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Rapid screening method for aluminum tolerance in maize (*Zea mays* L.)

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Short title: Rapid screening for aluminum tolerance in maize

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language, structure, and presentation are appeali
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Abstract

~~A significant decrease in maize grain yield due to a~~Aluminum toxicity is ~~considered to be~~ one of the most ~~important~~ significant agricultural problems in ~~for~~ tropical regions characterized by~~with~~ acidic soils~~,~~ ~~as~~because it leads to a significant decrease in maize grain yield through the inhibition of root growth. Breeding and selection for aluminum tolerance~~Genetic improvement~~ is a useful approach ~~to~~ for increasing~~e~~ maize yield in acidic soils;~~,~~ ~~but~~ however, this requires a rapid and reliable method ~~to~~ for discriminating~~e~~ between aluminum-tolerant (Al-tolerant) and aluminum-sensitive (Al-sensitive) genotypes. Therefore, i~~I~~n ~~our work~~this study, we investigated the feasibility of using hematoxylin staining (HS) of the root tissue to detect aluminum~~Al~~ ~~-~~tolerance in~~t~~ plants at the seedling stage. ~~The~~ An original population of maize seedlings and ~~along with~~ two populations obtained after one cycle of divergent selection for aluminum tolerance were evaluated. ~~by for~~T~~t~~he~~ir~~ net root growth (NRG) of the seedlings was evaluated ~~and HS~~ after 7 days of growth in nutrient solution containing aluminum, following which ~~and~~ HS of the root tissue was performed. ~~Results showed a n~~A negative correlation was observed between NRG and HS ~~-~~in all populations, ~~in which~~with Al-~~-~~sensitive plants, characterized by low NRG, exhibiting~~ed~~ more intense staining than Al-tolerant plants. These results indicate that HS of the root tissue is a useful procedure for the rapid screening and selection of~~ng~~ Al-tolerant maize seedlings.

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Introduction

Aluminum toxicity is ~~the ~~a major factor limiting plant growth in the acidic soils ~~that~~ of ~~comprise~~ large agricultural areas~~, principally in~~ primarily in tropical and subtropical regions [1] ~~(Kochian, 1995)~~. The main effect of aluminum toxicity in higher plants is thought to be root growth inhibition. ~~, and Sseveral approaches have been suggested to increase grain yields in these soil s with high levels of aluminumtypes, closely related to root growth inhibition, which is considered to be the main effect of aluminum toxicity in higher plants.~~ ~~The degree of Al-induced root growth inhibition can be used to screen plants at the seedling stage for their relative aluminum sensitivity (Delhaize and Ryan, 1995; Kochian, 1995). There is considerable genetic variability in sensitivity in the major crops and the evaluation of root elongation in nutrient solution has been useful in developing Al-tolerant varieties (Delhaize *et al.*, 1993; Pellet *et al.*, 1995).~~

Higher plants ~~make~~ use ~~of~~ two main mechanisms to preclude~~avoid~~ the effects of aluminum~~Al~~ toxicity.~~,~~ ~~one being~~The first is an exclusion mechanism~~,~~ by which the influx of aluminum through ~~is prevented from crossing~~ the ~~plasmalemma~~ plasma membrane~~s~~ into the cytoplasm is inhibited in the ~~of~~ root cells ~~and reaching the root cytoplasm~~. This mechanism involves the binding of aluminum ions to the ~~by~~ cell ~~-~~wall [1]~~binding (Kochian, 1995)~~, the release of organic acids (especially citrate and malate) [2-4]~~(Miyasaka *et al.*, 1991; Delhaize *et al.*, 1993; Pellet *et al.*, 1995)~~, and modification of the pH of the rhizosphere

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[5]~~(Miyasaka *et al*., 1989)~~. If aluminum ions do~~es~~ permeate ~~cross~~ the ~~plasmalemma~~plasma membrane, ~~it is excluded~~ they are effluxed from the cell by ~~the~~ an ATPase pump located in the ~~plasmalemma~~ plasma membrane [1]~~(Kochian, 1995)~~. The ~~other~~ second mechanism is an internal response~~,~~ characterized by the production of specific proteins capable of forming complexes with the toxic aluminum ions [6,7]~~(Aniol, 1984; Basu *et al*., 1994)~~.

Several approaches have been suggested to increase grain yields in soils with high levels of aluminum. There is considerable genetic variability in the sensitivities of major crops to aluminum, and the ~~S~~selection and breeding of plants for aluminum tolerance are considered important approaches for increasing grain yields in acidic soils. While ~~F~~field trials have ~~proved~~been shown to be effective in selecting aluminum-tolerant (Al-~~resistan~~tolerant) plants,~~ce~~ ~~but~~ they are ~~very~~ expensive and time-consuming. A rapid and reliable screening system is therefore needed to discriminate between aluminum-sensitive (Al-sensitive) and Al-~~resistan~~tolerant genotypes.

