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Hematoxylin staining as a phenotypic index for aluminum tolerance selection in tropical maize (*Zea mays* L.)

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Abstract Hematoxylin staining is an early indicator of Aluminum (Al) toxicity effects on the apices of young, developing roots grown in nutrient solution. In this work, the potential of this technique as a reliable and reproducible phenotypic index for Al tolerance in tropical maize genotypes was assessed, with its performance systematically compared to two other parameters widely used in breeding programs – relative seminal-root length (RSRL) and net seminal-root length (NSRL). Seeding roots from contrasting genotypes for Al sensitivity stained remarkably different after 24- and 48-h and 7-day exposures to 222 μ*M* Al in nutrient solution, with the Al-dye complex being detected in both the outer (epidermis) and inner (cortex) portions of the roots from the sensitive cultivar. Hematoxylin staining was compared to the RSRL and NSRL parameters using 20 families from the third generation of selfing (S3) following the cross between two contrasting inbred lines that had been previously classified by the RSRL index in an independent procedure. The coloration technique showed the highest capacity to discriminate among tolerant and sensitive genotypes and displayed significant correlation coefficients to the other two indexes. Evaluation of the results from diallel crosses involving nine inbred lines proved that hematoxylin staining was also particularly adequate for identifying expressive hybrid vigor, as demonstrated by the general (GCA) and specific (SCA) combining ability estimates obtained by using the three indexes simultaneously. Hence, hematoxylin staining of Al-stressed root apices appears to be a powerful tool to assist in Al-tolerance selection in tropical maize breeding programs.

Key words Aluminum tolerance · Hematoxylin · Plant breeding · Seminal-root length

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Introduction

Acid soils are found in large areas of tropical and temperate climates and are a major limitation to agriculture in these regions. For example, in Latin America only they comprise approximately one billion hectares (Howeler 1991). In soils with neutral or slightly acid pH, aluminum (Al) is primarily found as oxides or silicates that are inert to plant roots. However, at values of pH <5.0, toxic forms of Al are released into the soil solution at levels that damage root growth, thus affecting the whole plant development (Foy et al. 1978; Kochian 1995). Roots exposed to toxic Al tend to become short, thick and underdeveloped, thereby reducing nutrient uptake and increasing susceptibility to drought (Bona et al. 1991; Llugany et al. 1994; Sasaki et al. 1996). There is extensive genetic variability with respect to Al tolerance in plants, at both the inter- and intra-species levels (Kochian 1995; Ishikawa and Wagatsuma 1998). In maize, the majority of commerical genotypes are sensitive to regular levels of toxic Al, such that breeding for more adapted cultivars seems to be the best strategy to improve farming of this crop in regions with acid soils (Howeler 19991; Kochian 1995).

The genetic control of Al tolerance in maize is likely due to a quantitative mode of inheritance (Magnavaca et al. 1987 a), but a more precise knowledge of the physiological aspects involved is still lacking. Based on work with other species, it is possible that only a few genes have a major influence on this trait, with genetic modifiers having smaller effects (Kochian 1995; Pellet et al. 1996; Larsen et al. 1998; Degenhardt et al. 1998). Several methods have been developed for evaluating Al tolerance, and

these have also contributed to the elucidation of physiological processes underlying this trait (Polle et al. 1978; Ruiz-Torres and Carver 1992; Moustakas et al. 1993; Zhang et al. 1994). Among those methods, those assessing root growth in nutrient solutions are the most attractive as they provide adequate forms of Al stress, thereby allowing preliminary screening of a large number of genotypes in a small area and, consequently, decreasing the number of promising genotypes to be further analyzed under field conditions (Polle et al. 1978; Magnavaca et al. 1987 b; Ruiz-Torres and Carver 1992). Relative seminal root length (RSRL) has been frequently used as a suitable phenotypic index for Al tolerance in maize seedlings cultivated in nutrient solution (Magnavaca et al. 1987 b). Nevertheless, it should not be used as the single criterion since it may be misleading in genotypes that accumulate high amounts of Al in the aerial part of the plant (Massot et al. 1992; Moustakas et al. 1993; Foy and Peterson 1994). The Al concentration in root tips and the production of callose induced by Al in nutrient solution have also been investigated in maize as early indicators of Al sensitivity among genotypes (Llugany et al. 1994; Zhang et al. 1994).

