Output 3. Grass and legumes genotypes with superior adaptation to edaphic and climatic constrains are developed

3.1 Genotypes of *Brachiaria* with adaptation to edaphic factors

Highlights

- Through collaborative research with the Yamagata University, Japan, we showed that the high level of aluminum resistance in *Brachiaria decumbens* cv. Basilisk (signalgrass) is associated with less permeability of the plasma membrane of the root-tip portion.

- Confirmed the applicability of a new simple, rapid and reliable technique of methylene blue (MB) staining for the discrimination of aluminum (Al)-resistant protoplasts from a wider range of plant species, cultivars and lines and showed higher level of Al resistance in signalgrass compared with field crops.

- Showed that phosphoenolpyruvate carboxylase is activated in the *Brachiaria* hybrid cv. Mulato under low P supply and low pH conditions and that this increased activity could contribute to its adaptation to tropical acid soils with low P availability.

- Developed and validated a hydroponic screening procedure that uses vegetative propagules (stem cuttings) of *Brachiaria h*ybrids to rapidly evaluate root vigor and aluminum resistance, two key components of edaphic adaptation.

- Screened 139 apomictic/sexual hybrids of *Brachiaria* using a hydroponic screening method and identified 9 hybrids (BR04NO1018, 1552, 1900, 2110, 2128, 2166, 2179, 2201 and 2681) that were superior to *Brachiaria decumbens* parent in terms of aluminum resistance.

- Showed that the *Brachiaria* hybrid, cv. Mulato II performed well into the fourth year after establishment in the Llanos and its superior performance at 42 months after establishment was associated with its ability to acquire greater amounts of nutrients from low fertility soil.

3.1.1 Mechanisms of edaphic adaptation of *Brachiaria*

3.1.1.1 Defining physiological mechanisms and developing screening methods

Contributors: T. Wagatsuma, M. S. H. Khan, K. Tawaraya, K. Igarashi, T. Murayama, T. Kawamura (Yamagata University, Japan), S. Ishikawa (NIAES, Japan), M. Uemura (Iwate University, Japan), P. Wenzl (DarT P/L, Australia) and I. M. Rao (CIAT)

Rationale

Within the genus *Brachiaria*, signalgrass was found to be outstandingly resistant to aluminum (Al) as a result of unknown mechanisms independent of secretion of organic acid anions. Previous research by Wagatsuma and coworkers from the Yamagata University, Japan showed direct evidence of the contribution of plasma membrane (PM) lipids to Al resistance, i.e., the reduced permeabilization of plasma membrane as a mechanism of Al resistance in triticale. Here, we report a new feature of Al-resistance based on the PM lipid layer of signalgrass.

Materials and Methods

Al accumulation was observed after hematoxylin staining of root-tip portions treated for 24 h with 20 μM $AlCl_3$ in 0.2 mM $CaCl_2$ (pH 4.9) for other plant species or 100 μM $AlCl_3$ in 0.2 mM $CaCl_2$ (pH 4.2) for the two *Brachiaria* species (*B. decumbens* and *B. ruziziensis*). PM permeability was determined by fluorescein diacetate (FDA)-propidium iodide (PI) fluorescence microscopy (ex 450-490 nm, em 520 nm). PM lipids were obtained using the newly established technique and their composition was analyzed qualitatively by high performance thin layer chromatography (HPTLC). Flavonoid deposition was observed by fluorescence microscopy after staining with Naturstoffreagenz A (NA) (0.1% [w/v] 2-aminoethyl diphenyl borate (ex 390nm, em 450nm). Permeability experiments were carried out by using nylon microcapsule coated with different compositions of lipid molecules or catechin.