The degree of aluminum~~Al~~-induced root growth inhibition can be used to screen plants at the seedling stage for their relative aluminum sensitivities [1,8], wherein ~~and~~ root elongation in nutrient solutions, as determined by net root growth (NRG), is used to identify ~~aluminum-tolerant (~~Al-tolerant~~)~~ varieties of several crops [3,4]. ~~The selection of seedlings in nutrient solution is a rapid screening method based on net root growth (NRG), developed to screen for aluminum tolerance in several crops.~~ A rapid method ~~using~~ involving hematoxylin staining (HS) has been widely used for the direct visualization and localization of aluminum in root tissues [9]~~(Rincón and Gonzales, 1992)~~. ~~It~~ HS results in the development of an intense blue coloration in the root tips of sensitive genotypes ~~and is a useful approach for macroscopically detecting aluminum accumulation in root tips by the formation of an intense~~

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~~blue coloration in the root tips of sensitive genotypes~~. Theis reaction ~~occurs by~~involves the oxidation (in the presence of $NaIO_3$) of hematoxylin to hematyn, which, in the presence of aluminum, stains ~~produces~~ nucleic acids [10]~~coloration (Polle *et al*., 1978)~~. The reaction of hematoxylin with aluminum-stressed roots has been used by several researchers in studies involving~~in different~~ various crop species~~,~~ such as wheat (*Triticum aestivum* L.) [9-12]~~(Polle *et al*., 1978; Carver *et al*., 1988; Rincón and Gonzales, 1992; Tice *et al*., 1992)~~, soybean (*Glycine max* Merrill) [13-15]~~(Fonseca Jr., 1982; Fonseca Jr. *et al*., 1982; Spehar and Makita, 1994)~~, the common bean (*Phaseolus vulgaris* L.) [16]~~(Braccini *et al*., 1996)~~, and maize (*Zea mays* L.) [17]~~(Cançado, 1997)~~. However, information

~~Iabout~~ regarding the use~~tilization~~ of ~~hematoxylin stain~~HS in screening for aluminum tolerance in maize ~~(*Zea mays* L.) is~~remains scarce. ~~and the work reported i~~In this ~~paper~~ study, therefore, we sought~~was undertaken~~ to determine the effectiveness of this method~~ology~~ ~~for~~ in the rapid screening of ~~detecting~~ Al-tolerant and Al-sensitive genotypes of maize and found that it was a promising approach.

Materials and methods

Maize populations

SIKALQ, t~~T~~he tropical maize population used in this study, ~~SIKALQ,~~ is the result of an introgressive cross between the local population ESALQ-PB23A (50% ESALQ PB2 and 50% ESALQ PB3; yellow endosperm) and the exotic variety SIKUANI ICA V-110 [18]~~(Giaveno *et al*., 1998)~~. For this experiment, 2,000 ~~Two thousand seedlings of tropical~~

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~~maize population~~ SIKALQ seedlings were ~~grown in a greenhouse for seven days in nutrient solution at pH 4.2 and 5 ppm of aluminum as described by Furlani and Furlani (1988).~~ acquired. ~~The population SIKALQ is the result of an introgressive cross between the local population ESALQ-PB23A (50% ESALQ PB2 and 50% ESALQ PB3; yellow endosperm) and the exotic variety SIKUANI ICA V-110 (Giaveno *et al*., 1998).~~ Seedlings having s~~S~~econdary roots that were ~~of seedlings with~~ similar in length to the principal root were eliminated by hand, following which ~~before~~ ~~and~~ the length of ~~the~~ each seedling's principal root was measured to obtain the initial root length (IRL). ~~After~~ Seedlings were grown for 7 days in a greenhouse in a nutrient solution (pH 4.2) containing ~~including~~ 5 -ppm aluminum, as described by Furlani and Furlani [19]~~seven days~~. After removal of~~,~~ the seedlings ~~were removed~~ from the nutrient solution, ~~and~~ the final length of the principal root (FRL) of each plant was measured. The NRG ~~for individual~~of each plant~~s~~ was calculated using the following equation:~~as~~ NRG = FRL ~~-~~– IRL.