Hematoxylin, a dye commonly used in cytogenetic studies, has also been used as a precocious, non-destructive way of studying Al sensitivity in plant species (Polle et al. 1978; Carver et al. 1988; Rincón and Gonzales 1992; Delhaize et al. 1993; Wagatsuma et al. 1995), including maize (Guevara et al. 1992; Ryan et al. 1993; Jorge and Arruda 1997). This dye has the property of turning blue when it forms a complex with Al so that the penetration and retention of this ion in the roots can be assessed (Polle et al. 1978; Delhaize et al. 1993). Therefore, the color intensity of stained root apices grown in nutrient solution can be a direct and quantitative measure of Al sensitivity (Ruiz-Torres and Carver 1992) because susceptible genotypes tend to accumulate higher amounts of Al in their root tissues (Polle et al. 1978; Carver et al. 1988). An important aspect of this technique is that the reaction between hematoxylin and Al is specific, such that other stressing factors would exert a minimal effect, if any, on the evaluation processes of the Al effects. In wheat, this technique proved conducive in identifying tolerant and sensitive genotypes after a very short exposure time of seedlings to Al, well before differences in the seminal root length become detectable (Delhaize et al. 1993). In the investigation presented here, the feasibility of using hematoxylin staining of roots to identify tolerant and sensitive tropical maize genotypes was examined. Our main objective was to compare this method systematically and rigorously with other commonly used phenotypic indexes in order to assess its detection sensitivity, reproducibility and efficacy as a leading (or sole) parameter for selection. The results suggested that hematoxylin staining of root apices is an easier, rapid and more reliable method than any other known method for discerning among Al-tolerant and Al-sensitive tropical maize genotypes.

Materials and methods

Plant material

All lines used in the experiments belong to the collection of the maize breeding program of the National Research Center of Maize and Sorghum – EMBRAPA, Brazil. Two contrasting inbred lines, L1143 (tolerant) and L53 (sensitive), were used for the initial hematoxylin staining experiments. For experiments involving comparisons with other phenotypic indexes (relative seminal root length, RSRL, and net seminal root length, NSRL), two contrasting lines, L1327 (tolerant) and L53 (sensitive), as well as ten recombinant-tolerant and ten recombinant-susceptible lines derived from the third generation of selfing (S3) from the cross L1327 × L53 were used. The open-pollinated variety 'CMS36' was used as a tolerant control. Finally, the three indexes were evaluated for nine inbred lines developed in the EMBRAPA's breeding program (L11, L13, L16, L20, L22, L36, L64, L723 e L724) and for the F_1 individuals corresponding to diallel crosses among them.

Germination and seedling cultivation in nutrient solution

Maize seeds were treated with 0.1% (w/w) Captan 750 TS fungicide and set to germinate inside paper rolls wetted with distilled water, under continuous aeration. Seven days after sowing, plantlets were chosen based on their uniformity and lack of visible damage in their roots and transferred to nutrient solution, either in plastic 1.5-l pots (hematoxylin staining experiment) or in plastic 8.5-l trays (S3 families and diallel crosses experiments), with continuous aeration. The nutrient solution consisted of (g/l): $Ca(NO_3).4H_2O$, 270; KCl, 18.6; K_2SO_4, 44; KNO_3, 24.6; KH_2PO_4, 17.6; NH_4NO_3, 33.8; $Mg(NO_3).6H_2O$, 142.4; $Fe(NO_3)_3.9H_2O$, 20.3; HEDTA, 13.4; $MnCl_2.4H_2O$, 2.34; H_3BO_3, 2.04; $ZnSO_4.7H_2O$, 0.88; $CuSO_4.5H_2O$, 0.2; $Na_2MoO_4.2H_2O$, 0.26. When present, the Al stress was achieved by adding $KAl(SO_4)_2.12H_2O$ at a final concentration of 222 μ*M* Al. The seedlings in nutrient solution were cultivated in a growing chamber at a photoperiod of 14 h light : 10 h dark. The light conditions were approximately 340 μmoles m^{-2} s^{-1} of light intensity, 30 °C and 70% relative air humidity; the dark conditions were 22 °C and 90% relative air humidity. The seedlings were individually placed in circular holes (3 cm in diameter) on a support above the solution and then locked in place by small sponges. The pH of the solution was set to 4.0, which was monitored daily and adjusted when necessary for longer cultivation periods.

Hematoxylin staining

The basic protocol was based on Polle et al. (1978). The roots of seedlings cultivated for 24 h, 48 h and 7 days, in the presence or absence of Al, were gently shaken in 200 ml distilled water for 15 min. The water was then replaced by 200 ml of aqueous hematoxylin solution [0.2% hematoxylin (Merck) and 0.02% potassium iodide, w/v] and left at the same slow agitation for 20 min. Finally, the solution was replaced again by 200 ml water, thereby repeating the first step. The root apices were excised and photographed under both stereoscopic and light microscopes. Transversal sectioning of the apices were also performed in order to be able to observe the presence of hematoxylin-Al complexes in internal tissues. After staining, the roots were evaluated visually by four different referees and then returned to trays containing nutrient solution. To score the intensity of staining, we used an arbitrary 0-to-5 scale, in which the total absence of color was assigned a zero, and maximal staining, a 5. The average score of the four referees was considered for analysis. Hematoxylin staining of root apices was performed after a 48-h exposure to Al for the S3 families and after 7 days of exposure for the diallel crosses.

