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# A Greenhouse Method to Screen Brachiariagrass Genotypes for Aluminum Resistance and Root Vigor

Peter Wenzl,* Adriana Arango, Alba L. Chaves, María E. Buitrago, Gloria M. Patiño, John Miles, and Idupulapati M. Rao


## ABSTRACT

***Brachiaria*** **species are widely sown on the infertile and Al-toxic soils of neotropical savannas. Breeding programs seek to combine edaphic adaptation with other traits in interspecific hybrids. Edaphic adaptation is difficult to assess because it is only manifest in pasture persistence across several growing seasons. We developed and validated a solution-culture technique that uses rooted vegetative propagules from mature plants to assess two key components of edaphic adaptation: root vigor and Al resistance. Root vigor was assessed by measuring growth of adventitious root systems in 200 μ*M* $CaCl_2$ (pH 4.2). Aluminum resistance was assessed by comparing root growth in this solution vs. root growth in an identical solution that also contained 200 μ*M* $AlCl_3$. The well-adapted parent (*Brachiaria decumbens* cv. Basilisk) was superior to the less-adapted parent (*B. ruziziensis* clone 44-02), and both traits segregated as expected in a set of 44-02 × Basilisk hybrids. A simplified version of this technique, which exclusively relies on visual inspection, has been implemented in our breeding program to facilitate progress toward edaphic adaptation.**


*Brachiaria* species are the most widely sown tropical forage grasses, occupying up to 70 million ha of South American savannas (Fisher and Kerridge, 1996). The Centro Internacional de Agricultura Tropical (CIAT) and the Empresa Brasileira de Pesquisa Agropecuária (EMBRAPA) are seeking to develop apomictically reproducing interspecific hybrids by combining traits of three parental species: acid-soil adaptation and spittlebug resistance of *B. decumbens* and *B. brizantha*, respectively (both tetraploid apomicts), and sexual reproduction of a tetraploidized, sexual biotype of *B. ruziziensis* (???Sweene et al., 1981), which lacks both agronomic traits (Miles and do Valle, 1996; Miles et al., 2004). Efficient screening methodologies are required to recover the desired traits through stepwise accumulation of favorable alleles in subsequent cycles of recombination and selection. There is a need to develop a greenhouse-based method to assess edaphic adaptation of large segregating populations.

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Some brachiariagrasses such as Basilisk are well-adapted to the soils of neotropical savannas (Paulino et al., 1987; Rao et al., 1996, 1998). 44-02 pastures, by contrast, tend to degrade within a few years after establishment (Rao et al., 1996; Miles et al., 2004). The acid and infertile savanna soils are characterized by a combination of nutrient deficiencies (most significantly P, but also Ca, Mg, Mo, and sometimes N and K) and mineral toxicities (Al, occasionally Mn) (Rao et al., 1993; Sánchez, 1997). Edaphic adaptation presumably is an aggregate trait expressed in mature plants which comprises physiological components conferring adaptation to these and possibly other stress components.

The objective of this study was to establish and validate a procedure to evaluate the edaphic adaptation of breeding materials using vegetative propagules (stem cuttings) grown in solution culture. The procedure was designed to quantify two key component traits: root vigor and Al resistance. Vigorous growth of roots, particularly fine roots, increases a plant's nutrient foraging capacity and improves its ability to extract nutrients from infertile soils (Marschner, 1995; Rao et al., 1999). Aluminum toxicity was incorporated as a selection target because previous experiments had confirmed that brachiariagrass genotypes differ for this trait (Wenzl et al., 2001).

We initially tested the procedure by monitoring, during up to 3 wk, the growth of the adventitious root system of stem cuttings from the three parental genotypes (Basilisk, *B. brizantha* cv. Marandú, and 44-02). Having established its effectiveness, we evaluated a refined version of the procedure with *B. ruziziensis*, *B. decumbens*, and a group of 38 *B. ruziziensis* × *B. decumbens* hybrids, which were expected to segregate for edaphic adaptation because of the typically high heterozygosity level of apomicts such as *B. decumbens* (Asker and Jerling, 1992).