Results and Discussion

Root-tips of signalgrass accumulated the least Al among all plant species tested (Photo 14-B_1), indicating their strongest Al exclusion ability. The PM of the root-tip portion of signalgrass was less permeabilized after 9-h Ca treatment following 1-h Al treatment (Photo 15), indicating

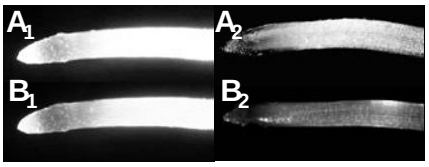

Photo 15. FDA-PI fluorescence microscopy. A: signalgrass, B: ruzigrass. Left: immediately after 1-h Al treatment, right: after 9-h Ca treatment following 1-h Al treatment (100 μM Al). Green fluorescence (white) shows an intact PM and red fluorescence (black) shows permeabilization of the PM.

the strong structure of the PM lipid layer even after elongation of the root-tip portion under normal conditions. More glucocerebrosides and free sterols were observed in the PM lipid of root-tips for Al-tolerant maize cv. Neodent 90 than that in Al-sensitive cv. KD850 (Figure 25), and more free sterols were observed in signalgrass than ruzigrass (data not shown). Since glucocerebrosides and free sterols are, in general, the main lipid components of the PM lipid layer, they can primarily regulate PM physics. Artificially mixed lipids containing larger amounts of glucocerebrosides and free sterols showed less permeability during capsule experiments (data not shown). Glucocerebrosides have longer fatty acyl chains (typically C_{24}) and exist primarily in the outer leaflet of the PM. Several OHs from glucose and hydroxyl-fatty acyl chains, amide and carbonyl groups and other oxygen sites of glucocerebroside have been

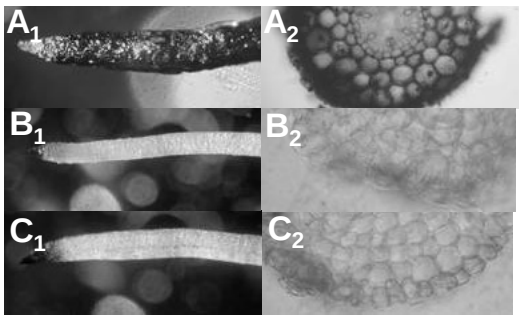

Photo 14. Hematoxylin staining of root-tip portions in A: an Al-sensitive wheat cultivar (Scout), B: signalgrass, C: ruzigrass. Left: portion 1 cm from the tip, right: section 2-3 mm from the tip. Strong purple (black in the picture) shows heavy deposition of Al.

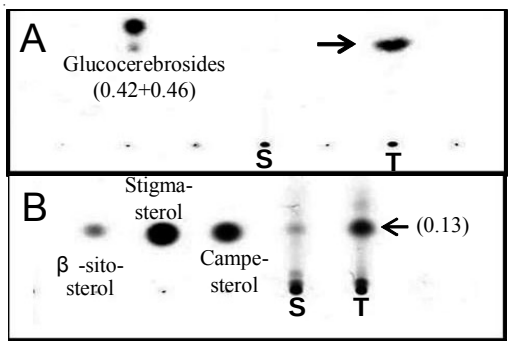

Figure 25. HPTLC analysis of PM lipids isolated from maize root-tips cultured under normal conditions. A: glucocerebrosides, B: free sterols. S: Al-sensitive cultivar (KD850), T: Al-tolerant cultivar (Neodent 90).

considered responsible for hydrogen bonding with neighboring phospholipids and formation of a transbilayer peg using long fatty acyl chains. Free sterols have also been considered responsible for formation of a more rigid lipid layer due to their planer steroid skeleton structure. All these considerations agree with the present capsule experimental data (not shown here).

It is concluded that a plasma membrane (PM) lipid layer containing large amounts of glucocerebrosides, free sterols, and flavonoids has superior resistance against permeabilization by Al ions. This is essential for Al resistance, which has been ascribed to reduced PM permeabilization. No difference was observed in phenylpropa1noid deposition between root-tips of the two *Brachiaria* spp. examined (data not shown). Flavonoids are synthesized in the cytoplasm and thereafter transported, in part, through the PM to the cell wall area. Further experiments are therefore needed in the future, especially to determine the localization of flavonoids within the PM lipid layer.

With all root sections, the outer surface of epidermal cells emitted strong yellowish-green fluorescence (Photo 16). Green fluorescence was recognized in the surface area of entire cortex cells in the root-tip portion of signalgrass. Flavonoids emit green fluorescence with NA staining, and several kinds of flavonoid compounds have been reported in the cell wall and vacuole using this technique. Flavonoids have been shown to penetrate membrane lipid interiors resulting in restriction of membrane fluidity. Many previous studies on animal nutrition, and membrane-rigidifying and tumor cell growth-inhibitory effects have been carried out in connection with the differential penetration activities of flavonoids. We observed reduced permeability in lipid mixture containing catechin (data not shown).