One cycle of divergent selection was completed by selecting the 10% most tolerant and 10% most sensitive seedlings based on NRG values. Selected seedlings ~~of~~ from both groups were transplanted to the field and randomly crossed to other plants of the same group to obtain Al-tolerant and Al-sensitive cycle-I sub-populations, ~~-~~as described by Giaveno *et al.* [18]~~,~~ and were designated as CI-AT ~~(Al-tolerant)~~ and CI-AS, respectively ~~(Al-sensitive) as described by Giaveno *et al*. (1998)~~.

~~Growth~~ NRG measurements

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~~Treatments were the three maize populations described above,~~ ~~and the~~ ~~s~~Seedlings ~~of~~ from the initial population (C0), as well as those from the two cycle-I subpopulations (CI-AT and CI-AS), ~~each population were grown in a growth chamber for seven days at a constant temperature of 24°C and a 16-h light and 8-h dark photoperiod.~~ ~~Treatments~~ were placed ~~into~~ in four 3.5-~~liter~~ L buckets containing the same nutrient solution as that used above [19]~~(Furlani and Furlani, 1988)~~ in a completely randomized block design, in which the buckets were considered ~~as blocks~~blocks and the populations were considered treatments. The buckets were then placed in a growth chamber for 7 days at a constant temperature of 24°C, -with~~and~~ a 16-h light/8-h dark photoperiod. At the end of this period, the~~an~~ NRG value of each seedling was calculated as described above. ~~and the Pearson's coefficient of correlation with HS calculated.~~ Values of NRG were analyzed by ANOVA, and population ~~the~~ means were compared ~~by~~ using ~~the~~ Duncan's test.

Hematoxylin staining of roots

A previous ~~Preliminary~~ study~~ies~~ using the tropical maize ~~*(Zea mays* L.)~~ populations CMS 36 (Al-tolerant) and BR 106 (Al-sensitive) ~~were carried out in growth chambers to assess~~determined the best combination of exposure time ~~of exposure~~ and aluminum~~Al~~ concentration for HS [20]~~(Giaveno and Miranda Filho, 1999)~~.

After 24 ~~-~~h of exposure to aluminum, seedlings were removed from the nutrient solution and stained by HS for visual detection of aluminum, using the method proposed by Polle *et al*. [10]~~(1978)~~ and~~,~~ modified by Cançado [17]~~(1997)~~. Briefly, ~~S~~seedlings were placed in flasks containing deionized water, ~~and~~ washed for 30 min o~~i~~n a horizontal shaker, and then

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stained with hematoxylin solution (2 g/L~~l of~~ hematoxylin and 0.02 g/L ~~l of~~ KIO$_3$) for 15 min. ~~The s~~Seedlings were washed again for 20 min in deionized water to remove excess ~~of~~ stain.

Stained root tips were evaluated using a visual scale ~~varying~~ ranging from 0 (no staining), indicating high aluminum~~Ally~~ toleranсe,~~t)~~ to 5 (strong staining), indicating high aluminum ~~Ally~~ sensitivity~~e)~~. HS ~~V~~scores~~alues of HS~~ were analyzed by ANOVA, and ~~the~~ population means were compared by ~~the~~ Duncan's test. Pearson's correlation coefficients between HS scores and NRG values were determined.

Results and d~~D~~iscussion

~~In previous work we have shown that 24 h of Al exposure was sufficient to result in staining when the roots were subsequently treated with hematoxylin (Giaveno and Miranda Filho, 1999).~~

In the present ~~work~~ study, the utility of HS as a rapid screening method for the determination of aluminum tolerance in maize seedlings was investigated. T~~t~~wo parameters, NRG (~~Figure 1~~Fig 1) and HS (~~Figure 2~~Fig 2), were ~~used~~ assessed in maize seedlings from three experimental populations to determine ~~measure~~ aluminum tolerance ~~in maize seedlings~~, and both parameters ~~showed~~ were found to be statistically different~~ce~~ between the CI-AS population and the other two~~s among the~~ populations (~~Table I~~Table 1). These results confirm~~ed~~ the effectiveness of the divergent selection method ~~for~~ based on NRG.

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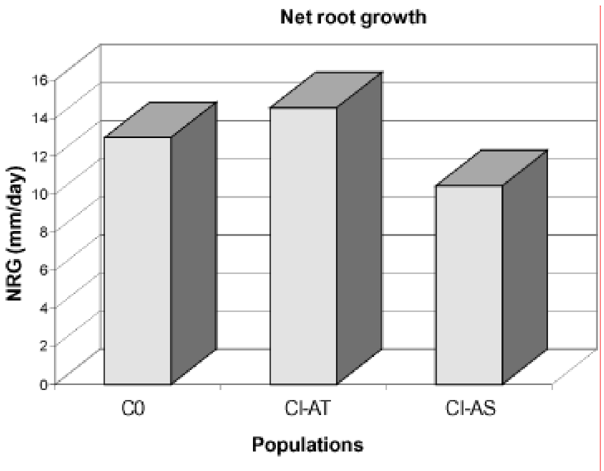

Figure 1 - Mean net root growth (NRG) values of the original (C0), Al-tolerant (CI-AT) and Al-susceptible (CI-AS) populations.