Assessment of the RSRL and NSRL phenotypic indexes

In the S3 families and diallel cross experiments, the seminal root length of seedlings was measured, both at the time of transferring them to nutrient solution with Al and 7 days thereafter (initial and final seminal root length, ISRL and FSRL, respectively). The NSRL was taken from the difference between FSRL and ISRL, and the RSRL was obtained from dividing the corresponding NSRL by ISRL (Magnavaca et al. 1987 b).

Experimental design and assessment of combining abilities

For the experiment with the S3 families, the experimental plan used was randomized blocks with 23 treatments (genotypes) per replication and three replications, in a total of 69 experimental units; each unit consisted of seven seedlings. For the studies on the diallel crosses, the averages of RSRL and NSRL, as well as the hematoxylin staining scores, were used in the 'method two' of combinatory analysis proposed by Griffing (1956) for estimation of the general combining ability (GCA) and species combining ability (SCA) among the lines; the corresponding mathematical model is:

$$Yij = m + gi + gj + sij + \varepsilon ij,$$

where Y_{ij} = mean of the hybrid ($i \neq j$) or the parents ($i = j$); m = general mean; g_i and g_j = effects of the general combining ability of the i and j progenitor, respectively; s_{ij} = effect of the specific combining ability for the crosses between the i and j progenitors (assuming $s_{ij} = s_{ji}$); and ε_{ij} = average experimental error. The experimental design was again randomized blocks, with 42 treatments (crosses) and three replications, in a total of 126 experimental units, each with seven seedlings. The statistical analyses were all performed using the MSTAT-C software package, version 1.41 (Michigan State University).

Results and discussion

Hematoxylin staining of root apices from Al-tolerant and Al-sensitive tropical maize genotypes

A simple visual inspection of stained root apices from contrasting phenotypes revealed that hematoxylin was sufficient to discriminate between Al-tolerant and Al-sensitive genotypes after a 24-h exposure of their seedlings to 222 μ*M* Al in nutrient solution (Fig. 1). The sensitive L53 line showed an intense blue color in the roots coupled with a severe epidermal degradation that extended from the elongation zone up to the root tip (Fig. 1 a, c). This pattern of injury was inclear agreement with previous observations in pea-root apices (Wagatsuma et al. 1995). In contrast, the L1143 tolerant line did not display those signs of physical damage in the root epidermis, although a region right above the apex did appear to be narrower, with an intense blue color (Fig. 1 b, d); however, no physical damage was detected in this constricted zone. After a 7-day stress under the same Al concentration, the staining patterns remained the same but tissue degradation was higher indicating that longer exposures of sensitive roots to Al proportionally increase the physical damage. For the tolerant line, longer exposures to Al did not affect the integrity of the root apices, and the distance between the root apex and the constrained zone was greater than in the 24-h stressed plants. Based on this, we assume that such a compression occurs only once, in the beginning of development of roots exposed to Al in nutrient solution. The presence of such a constricted area with an intense blue color strongly suggests that at least an initial and minimal absorption of Al in the developing root of a tolerant genotype can also occur, which is a proposal that conforms nicely to previous data obtained for wheat (Zhang and Taylor 1989). The location and timing of the appearance of this constricted zone would be, therefore, in agreement with earlier results that identified the root meristem as the primary site of Al toxicity (Ryan et al. 1993), with a short time of exposure to Al being sufficient to discriminate genotypes by hematoxylin staining (Delhaize et al. 1993). We postulate that the tolerant genotype was able to recover from the initial absorption and damage caused by Al, leaving behind only a ''footprint'' of this process, i.e. the blue-stained constrained zone (Fig. 1 b, d). When the nutrient solution lacked Al, there was no difference in the growing behavior and staining patterns of roots from both genotypes (data not shown). Light microscopy analysis on transversal sections of stained L53 root apices showed that the reaction with hematoxylin is not merely superficial, since the dye also penetrated and stained the cortex region (Fig. 1 e). This agrees with previous results in pea roots where the epidermal and outer cortical cells were stained by hematoxylin, appearing mostly destroyed and irregularly arranged, thus suggesting this region as a site for the deleterious action of Al (Wagatsuma et al. 1995). The same was not true for the tolerant line L1143 in which no apparent blue color was detected in inner tissues (Fig. 1 f). It is noteworthy that in a previous investigation with maize seedlings no significant differences in the staining pattern with hematoxylin was observed between sensitive and tolerant cultivars (Ryan et al. 1993). However, one possible explanation is that the cultivars used in those studies did not represent extremes of tolerance or susceptibility to toxic Al (Ryan et al. 1993), as opposed to the situation in our case. Another possibility for this discrepancy is a distinct reaction to the dye caused by a germplasm effect of tropical maize genotypes. Our results, taken together with other previous data in maize (Guevara et al. 1992), clearly indicate that this dye is in fact able to distinguish, early in root development, Al-tolerant tropical maize genotypes from Al-sensitive gones.