In summary, Al accumulation in the root-tip portion of signalgrass was lowest of all plant species examined in this report. The plasma membrane (PM) of the root-tip portion in signalgrass was less permeabilized than that in the less Al-tolerant ruzigrass after re-elongation of roots following short-term Al treatment. The PM of root-tips in an Al-tolerant maize cultivar was shown to contain larger amounts of glucocerebrosides and free sterols than an Al-sensitive maize cultivar. The permeability of nylon capsules coated with PM lipids isolated from root-tips of the Al-tolerant maize cultivar was lower than that of capsules coated with PM lipids from the Al-sensitive cultivar. Using Naturstoffreagenz A (2-aminoethyl diphenyl borate) fluorescence microscopy, larger amounts of flavonoids were found deposited in the root-tip portion of signalgrass compared to the other portion and species examined. Artificially mixed lipids containing larger amounts of glucocerebrosides, free sterols and catechin showed less permeability in Al medium. We propose a new feature of Al exclusion based on the special composition of the PM lipid layer.

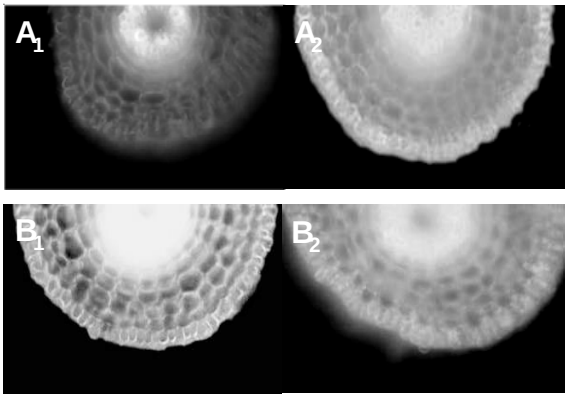

Photo 16. NA-stained fluorescence microscopy of roots grown under normal conditions. A: signalgrass, B: ruzigrass. Left: 2-3mm from the root tip, right: ca. 2cm from the root tip. Green (dark) fluorescence (A_1) shows higher deposition of flavonoids, and whitish blue (white) fluorescence shows autofluorescence by other phenolic compounds.

3.1.1.2 Methylene blue stainability of root-tip protoplasts as an indicator of aluminum resistance in a wide range of plant species, cultivars and lines

Contributors: T. Wagatsuma, M. S. H. Khan, T. Yamamoto, T. Kawamura, K. Hosogoe, K. Tawaraya (Yamagata University, Japan), S. Ishikawa (NIAES, Japan), P. Wenzl (DarT P/L, Australia), and I. M. Rao (CIAT)

Rationale

Several mechanisms of aluminum (Al) resistance in plants have been reported, (i.e., exudation of organic acid (OA) anions in wheat, cell wall properties in wheat, microtubule properties in cultured tobacco cells, plasma membrane strength (PMS) and root phenolics in woody plants, and plasma membrane (PM) intactness in several plant species). Although exudation of OA anions is accepted as a primary and widely applicable strategy for many plant species, no involvement of OA anions exudation in Al resistance has been reported in rice and sorghum, *Brachiaria* spp., rice, pea, and triticale.

It is generally believed that Al resistance is controlled by multiple genes with different degrees of contribution to Al resistance. Although intensive research work has been carried out using *Arabidopsis* on the identification of Al-tolerant genes and on making transgenic Al-tolerant *Arabidopsi*, Al tolerance of *Arabidopsis* is accepted to be a rather sensitive level lower than that of Al-sensitive wheat line ES3. Contrary to this, *B. decumbens* has been demonstrated to tolerate extraordinarily high concentration of Al ions. Considering no involvement of OA anions release in the extraordinary Al resistance of *B. decumbens*, it is suggested that there may be another higher Al-resistant strategy operated commonly and primarily in a wide range of plant species, cultivars and lines.