## MATERIALS AND METHODS

### Plant Material

The three main parents of the *Brachiaria* breeding program (Basilisk, Marandú, and tetraploid 44-02) and 38 different *B. ruziziensis* × *B. decumbens* hybrids were used to validate the screening procedure. These 41 genotypes had been propagated vegetatively on soil from an experimental station in Santander de Quilichao (altitude = 990 m; Oxisol–Plinthic Kandiudox; Cauca Department, Colombia) for several years. Soil from the same site was used to grow enough tillers to produce sufficient numbers of stem cuttings for this study. Chemical characteristics of this soil were: pH 4.6 at a soil-to-water ratio of 1:1, 14.7 mg $kg^{-1}$ Bray-II extracted P, 1.8 $cmol_c$ $kg^{-1}$ KCl-extracted Al, 0.15 $cmol_c$ $kg^{-1}$ Bray-II extracted K, 2.3 $cmol_c$ $kg^{-1}$ KCl-extracted Ca, and 1.3 $cmol_c$ $kg^{-1}$ KCl-extracted Mg.

**Abbreviations:** RL, total root length.



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Before planting, the soil was mixed with sand at a 3:1 ratio, and the mixture was fertilized with (milligram of element per kilogram of soil–sand mixture): 20.6 N (urea), 25.8 P (triple superphosphate), 51.6 K (KCl), 34.0 Ca (dolomitic lime), 18.0 Ca (triple superphosphate), 14.6 Mg (dolomitic lime), 10.3 S (elemental sulfur), 1.0 Zn ($ZnCl_2$), 1.0 Cu ($CuCl_2$), 0.05 B ($H_3BO_3$), and 0.05 Mo ($Na_2MoO_4 \cdot 2\ H_2O$) (Rao et al., 1992). The fertilized soil–sand mixture was distributed into 41 containers, each holding 40 kg. Approximately 10 tillers from each of the 41 genotypes were potted into the containers (one genotype per container).

Forty-five days after potting, vegetative propagules were produced from tillers with three to five leaves and not more than three nodes. The tillers were detached below the lowest node above soil level, and all but the youngest three leaves were removed. The remaining leaves were pruned to approximately 2 cm to reduce transpiration. The resulting stem cuttings were used for solution-culture experiments in the greenhouse. The tillers remaining in the pots were pruned and the soil–sand mixture was fertilized with an identical amount of urea and triple superphosphate as had been used for potting. After 23 to 25 d, a second set of stem cuttings was generated, and the soil–sand mixture was fertilized again with urea and triple superphosphate. After producing a third set of stem cuttings under identical conditions, the remaining tillers were pruned and repotted into a fresh soil–sand mixture that had received a full fertilization. This repotting cycle was repeated four times in the course of this study.

## Growth Conditions

All growth experiments with nutrient solutions were performed in the greenhouse at CIAT headquarters (3°30′ N, 76°21′ W; altitude = 965 m). Typical conditions in the greenhouse were 19 to 36°C, 48 to 96% relative humidity, and 1100 $\mu mol\ m^{-2}\ s^{-1}$ maximum photon-flux density during the day.

## Rooting of Cuttings

The bases of the stem cuttings produced from tillers of potted plants were inserted into 1.5-cm-thick polyurethane foam discs (diam. = 4 cm) and transplanted to racks floating on a large volume of aerated, low-ionic-strength nutrient solution (Fig. 1). This solution, known to support close-to-maximum growth of *Brachiaria* seedlings (Wenzl et al., 2003), contained (in $\mu M$): 500 $NO_3^-$, 50 $NH_4^+$, 300 $K^+$, 300 $Ca^{2+}$, 150 $Mg^{2+}$, 160 $Na^+$, 5 $H_2PO4^-$, 286 $SO_4^{2-}$, 5 $Fe^{3+}$, 1 $Mn^{2+}$, 1 $Zn^{2+}$, 0.2 $Cu^{2+}$, 6 $H_3BO_3$, 5 $SiO_3^{2-}$, 0.001 $MoO_4^{2-}$, 5 $H_2$–$EDTA^{2-}$, 332.4 $Cl^-$ (excluding HCl) and 67.8 HCl to adjust the pH to 4.20.