Comparative assessment of hematoxylin staining with RSRL and NSRL

In order to evaluate the efficiency of this staining procedure as a phenotypic index relative to other parameters commonly used to assist selection of Al-tolerant maize genotypes, namely the relative seminal root length, RSRL, and the net seminal root length, NSRL (Magnavaca 1987 b; Ruiz-Torres and Carver 1992), we selected ten tolerant and ten sensitive lines from the third generation of selfing (S3) of the cross between the contrasting

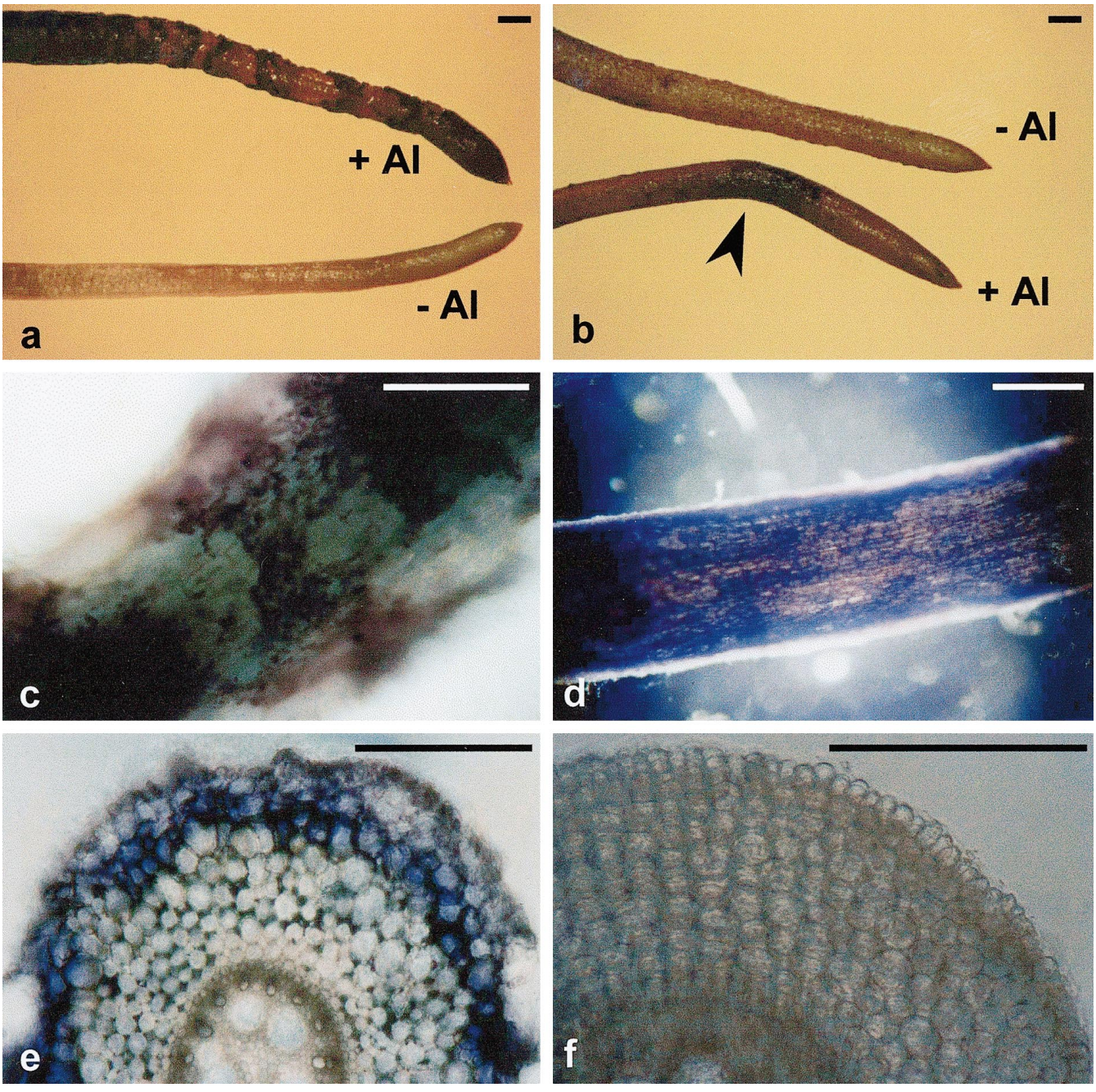

Fig. 1a—f Maize seedling-root apices stained with hematoxylin after a 24-h exposure to 0 or 222 μ*M* Al in nutrient solution. **a** Root apices of the line L53 in the presence and absence of Al. **b** Apices of line L1143 in the presence and absence of Al; *arrowhead* indicates the dark-stained constricted zone. **c** Closer view of the root epidermis of susceptible line L53 exposed to Al showing conspicuous tissue degradation. **d** Closer view of the dark-stained constricted zone of L1143 root exposed to Al showing no visible signs of physical damage as in the sensitive line. **e** Transversal section of a L53-root apex exposed to Al and stained with hematoxylin showing dye penetration into epidermal and cortical tissues (possible sites of Al presence). **f** Transversal section of a L1143 root apex exposed to Al and stained with hematoxylin showing no staining in both types of tissues, which indicates the absence of detectable Al. *Bars:* 1.0 mm

lines L1327 and L53. These contrasting inbred lines have been so classified by the RSRL index in a previous and independent procedure. As seen from Table 1, hematoxylin staining of root apices of all 20 lines enduring a 48-h exposure to Al appeared to work better than the other two indexes with respect to a phenotypic-to-genotypic classification. Statistical analysis of the scoring values obtained for hematoxylin coloration allowed the clear-cut