We developed a new simple, rapid and reliable technique for the discrimination of Al-tolerant protoplasts: methylene blue (MB) is strongly adsorbed on the plasmalemma of root cells in the tip portion of Al-sensitive plant species among 4 plant species, i.e., rice, oats, maize and pea. The objectives of the present research were to ascertain the applicability of this technique to a wide range of plant species, cultivars and lines and to identify Al-resistance mechanisms operated commonly and primarily in a wide range of germplasm.

Materials and Methods

Seeds of rice (*Oryza sativa* L. cv. Reikoushindan-kurodani and cv. Sasanishiki, *O. glaberrima* L. line W492), maize (*Zea mays* L. cv. Neodnet90 and cv. Golddent KD850), wheat (*Triticum aestivum* L. cv. Kitakami B, cv. Hachiman, cv. Atlas 66, cv. Scout 66, lines ET8 and ES8), barley (*Hordium vulgare* L. cv. Nozomi-nijo), and triticale (*Triticosecale* Wittmark cv. Currency lines ST22 and ST2) were soaked in tap water under aeration for 24 h in the former 2 species and for 12 h in the latter 3 species at 27°C. The seeds of buckwheat (*Fogopyrum esculentum* Moench cv. Shinshu-1) and pea (*Pisum sativum* L. cv. Harunoka) were surface-sterilized for 10 min with 0.5% (v/v) NaClO mixed with 3 drops of Tween 80. The seeds of signalgrass (*Brachiaria decumbens* Stapf cv. Basilisk) and ruzigrass (*B. ruziziensis* Germain and Evrard cv. Common) were surface-sterilized for 1 min with 70% ethanol, and next for 10 min with 2% (v/v) NaClO mixed with 3 drops of Triton X-100. All these seeds were germinated on a nylon screen that was put on a polypropylene container filled with 8 liters of tap water under aeration at 27°C. Seedlings with roots approximately 4 cm in length were used in the experiments. Seedlings were treated with 0.2 mM $CaCl_2$ for 6 h (pH 4.9), and then without (control) or with (+Al) 20 µM $AlCl_3$ in 0.2 mM $CaCl_2$ for 24 h under conditions maintaining pH at 4.9 for the whole duration under aeration at 27°C. Al tolerance was defined as the relative net elongation of the longest root from each 10 seedlings between control and +Al treatments.

The isolation and purification of protoplasts from root-tips were carried out as follows. Briefly, apical 1 cm segments were digested with a medium composed of 0.6 M mannitol, 2% (w/v) Cellulase Onazuka RS, 0.1% Pectolyase Y-23, 0.05% (w/v) BSA, 1 mM $CaCl_2$, and 0.5 mM DTT (pH 5.6). The digested tissues separated by a nylon cloth were gently agitated and washed in suspension medium, and filtered. All filtrates were centrifuged at 200×g. The pellet of crude protoplasts was gently suspended in 30% (w/v) Ficoll in 0.6 M mannitol and 2 mM Tris-MES (pH 6.5). A discontinuous gradient was formed by successive layering of solutions of 8, 5 and 0% Ficoll in 0.6 M mannitol and 2 mM Tris-MES (pH 6.5). Purified protoplasts were collected after centrifugation at 380×g from the interface between the layers of 0% and 5% Ficoll. Isolation and purification was replicated separately in twice or three times.

The purified protoplasts were stained for 3 min with 0.1% (w/v) MB solution in 0.6 M mannitol (pH 4.2). The stained protoplasts were observed under a light microscope and photographed in identical conditions (light intensity: 6.0; fixed exposure; image quality, fine) with digital camera.
Image analysis was carried out by image analysis system. Debris and aggregated protoplasts in each picture were removed by Adobe Photoshop 6.0. File format was converted from Joint Photographic Experts Group (JPEG) to Moving Picture Coding Experts Group (MPEG). The original color pictures were converted to monochrome pictures, and they were treated as binary images. The area at threshold 125, 110, 95 or 85 for each treated image was measured in the conditions of 8-neighbor and minimum pixel of 50. The whole area was expressed as pixel number. For each plant cultivar or line, 5 pictures were selected and analyzed.