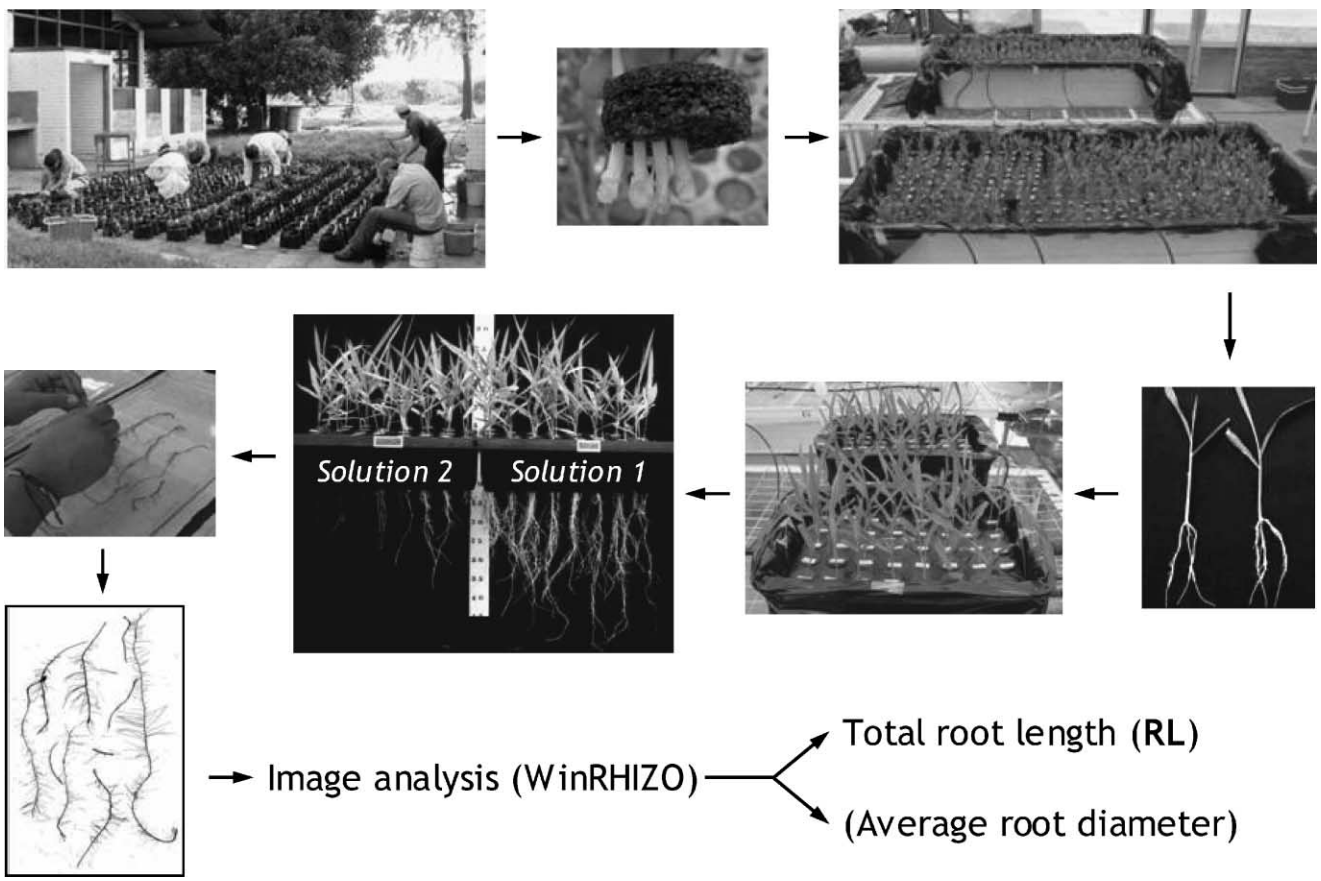


**Fig. 1. Procedure to identify acid soil-adapted *Brachiaria* genotypes. Plants were propagated in a mixture of soil and sand (3:1). Vegetative propagules (stem cuttings), excised from these plants, were floated at the surface of a low-ionic-strength nutrient solution to produce adventitious roots. After 9 d, pairs of rooted stem cuttings were selected for homogeneity. One propagule of each pair was transferred to Solution 1 (200 $\mu M$ $CaCl_2$, pH 4.20), the other to Solution 2 (200 $\mu M$ $CaCl_2$, 200 $\mu M$ $AlCl_3$, pH 4.20). Twenty-one days after transfer, roots were separated from stems, stained, and scanned on a flatbed scanner to determine total root length (RL) and average root diameter. Genotypes with vigorous root growth were identified based on RL in Solution 1. Aluminum-resistant genotypes were identified based on RL in Solution 2 after removing the variance component that was due to differences in root vigor.**

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### Testing the Two Treatments

After 9 d, 12 pairs of rooted stem cuttings from the three parents of the breeding program were selected for within-pair homogeneity (Fig. 1). One propagule of each pair was transferred to Solution 1 (200 $\mu M$ $CaCl_2$, pH 4.20), the other to Solution 2 (200 $\mu M$ $CaCl_2$, 200 $\mu M$ $AlCl_3$, pH 4.20). The pH of both solutions was adjusted by adding calculated quantities of HCl (64.9 $\mu M$ for Solution 1; 39.2 $\mu M$ for Solution 2) and measured with a pH electrode designed for low-ionic-strength solutions (see Wenzl et al., 2003, for details). The two groups of 36 stem cuttings (12 per parent) were grown in two plastic trays wrapped in black polyethylene bags, which held 20 L of the two solutions (Fig. 1). The solutions were continuously aerated and renewed every second day to minimize pH changes (the pH increased by up to 0.15 units in 2 d when roots were bigger). Root growth was monitored by measuring the length of the longest root every third day, for up to 21 d. The whole experiment was performed twice.