separation of the lines tested in two groups, tolerant and sensitive, whereas for the other indexes, some genotypes ended up being classified in the opposite group (Table 1). The fact that the RSRL index did not performed in the same way relative to its preliminary classification of genotypes (see above) possibly reflects

Table 1 Mean values of RSRL, NSRL and hematoxylin-staining scores for 20 genotypes differing for Al response that had previously been classified by the RSRL index [a]

RSRL			NSRL			Hematoxylin		
Line	Mean [b]		Line	Mean		Line	Mean	
L1327^{T}	0.7534	a	CMS36^{T}	7.770	a	L46^{T}	0.667	a
L188^{T}	0.7449	a	L175^{T}	6.950	a,b	L1327^{T}	0.750	a
L46^{T}	0.7254	a	L88^{T}	6.693	a,b,c	L45^{T}	0.917	a
L58^{T}	0.6859	a,b	L58^{T}	5.860	a,b,c,d,e	L21^{T}	1.000	a
L160^{T}	0.6107	a,b,c	L18^{T}	5.857	a,b,c,d,e	L175^{T}	1.083	a
L18^{T}	0.5965	a,b,c,d	L1327^{T}	5.763	a,b,c,d,e,f	L16^{T}	1.250	a,b
L45^{T}	0.5530	a,b,c,d,e	L46^{T}	5.570	a,b,c,d,e, f	L18^{T}	1.250	a,b
L175^{T}	0.5234	a,b,c,d,e,f	L160^{T}	5.383	a,b,c,d,e,f	L88^{T}	1.583	a,b
L21^{T}	0.4933	a,b,c,d,e,f	L45^{T}	5.330	a,b,c,d,e,f	CMS36^{T}	1.643	a,b
L124^{S}	0.4117	b,c,d,e,f,g	L124^{S}	4.453	b,c,d,e,f,g	L58^{T}	1.667	a,b
CMS36^{T}	0.4111	b,c,d,e,f,g	L21^{T}	4.430	b,c,d,e,f,g	L160^{T}	2.250	b,c
L122^{S}	0.3856	c,d,e,f,g	L27^{S}	4.380	b,c,d,e,f,g	L124^{S}	2.833	c,d
L197^{S}	0.3567	c,d,e,f,g	L53^{S}	4.167	c,d,e,f,g	L27^{S}	3.000	c,d,e
L16^{T}	0.3561	c,d,e,f,g	L126^{S}	3.737	d,e,f,g	L122^{S}	3.083	c,d,e
L27^{S}	0.3317	c,d,e,f,g	L98^{S}	3.523	e,f,g	L229^{S}	3.167	c,d,e,f
L98^{S}	0.3298	c,d,e,f,g	L33^{S}	3.500	e,f,g	L201^{S}	3.500	d,e,f,g
L201^{S}	0.3101	d,e,f,g	L122^{S}	3.453	e,f,g	L197^{S}	3.583	d,e,f,g
L33^{S}	0.2986	e,f,g	L201^{S}	3.427	e,f,g	L33^{S}	4.083	e,f,g
L29^{S}	0.2648	f,g	L197^{S}	3.263	e,f,g	L126^{S}	4.083	e,f,g
L53^{S}	0.2595	f,g	L29^{S}	3.237	e,f,g	L98^{S}	4.250	f,g
L126^{S}	0.2505	f,g	L16^{T}	3.167	f,g	L29^{S}	4.500	g
L229^{S}	0.1917	g	L229^{S}	2.690	g	L53^{S}	4.500	g