Thin section was cut by hand with razor blade at ca. 3 mm from root-tip of 3-4 cm root of maize (cv. Golddent KD850). The section stained preliminarily for 1 h with DAPI (4′,6-diamidino-2-phenylinodol), was put inside of the enclosed area made by cover slip (0.2 mm in thickness) which was adhered with manicure to the slide glass. The enclosed space was filled with digesting medium of the same composition as described above. Digestion process was observed with a fluorescence microscope equipped with a UV filter (Nikon, excitation filter 450-490nm; barrier filter 520 nm) at 15, 45 and 75 min after the start of digestion.

Seedlings other than two *Brachiaria* spp. with roots approximately 4 cm in length were pretreated with 0.2 mM $CaCl_2$ for 6 h (pH 4.9), then treated with 20 µM $AlCl_3$ in 0.2 mM $CaCl_2$ (pH 4.9) for 1 h, and next regrown in 0.2 mM $CaCl_2$ (pH 5.2) for 9h. Two *Brachiaria* spp. were treated with 100 µM $AlCl_3$ in 0.2 mM $CaCl_2$ (pH 4.2) for 2 h. After the Al treatment and regrowth, roots were stained for 10 min with fluorescein diacetate-propidium iodide (FDA-PI). In case of rice, wheat, triticale and *Brachiaria* spp., concentrations were FDA (12.5 mg L^{-1})-PI (5 mg L^{-1}), and in case of pea and maize, these were FDA (12.5 mg L^{-1})-PI (15 mg L^{-1}). After the removal of extra dyes with deionized water, the root-tips were observed under a fluorescence microscope equipped with a UV filter, and photographed by digital camera (Nikon, Coolpix 950). The root-tip portions and their hand sections 2-3 mm from root apices after 24-h treatment with Al were stained with hematoxylin and observed by light microscopy.

Results and Discussion

Under similar stress conditions (20 µM $AlCl_3$, for 24 h), Al tolerance among plant species was in general in the following order: two *Brachiaria spp*.>buckwheat, rice, maize>wheat, pea, triticale>barley. Although there were wide differences in Al tolerance among cultivars or lines within the same plant species, this order agrees with the general tendency: two *Brachiaria spp*. were most tolerant but, on the other hand, barley was most sensitive to Al. Between the two *Brachiaria spp*, signalgrass was more tolerant than ruzigrass. Within *Oryza* spp., *O. glaberrima* was most sensitive and within *O. sativa*, cv. Reikoshindan-kurodani was more tolerant than cv. Sasanishiki. For other plant species, Al

tolerance among cultivars or lines was as follows: in wheat, cv. Atlas 66>line ET8, cv. Kitakami B>cv. Hachiman, line ES8, cv. Scout 66; in triticale, line ST2> line ST22.

Although permeabilities of the PM in root-tips of wheat just after 1-h of Al treatment were greater in lines ET8 and ES8, those after re-elongation in 0.2 mM $CaCl_2$ were greater in cv. Scout 66 and line ES8. Among *Brachiaria spp*., rice species (cv. Sasanishiki and *O. glaberrima*), triticale line (ST2 and ST22) and maize cultivars (Neodent 90 and Godldent KD850), the same tendency was observed, i.e., the more PM permeabilization in Al sensitive plant. These results confirm an essential role of the PM intactness of root-tip cells in early stage Al resistance of a variety of plant species.

Al accumulation in root-tips was in the following order: *Brachiaria spp*< rice < wheat, triticale. Among wheat cultivars and lines, Al-sensitive cv. Scout 66 and line ES8 accumulated more amount of Al in root-tips than Al-tolerant cv. Atlas 66 and line ET8. In Al-sensitive wheat cultivar and line, Al was accumulated most heavily in epidermis and also heavily in cortex, but on the contrary, in Al-tolerant wheat cultivar and line, Al was accumulated mainly in epidermis and endodermis. Although Al localization in a cell was recognized mainly in the cell surface area in Al-tolerant plants, higher distribution was also observed in symplastic area in Al-sensitive plants. These results indicate that the permeabilized PM of Al-sensitive plants permits a greater movement of Al into the symplast.

