this study can be used in future breeding program of olive in Brazil since this crop is in expansion due to its economic importance.

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