[a] The previous genotypic classification of the 20 lines (T, Al tolerant; S, Al sensitive) was performed in the maize breeding program of the CNPMS/EMBRAPA. Line CMS36 was used as reference for Al tolerance

[b] Means within a column followed by the same letter are not significantly different at $P < 0.05$ (Tukey test)

a pitfall of phenotypic parameters that use 'growth' (of roots in this case) as the basis for assessment. These indexes are always subject to a higher degree of variation from experiment to experiment that is likely caused by different physiological states of the plants. A regression analysis for all three parameters in a pairwise fashion yielded statistically significant correlation coefficients (Fig. 2), thus indicating that the coloration of root-apice with hematoxylin can be employed, without restriction, as an informative phenotypic index of Al tolerance. The negative coefficients for the regression involving staining scores and the root growth-derived parameters, RSRL and NSRL, is obviously explained by the nature of the procedures: Al tolerance associates with increased root lengths, but also with decreased color intensity generated by hematoxylin, which is a consequence of less Al presumably being taken up by tolerant genotypes (Delhaize et al. 1993).

Performance of the hematoxylin-staining index in diallel crosses

A fundamental aspect and maize breeding is the search for the best combinations between inbred lines that express the most 'hybrid vigor'. Hence, it is of prime importance to evaluate the performance of hematoxylin staining with respect to the estimation of general and specific combining abilities between genotypes, GCA and SCA, respectively, since it is of interest to identify hybrid combinations with favorable SCAs that involve at least one progenitor with a high GCA for a given trait. Nine tropical maize inbred lines (Table 2) and non-reciprocal diallel crosses among them (data not shown) were used for the simultaneous assessment of the combining values furnished by the three indexes under study. Despite the contention that using older roots for staining tends to mask differences in genotypic behavior under Al stress (Delhaize et al. 1993 and references therein), we were interested in analyzing the extent of this possible masking effect as a way of assessing the potential of the technique under sub-optimal circumstances. In terms of GCA values toward Al tolerance and after a 7-day exposure to Al in nutrient solution, line L13 ranked first with hematoxylin staining, second with NSRL and third with RSRL, whereas L724 ranked first with NSRL and RSRL and fourth with heamtoxylin (Table 2). On the other hand, line L36 ranked at the other extreme (toward Al sensitivity) for NSRL and hematoxylin, but in the third position from the extreme with RSRL. Although the three indexes did not provide a strict match in terms of GCA values, the ranking of lines based on hematoxylin staining was mostly coincident with at least one of the other two indexes, even when roots of well-developed seedlings were used. Possible causes of the ranking variation observed for lines and indexes are physiological variabilty (for the growth-based indexes) and bias in the scoring process (for hematoxylin