The isolated protoplasts were composed of a variety of protoplasts with differences in MB stainability. Methylene blue [3,7-bis(dimethylamino) phenothiazin-5-ium chloride] has a positive charge with amino and imino groups as auxochromous groups, reacts with sites with negative charges of PM, and assumes a blue color under aerobic conditions. We already reported that MB could be used as a colored analog to non-colored Al ions, and that the more Al sensitive plant species had greater number of heavily-stained protoplasts. The area at threshold 125 almost shows the total area of protoplasts stained with MB, but, on the other hand, the area at threshold 95 shows the total area of protoplasts stained heavily with MB. We defined the proportion of the heavily-stained area at threshold 95 to the whole stained area at threshold 125 as MB stainability (%) of the whole protoplasts. MB stainability was negatively correlated with Al tolerance among wider range of plant species, cultivars and lines (Figure 26, $y= 45.7e^{-0.017x}$, $R^2= 0.577$**). Within the same plant species, negative relationship between Al tolerance and MB stainability was recognized in maize (Al tolerance: cv. Neodent 90>cv. Golddent KD850; MB stainability: in reverse order) and in wheat (Al tolerance: cv. Kitakami B, line ET8>cv. Scout 66, cv. Hachiman>>line ES8; MB stainability: in reverse order). Within rice species and cultivars, the relationship between the above two phenological characteristics was reverse to the former relationship (Al tolerance: cv. Reikoshindan-kurodani>cv. Sasanishiki> *O. glaberrima*; MB stainability: in the same order). Within triticale, Al tolerance was higher in line ST2, though no differences were observed in MB stainiabilities.

There are no clear explanations to these complex relationships among plant species, cultivars and lines. The whole relationship in Figure 26 using 18 plant species, cultivars and lines agree with the former result using only 4 plant species.

The protoplasts used for MB stainability (Figure 26) are expected to be isolated mainly from the outer and center cortices and epidermis and partially from the center of stele. As epidermis, outer and center cortices are the primary cells for Al accumulation in roots, the isolated protoplasts used for MB stainability were accepted to be appropriate for Al research.

MB was observed not only on the protoplast surface but also inside of the protoplast. Surface MB is considered to be the adsorbed MB on the negative sites of PM and inside MB to be permeated MB. This means the similarity in the behaviour of MB to Al ions. Negative correlation between Al tolerance and MB

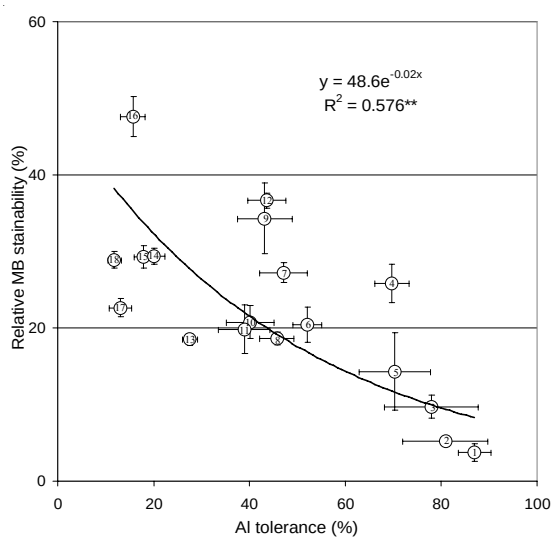

Figure 26. Relationship between Al tolerance among wider range of plant species and MB stainability of protoplasts. Al treatment: 20 μM $AlCl_3$ (pH 4.9) for 24 h. Al tolerance: ratio of the net root elongation of the longest root in Al treatment to that in control. MB stainability of protoplasts (%): ratio of the heavily-stained area at threshold 95 to the whole stained area at threshold 125. 1: signalgrass, 2: ruzigrass, 3: maize (cv. Neodent 90), 4: rice (cv. Reikoushindan-kurodani), 5: buckwheat, 6: rice (cv. Sasanishiki), 7: wheat (cv. Atlas 66), 8: Maize (cv. Golddent KD850), 9: triticale (line ST2), 10: wheat (line ET8), 11: wheat (cv. Kitakami B), 12: pea, 13: *Oryza glaberrima*, 14: Barley, 15: wheat (cv. Hachiman), 16: wheat (line ES8), 17: triticale (line ST22), 18: wheat (cv. Scout 66).

stainability (Figure 26) among a range of plant samples with wider differences in Al resistance finally suggests the importance not only of the less negativity of cell surface but also of the PM strength as has already been defined as the intactness of PM permeability for Al resistance and that this idea can be applicable to a variety of plant samples. Higher significance level found in the exponential regression line (Figure 26) suggests the greater Al permeation in Al-sensitive plants. Al permeation has been suggested to be dependent mainly of the lipid composition of PM.