### Testing the Refined Screening Procedure

44-02, Basilisk, and the 38 *B. ruziziensis* × *B. decumbens* hybrids were included in 10 successive, partly overlapping experiments. For each experiment, one to three pairs of stem cuttings of each genotype were prepared. The cuttings were rooted and transplanted to one of the two solutions, as described in the previous sections. At harvest, after 21 d of growth, roots were separated from aerial parts. The roots were stained for 24 h in an aqueous solution containing 0.1% (w/v) methylene blue and 0.1% (w/v) neutral red, washed, submersed in a thin layer of water and scanned on a flatbed scanner at 300 dpi because pilot experiments had confirmed that this resolution was sufficient to capture even the finest roots (Fig. 1). The images were analyzed with WinRHIZO software (Régent Instruments Inc., Québec, Canada) to measure total root length (RL) for each individual root system. The aerial parts were dried at 60°C for 48 h and their dry weights record.

### Staining Root Apices of Selected Genotypes with Hematoxylin

During the last experiment, a small number of root apices were excised from adventitious roots of stem cuttings from *B. ruziziensis*, *B. decumbens*, and two hybrids with contrasting levels of Al resistance (all grown in Solution 2 for 12 d). The apices were stained with hematoxylin {7,11b-dihydrobenz[b]-indeno[1,2-d]pyran-3,4,6a,9,10(6H)-pentol} according to Owbny??? (1993). Zones of Al-induced damage were visualized by fixing apices in a 1:1 mixture of 3.7% (v/v) phormol (???, pH 7.4) and glutaraldehyde (1,3-diformylpropane), and cutting 70-$\mu$m-thick longitudinal sections.

### Data Analysis

The pooled RL data from the 10 experiments designed to test the refined screening procedure were log transformed (???Causter and Venus, 1981) and adjusted for harvest mean and the dry weight of stem cuttings (Beck et al., 2003; Zeegers et al., 2004). This procedure removed 40.5% of the RL variance in Solution 1 and 32.2% in Solution 2. The removed variance components presumably were due to differences among replicate experiments in growth conditions as well as differences in the amount of carbohydrates and nutrients supplied by stem cuttings to roots.