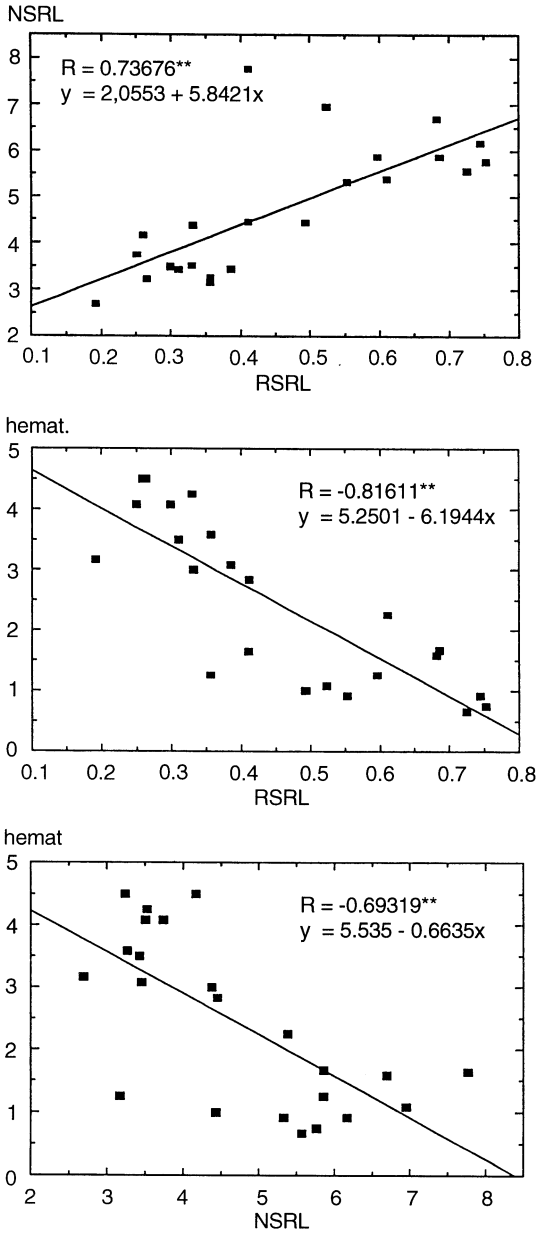

Fig. 2 Pairwise correlation analyses among NSRL, RSRL and hematoxylin-staining (*hemat.*) scores for the 20 S3 families experiment. Statistical significance for the regression coeficient (R) was assessed by Student's *t* test (** $P < 0.01$)

staining). Alternatively, the use of well-developed roots (7 days old) for hematoxylin analysis might have prevented a more precise scoring, which would agree with arguments against applying this dye too late in root development (Delhaize et al. 1993). Nevertheless, the comparative assessment of hematoxylin staining in the determination of SCAs from incomplete (non-reciprocal) diallel crosses involving the same lines essentially confirmed the trends expected by their GCAs estimated by all three parameters (data not shown).

The ANOVA for the combining analysis among the nine inbred lines using the three indexes in the diallel crosses suggested that the staining scores are able to detect statistical differences ($P < 0.05$) among treatments at the GCA level, performing better than the RSRL which did not detect significance for the treatments (Table 3). This is in contrast with data previously obtained for wheat, in which both RSRL and hematoxylin detected statistically significant differences at the GCA level (Ruiz-Torres and Carver 1992). However, in our study only the NSRL index was able to detect statistical significance at both the GCA and SCA levels (Table 3). One possible cause for a lack of statistical significance at the SCA level for hematoxylin staining may have been simple experimental variation caused by subjectivity in scoring color intensities (see above); consequently, attempts to further increase the precision of the hematoxylin scoring procedure are currently underway. Yet, the coefficient of variance (C.V.) displayed by this technique was the lowest among the different indexes (Table 3). Taking into account that the seedlings were less homogeneous in one of the experimental blocks, it is noteworthy that a statistically significant effect of blocking ($P < 0.01$) was apparent only for hematoxylin staining (Table 3). This feature could interstingly imply that relative to the other growth-based indexes, this parameter has also a higher capacity of detecting differences in plant vigor, reflected by subtle alterations in Al uptake within a same genetic background. Taken altogether, these results warrant further investigation of a much earlier application of hematoxylin in root apices (between 4 and 18 h, for example) to verify the performance on the estimation of general and specific combining abilities. We hypothesize that, under such conditions, this staining method will be able to detect statistically significant differences at the SCA level. If we consider the use of the three indexes simultaneously, the GCA and SCA values obtained in our experimens are in perfect agreement with previous results obtained by Parentoni et al. (1996), who worked with mostly the same inbred lines in field experiments evaluating the Al-response trait.

Finally, a regression analysis including the three parameters was performed with the results obtained in the diallel crosses (Fig. 3), similarly to what was accomplished for the S3 families. Again, the correlation coeffi-

Table 2 General combining ability (GCA) estimates [a] for nine maize inbred lines produced by the NSRL, RSRL and hematoxylin-staining indexes

Lines	NSRL	RSRL	Hematoxylin
L11	–541.6	–61.4	–179.8
L13	762.8	36.5	–423.7
L16	288.0	–12.9	–282.1
L20	–275.1	–17.7	221.1
L22	–60.8	–29.7	60.4
L36	–970.2	–27.6	575.9
L64	–432.3	18.6	124.2
L723	177.4	38.4	84.8
L724	929.7	55.1	–126.3

[a] For a better visualization of the differences, the actual estimated GCA values were multiplied by 10^3 to furnish the values presented in the table

Table 3 ANOVA for the diallel crosses experiment [a]

Source of variation	*df*	Mean square		
		NSRL	RSRL	Hematoxylin
Blocks	2	0.4770	0.00700	1.7310 **
Treatments	41	1.5242 **	0.00785	0.3485 *
model (GCA)	8	3.9120 **	0.01518 *	0.9262 *
deviation (SCA)	33	0.9453 **	0.00608	0.2085
Experimental error	82	0.4235	0.00600	0.2135
Total	125			
C.V. (%)		16.97	30.48	12.66
Mean		3.8361	0.2436	3.6843