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In general, horizontal lines are not included in published graphs. I also recommend removing the 3D effect to improve the clarity of the graph valu Finally, I recommend adding error bars to reflect standard error of the mean. If you do this, please add the following to the Fig 1 legend after the Fig title: "Error bars indicate SEM".

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Fig 1. Mean net root growth (NRG) values of the original (C0), Al-tolerant (CI-AT), and Al-susceptible (CI-AS) populations.

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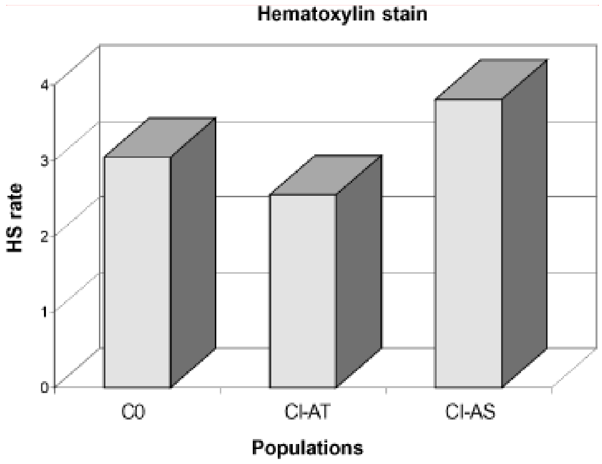

Figure 2 - Mean hematoxylin staining (HS) values of the original (C0), Al-tolerant (CI-AT) and Al-susceptible (CI-AS) populations.

Fig 2. Mean hematoxylin staining (HS) scores of the original (C0), Al-tolerant (CI-AT), and Al-susceptible (CI-AS) populations.

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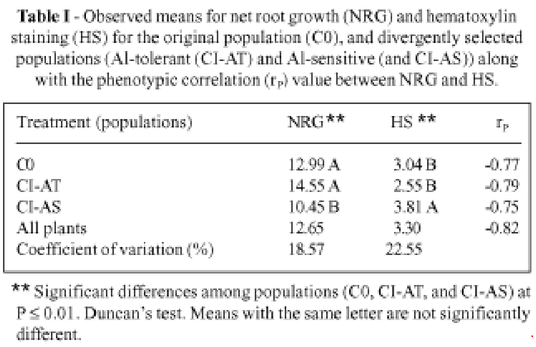

Table I - Observed means for net root growth (NRG) and hematoxylin staining (HS) for the original population (C0), and divergently selected populations (Al-tolerant (CI-AT) and Al-sensitive (and CI-AS)) along with the phenotypic correlation (r_p) value between NRG and HS.

Treatment (populations)	NRG**	HS **	r_p
C0	12.99 A	3.04 B	-0.77
CI-AT	14.55 A	2.55 B	-0.79
CI-AS	10.45 B	3.81 A	-0.75
All plants	12.65	3.30	-0.82
Coefficient of variation (%)	18.57	22.55	

** Significant differences among populations (C0, CI-AT, and CI-AS) at P ≤ 0.01. Duncan's test. Means with the same letter are not significantly different.

Table 1. Observed means and Pearson's correlation coefficients (r_p) of net root growth (NRG) and hematoxylin staining (HS) scores of the original (C0), Al-tolerant (CI-AT), and Al-susceptible (CI-AS) populations.

Treatment (population)	**Mean NRG**	**Mean HS**	**r_p**
C0	12.99 A[a]	3.04 A	-0.77
CI-AT	14.55 A	2.55 A	-0.79
CI-AS	10.45 B	3.01 B	-0.75
All plants	12.65	3.30	-0.82
Coefficient of variation (%)	18.57	22.55	

[a] Different letters in the same column indicate significant differences among population means at P ≤ 0.01.

~~The phenotypic~~Pearson's correlation analysis ~~showed~~revealed a negative trend between the NRG and HS values for all populations ~~showed a negative trend~~ (Table 1 and ~~Figure 3~~Fig 3). ~~It could be explained by the fact~~This suggests that Al-~~susceptible~~ sensitive seedlings have ~~poor~~ low NRG values ~~as a consequence of~~owing to the presence of high

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quantities of accumulated aluminum in the root cap, ~~which ~~resulting~~s~~ in~~ and therefore, these genotypes show ~~ higher HS scores. ~~On the other hand~~In contrast, Al-tolerant seedlings ~~apparently have some~~possess a mechanism to ~~avoid ~~preclude aluminum toxicity, which~~therefore they presenting~~ resulted in higher NRG values and ~~low ~~reduced HS scores.