Aluminum resistance was quantified after regressing the adjusted logarithms of the RL values from the Al treatment (Solution 2) on those from the basal treatment (Solution 1) to remove the variance component reflecting the inherent differences in root vigor among the hybrids. The residual values after regression were expected to be a more informative measure of true Al resistance than the original values from the Al treatment if root vigor and Al resistance were not correlated (Zeegers et al., 2004). Lack of correlation between the two traits was independently confirmed by comparing the genotype means for the adjusted logarithm of RL in Solution 1 (root vigor) against the genotype means for an alternative Al-resistance index (the log-transformed ratio of RL in Solution 2 to RL in Solution 1); the two sets of hybrid means were indeed uncorrelated ($r^2 = 0.02$).

## RESULTS AND DISCUSSION

### Initial Evaluation of the Main Parents of the Breeding Program

The commercial brachiariagrass cultivars are widely propagated by stem cuttings, a feature that enables breeders to generate genetically identical clonal propagules for evaluation of phenotypic characters (Cardona et al., 1999). When the basal node of stem cuttings was incubated in a low-ionic-strength nutrient solution for 9 d, all tested *Brachiaria* genotypes typically produced two to four adventitious roots (Fig. 1). We initially used the three parental species (*B. ruziziensis*, *B. decumbens*, *B. brizantha*) to establish the effectiveness of the hydroponic solutions designed to simulate stress factors of the acid-soil syndrome. Solution 1 contained a low concentration of $Ca^{2+}$ to protect root plasma membranes, but lacked other nutrients (200 $\mu M$ $CaCl_2$, pH 4.20). Solution 2 was identical to Solution 1, but also contained 200 $\mu M$ $AlCl_3$. Root growth in Solution 1 should reflect the plants' ability to produce an extensive root system that explores a large volume of soil for nutrient uptake. A comparison of root growth between the two solutions should provide a measure of Al resistance.

Stem cuttings of all three parental genotypes continued to produce leaves during the duration of the experiment. Leaves of *B. ruziziensis*, but not the other two parents, tended to become slightly chlorotic toward the end of the experiment. Roots of *B. decumbens* and *B. brizantha* continued to elongate in Solution 1 for the entire period of evaluation (3 wk). Those of *B. ruziziensis*, by contrast, ceased to elongate after approximately 1 wk and were considerably shorter (Fig. 2, left panel). Presence of Al in Solution 2 strongly inhibited root elongation of *B. brizantha*, but had only little effect on roots of *B. decumbens*. Root growth of *B. ruziziensis* in this solution was negligible (Fig. 2, right panel).

The growth of the three *Brachiaria* genotypes in the two solutions coincides with well-established differences in adaptation to infertile, acid soils: *B. decumbens* is well-adapted, adaptation of *B. brizantha* is intermediate, and *B. ruziziensis* performs poorly (Paulino et al., 1987; Rao et al., 1996; Miles et al., 2004). The results of this experiment suggest that vigorous root development under nutrient deprivation and a high level of Al resistance may both contribute to the excellent edaphic adaptation of *B. decumbens*.

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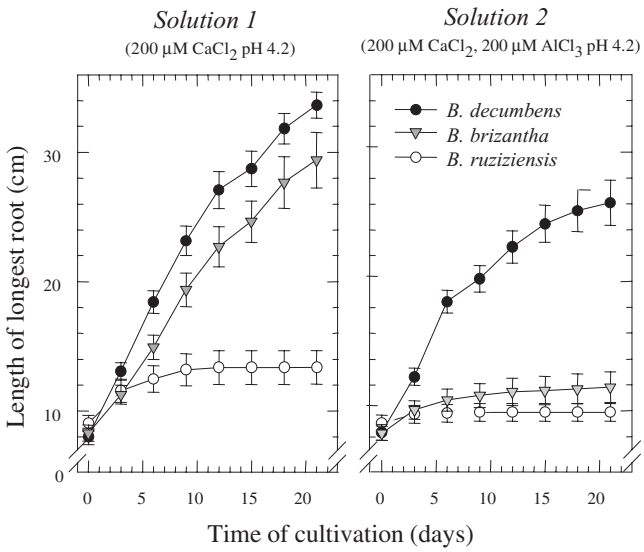