[a] Statistical significance for the differences among values was assessed by the *F*-test (* P <0.05, ** P <0.01)

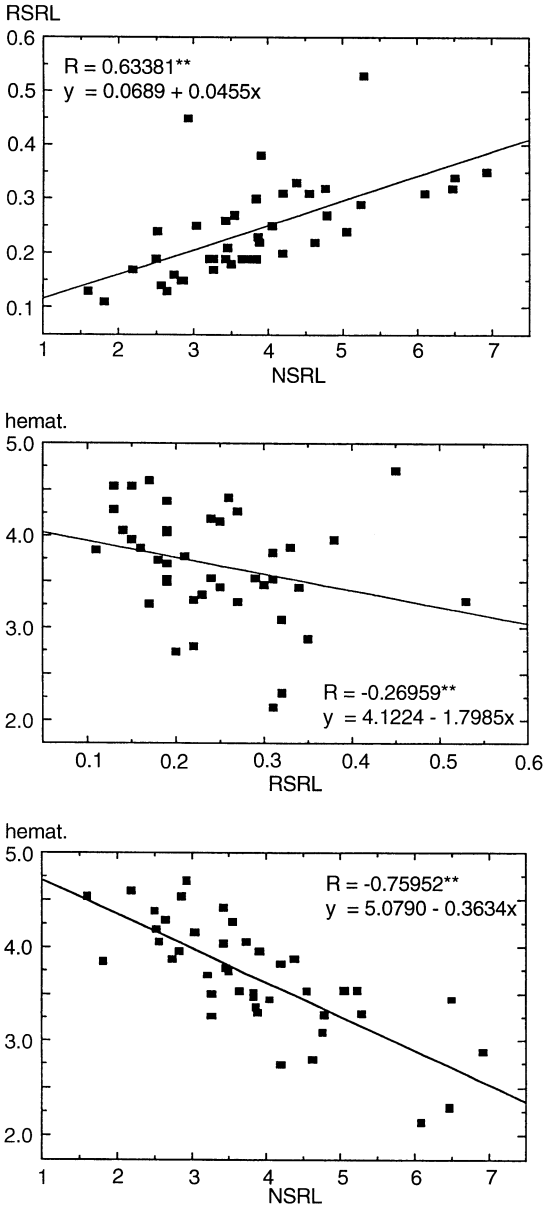

Fig. 3 Pairwise correlation analyses among NSRL, RSRL and hematoxylin-staining (*hemat.*) scores for the diallel cross experiments. Statistical significance for the regression coeficient (R) was assessed by Student's *t*-test (** P <0.01)

cients for hematoxylin staining and the other two indexes were very significant (P <0.01), indicating the feasibility of also applying this parameter to combining analysis among tropical maize inbred lines. Despite its significance, the R value achieved for the regression involving hematoxylin and RSRL can be considered to be rather low (–0.2696) when compared to the others (Fig. 3); an elevated experimental variability (C.V. = 30.48%) displayed by the RSRL index (Table 3) may account for such a low correlation coefficient. Such a high experimental variability for RSRL may have been a combined consequence of the higher heterogeneity of plants in one of the blocks plus the type of measurement of this index wich is inherently subject to physiological variance (see above).

Conclusions

Under the conditions of our study, the hematoxylin-staining technique demonstrated a higher capacity for discriminating Al susceptibility among genotypes and a higher experimental reproducibility than the other two indexes, RSRL and NSRL. This feature could probably be explained by a specific, 'plus-or-minus' type of action that is essentially independent of physiological variation, as well as by a high sensitivity of detection associated to a clear and easy scoring of the results. Moreover, based upon a similar performance relative to these other two phenotypic parameters commonly used in the definition of GCA and SCA values (RSRL and NSRL), it was possible to demonstrate an at least equal, if not higher, capability of hematoxylin coloration in serving as a reliable and informative phenotypic index for the discrimination of Al-tolerant genotypes in tropical maize. The technique appears to be very useful in the sense that a preliminary, precise and non-destructive assessment of genotypes can be achieved in a small area, which allows the number of promising germplasms to be subsequently evaluated in field experiments to be significantly decreased. Defining a more appropriate timing for root-apice staining as well as a more precise scoring procedure has the potential to augment even further the discriminatory capacity and accuracy of this technique. It becomes evident from the results presented here that hematoxylin staining of Al-

stressed root apices from seedlings grown in nutrient solutions, taken alone or in conjunction with other indexes, is capable of increasing the efficiency, precision and speed of selection, thereby serving as a robust tool to assist tropical maize breeding programs towards increased genotypic tolerance to acid soils.

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