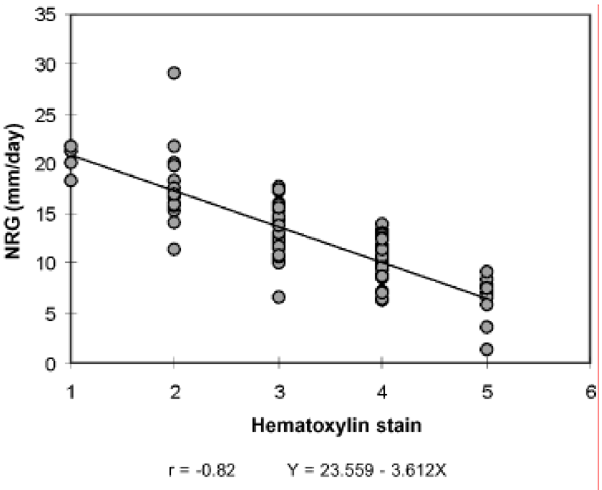

Figure 3 - Regression analysis of the data for all plants. NRG, Net root growth.

Fig 3. Regression analysis of net root growth (NRG) and hematoxylin staining (HS) score for all plants.

Our results also show a ~~possible ~~potential effect of selection ~~effect ~~on the phenotypic correlation ~~value in both selected populations~~between NRG and HS. In the tolerant population (CI-AT), there was a small increase in the phenotypic correlation coefficient (-0.792) when compared to that of the original population (-0.771). ~~On the other hand~~In contrast, a decrease in the correlation coefficient (-0.757) was observed in the sensitive

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population (CI-AS). However, such trends cannot ~~be~~ necessarily be assured in further selection cycles.~~,~~ ~~and w~~It is clear, however, ~~e can say~~ that ~~hematoxylin staining~~HS is strongly~~highly~~ negatively correlated ~~trait~~ with NRG in both the original and divergently selected populations.

~~Overall, our results are in agreement with those of other reports.~~, ~~such as that of~~ Cançado [17]~~(1997)~~ ~~who~~ reported a correlation coefficient ~~between HS and NRG~~ of -0.693 between HS and NRG, and a relative root growth of -0.816, ~~-~~using S_3 inbreed lines. Guevara *et al*. [21]~~(1992)~~ concluded that ~~hematoxylin staining~~HS was a good criterion for discriminating between Al-tolerant and Al-sensitive maize seedlings, and ~~.~~ t~~T~~hese results were partially confirmed by Ryan *et al*. [22]~~(1993)~~. Thus, o~~O~~verall, our results are in agreement with those of other reports.

Thus, ~~Our results lead us to~~w~~W~~e conclude that ~~hematoxylin staining~~HS is a suitable approach ~~to~~ for the screening of aluminum tolerance in maize seedlings, ~~because it~~ allowings for the rapid evaluation of a large number of genotypes without destroying the root apical meristem.

Acknowledgments

The authors are grateful to Dr. Aikihiko Ando (Departamento de Genética ESALQ-USP) for technical assistance. ~~Research and publication supported by FAPESP.~~

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~~RESUMO~~

~~A importante diminuição nos rendimentos de milho causados pela toxidez produzida pelo alumínio é considerada um dos mais importantes problemas nas regiões tropicais. O melhoramento genético é uma metodologia útil para aumentar os rendimentos do milho em solos ácidos, requerendo um método rápido e seguro que permita diferenciar os diferentes genótipos. O objetivo deste trabalho foi avaliar a possibilidade de utilizar a técnica da coloração com hematoxilina (HS) na detecção de plântulas tolerantes ao alumínio. Duas populações obtidas de um ciclo de seleção divergente e a original, foram avaliadas depois de sete dias em solução 14ntense1414 utilizando os parâmetros NRG (crescimento líquido da raiz principal) e HS. Os resultados apresentaram uma correlação 14ntense14 entre NRG e HS em todas as populações devido ao fato de que as plântulas suscetíveis, caracterizadas por um baixo NRG, apresentaram uma coloração mais 14ntense do que as tolerantes. Nossos resultados permitem concluir que a técnica de coloração com hematoxilina é um procedimento adequado para selecionar genótipos tolerantes ao alumínio em estado de plântula.~~

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