**Fig. 2. Initial test of the two treatments (without and with Al) using the three parental genotypes of the *Brachiaria* breeding program. The length of the longest root was recorded for up to 21 d of growth in the basal treatment (left panel) and the Al treatment (right panel).**

## Evaluation of a Set of Siblings

We next evaluated, in a series of replicate experiments, the 21-d growth performance of a broader range of genotypes. We selected 38 *B. ruziziensis* × *B. decumbens* F1 hybrids (full siblings), which were expected to segregate for edaphic adaptation because of the heterozygosity of apomictically reproducing *B. decumbens*. In this set of experiments we characterized root growth by measuring total root length (RL).

### Root Vigor

Total root length in Solution 1 ranged from 0.7 to 6 m. The latter value is quite remarkable considering the biomass of stem cuttings (mean dry weight: 0.42 g) and the fact that the growth medium contained only 200 μM $CaCl_2$. Not surprisingly, roots of brachiariagrasses in neotropical savannas represent a carbon sink of worldwide significance (Fisher et al., 1994).

The RL values showed a considerable degree of positive transgressive segregation, perhaps as a result of hybrid vigor (Fig. 3, left panel). The striking difference in RL between *B. ruziziensis* (and one of the hybrids) and the other genotypes probably was an artifact of the particular subset of hybrids used for this experiment, because a recent screening of a larger group of *B. ruziziensis* × *B. decumbens* hybrids has identified hybrids that are intermediate between those two groups of genotypes (data not presented). The performance of individual genotypes was consistent across harvests, as can be deduced from the mean standard error (0.034 log units) compared with the range of RL values measured for the group of 38 hybrids (0.933 log units) (Fig. 3, left panel). This result confirmed the suitability of Solution 1 for quantifying differences in root vigor among a set of full sibs. Root vigor as measured by this method probably comprises different physiological components expressed in plants at a physiologically mature, vegetative stage such as the frequency of initiation of adventitious roots, the tendency of nutrient-deprived plants to allocate carbon to roots rather than shoots, the efficiency with which nutrient reserves in stem cuttings are remobilized to sustain root growth, the branching of adventitious roots, and the growth of fine roots. Any of these components is likely to have an impact on a plant's adaptation to infertile soils.

### Aluminum Resistance

Aluminum resistance was less straightforward to measure because the inherent differences in root vigor had to be taken into account to quantify accurately the effect of Al toxicity on root development for different genotypes. A similar problem has been encountered previously with rye seedlings (Hede et al., 2002), but was much more pronounced in the case of vegetative *Brachiaria* propagules. We used a residual-variance approach to compute a root vigor-adjusted Al-resistance index (see Materials and Methods). The right panel in Fig. 3 displays the distribution of this index for the 38 *B. ruziziensis* × *B. decumbens* hybrids. The two genotypes with poor root vigor (*B. ruziziensis* and one hybrid; see left panel) showed quite different levels of Al-resistance (0.09 vs. 0.34 log units; right panel). The Al-resistance index, therefore, quantifies Al resistance even for genotypes with low root vigor, for which the greater relative contribution of the root length present at the initiation of treatments may introduce a bias toward higher resistance levels.

Aluminum resistance among the hybrids seemed to vary quantitatively. In agreement with the results from a seedling-based root elongation assay, Al resistance of *B.*

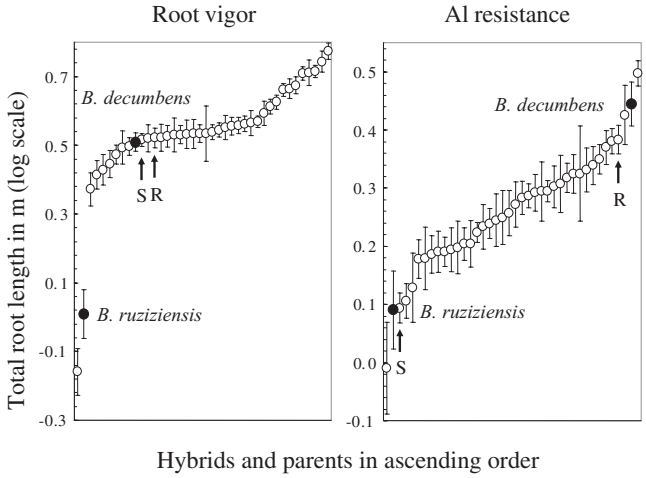


**Fig. 3. Segregation of root vigor (left panel) and Al resistance (right panel) in a group of 38 *B. ruziziensis* × *B. decumbens* F1 hybrids. Root vigor is the total root length (RL) in Solution 1 (200 μ*M* $CaCl_2$, pH 4.20), adjusted for the effects of stem-cutting biomass and harvest. Aluminum resistance is the stem-cutting-biomass and harvest-adjusted RL in Solution 2 (200 μ*M* $CaCl_2$, 200 μ*M* $AlCl_3$ pH 4.20), after removing the variance component caused by differences in root vigor. The two parents are highlighted by black symbols. The two hybrids with contrasting levels of Al resistance that were used for the hematoxylin-staining test (Fig. 4) are designated as "R" for Al resistant and "S" for Al sensitive.**

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*decumbens* was significantly superior to Al resistance of *B. ruziziensis* (Wenzl et al., 2001). In contrast to root vigor, the two parents were close to the two extremes of the Al-resistance distribution (Fig. 3, right panel). Though based on a limited number of segregants, this segregation pattern would be consistent with multiple genes contributing to Al resistance of adventitious root systems (a mixture of several root types), perhaps a similar situation as in species such as rice and probably maize (Nguyen et al., 2002; Kochian et al., 2004). The absence of positive transgressive segregation suggests that, in this collection of hybrids, *B. decumbens* provided most of the alleles that contributed importantly to Al resistance. A recent evaluation of 200 additional siblings has indeed confirmed these tentative conclusions (Buitrago et al., YEAR???, unpublished data).

Aluminum toxicity not only inhibits root elongation, but also induces lateral swelling of roots (Taylor, 1989). Aluminum-sensitive genotypes, therefore, should not only be characterized by a decrease in RL, but also an increase in root diameter. We found that the RL-based Al-resistance index was indeed negatively correlated with a similar index based on root diameter, which was also quantified by the image analysis software ($r^2 = -0.75$; data not presented). We also validated our method of quantifying Al resistance against the well-established hematoxylin-staining method, using the two parents and two hybrids that were close to the extremes of the range of Al-resistance levels (see arrows in Fig. 3). In agreement with our classification, root apices of Al-sensitive genotypes stained strongly; while those from Al-resistant genotypes remained clear (Fig. 4).

## Implementation in a Breeding Program

From the combined results of this study we conclude that our procedure screens brachiariagrass genotypes for traits that contribute to adaptation to infertile and acid soils (root vigor, Al resistance) at a physiologically mature, vegetative stage. This conclusion was further verified by testing *Brachiaria* hybrid 'Mulato', a recently released cultivar that had been selected for edaphic adaptation in field trials. The glasshouse screen revealed good root growth and intermediate Al resistance, consistent with its good vigor and responsiveness to applied nutrients on acid soils (data not presented).

We characterized root growth and root-system morphology in detail to test the effectiveness of the two experimental treatments (Solutions 1 and 2) in revealing genetic differences in edaphic adaptation. Preliminary data from a larger hybrid population suggest that this approach is also useful for identifying QTLs contributing to acid-soil adaptation (Buitrago et al., YEAR???, unpublished data). To maximize the number of segregants that can be screened, the procedure may be simplified. Plants could be cultivated in Solution 1 (basal treatment), but transferred to Solution 2 (Al treatment) a day or two before harvest, followed by hematoxylin-staining of root apices. This would enable breeders to assess separately root vigor (size of root system) and Al resistance (absence of staining), exclusively by visual inspection. Alternatively, plants could be cultivated in Solution 2 only, thus simultaneously selecting for both component traits (Fig. 2, right panel).

The latter approach may exclude potentially useful segregants for component traits. Yet this approach has significantly increased the efficiency of the *Brachiaria* breeding program at CIAT by enabling breeders to discard quickly a large number of nonadapted genotypes. In a more recent breeding cycle, 745 sexual segregants were screened for both edaphic adaptation and spittlebug resistance in the course of 6 mo, and the best 5% that combine both traits were used for further genetic recombination and improvement. Several hybrid-derived sexual genotypes that are markedly superior to the original sexual tetraploid *B. ruziziensis* have been identified using this procedure (data not presented).

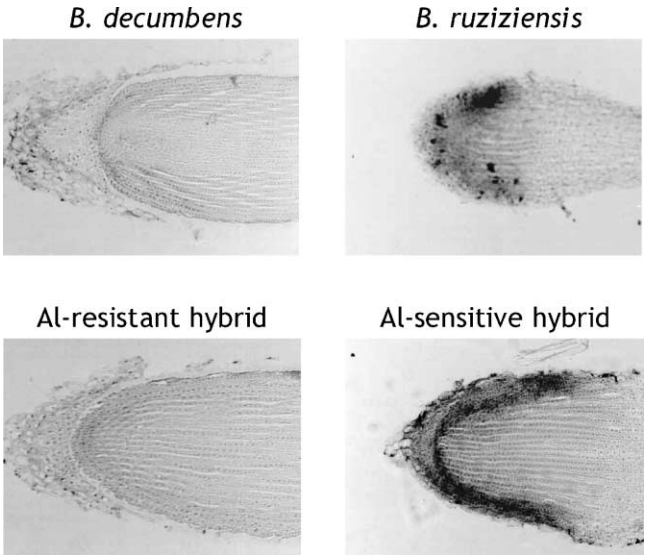


**Fig. 4. Hematoxylin-staining patterns for root apices of genotypes identified by the screen as Al-resistant (left side) and Al-sensitive (right side). The two *B. ruziziensis* × *B. decumbens* hybrids used for this test are also highlighted in Fig. 3.**

## Comparison with Other Screening Methods

Root vigor of mature plants does not appear to be a widely-used selection criterion in breeding programs targeting edaphic adaptation (see Annicchiarico and Piano, 2004, for an example in the context of drought tolerance). Root vigor of seedlings has received some breeding attention (Price et al., 1997), yet is unlikely to bear much relevance for edaphic adaptation of *Brachiaria*, which is manifest in the persistence of pastures across several growing seasons. Stem cuttings from mature plants are probably a more suitable material to assess long-term edaphic adaptation because the dramatic differences in root vigor between *B. ruziziensis* and *B. decumbens* were not expressed at the seedling stage (Wenzl et al., 2001).

Aluminum resistance is usually assessed in seedling-based assays, either by quantifying root elongation or apical callose concentrations, or by staining root apices with hematoxylin (Kerridge and Kronstad, 1968; Polle

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et al., 1978; Ruiz-Torres et al., 1992; Llugany et al., 1994; Cançado et al., 1999). Although seedling-based assays have been successfully applied to brachiariagrasses (Wenzl et al., 2001), poor germination of *Brachiaria* seeds at the surface of nutrient solutions and the poor viability of hydroponically-grown seedlings on transplantation to soil limit their applicability in a breeding program. The Al-resistance screen based on stem cuttings circumvents the transplantation problem and enables the concurrent assessment of root vigor of mature plants as a second component trait contributing to edaphic adaptation. Vegetative propagation also permits simultaneous assessment of a single genotype (clone) for other traits such as insect or disease resistance, nutritional quality, and seed production.

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