Although cell wall has been reported as one of the important apparatus for Al tolerance, the negative correlation between Al tolerance and MB stainability among a variety of plant samples strengthens the significant role of the PM strength in Al tolerance of wider range of plant species, cultivars and lines.

There are many known and possible factors that can affect membrane rigidity, fluidity and permeability in association with the composition of lipid molecules (phospholipids, sterols or glucocerebrosides), differences in acyl chains and lipid saturation, and polysaccharides or phenolic environment near membrane.

Further research is needed to clarify the role of lipid composition of PM and its genetic and molecular characteristics as the controlling factors for Al resistance.

In summary, we confirmed the applicability of a new simple, rapid and reliable technique of methylene blue (MB) staining for the discrimination of aluminum (Al)-resistant protoplasts from a wider range of plant species, cultivars and lines and identified a common strategy for Al resistance at an early stage.

A total of 10 plant species, i.e. two *Brachiaria* spp., two *Oryza* spp., buckwheat, maize, pea, triticale, wheat and barley were tested. Al resistance (based on relative net root elongation of the longest root) evaluated at 20 μM $AlCl_3$ (pH 4.9) for 24 h ranged widely from 10 to 88. Among cultivars and lines within the same species, Al accumulation in root-tip portion was greater in Al-sensitive plant, which corresponded to greater permeability of plasma membrane. MB stainability was negatively correlated with Al resistance.

This observation indicates the common importance of PM strength that was already defined as the intactness of PM permeability in addition to PM negativity for Al resistance of a wide range of plant species, cultivars and lines. Defining the role of lipid composition of PM will be an important future task in connection with PM strength.

3.1.1.3 Role of Phospho*enol*pyruvate carboxylase in the adaptation of *Brachiaria* hybrid cv. Mulato to low phosphorus acid soils

Contributors: H. Begum, M. Osaki, M. Nanamori, T. Watanabe, T. Shinano (Hokkaido University, Japan) and I. M. Rao (CIAT)

Rationale

Phosphorus (P) deficiency is an important factor limiting forage production in tropical and sub-tropical soils. In these soils, P forms insoluble compounds with a number of di- and tri-valent cations (*e.g.*, Al^{3+}, Fe^{3+}) and it is the least readily available nutrient in the rhizosphere. Correcting P deficiency with application of P fertilizer is not a viable approach for resource-poor farmers in the tropics and sub-tropics, especially on soils with high P-fixing capacity. Under such conditions, the integration of field crops with forage cultivars that can make most efficient use of the P supplied as maintenance fertilizer application represents a key element of sustainable crop-livestock systems in the tropics. Genetic variations in P uptake efficiencies have been widely reported in several food and feed crops. Plant traits responsible for P uptake efficiency include rhizosphere acidification, root exudation of organic acid anions and phosphate mobilizing enzymes, root morphology, uptake kinetics and symbiotic association with mycorrhizal fungi.

Plants have evolved two broad strategies for improving P acquisition and use in nutrient-limiting environments: (1) those aimed at efficient P use; and (2) those directed toward enhanced P acquisition or uptake. Processes that use efficiently the acquired P involve decreased growth rate, increased growth per unit of P uptake, remobilization of internal Pi, modification in internal carbon metabolism that bypass P-requiring steps, and alternative respiratory pathways. By comparison, processes that lead to increased P uptake include enhanced secretion of phosphatases and exudation of organic acids, changes in root morphology and enhanced expression of Pi transporters.

To enhance P uptake, because of their high affinity for divalent and trivalent cations, organic acid anions released from the roots are thought to displace P from insoluble complexes, making it more available for uptake by plants. Organic acid anions also play an important role in detoxification of Al both externally and internally. Enhanced expression and activity of phospho*enol*pyruvate carboxylase (PEPC) has been linked with P deficiency-induced biosynthesis and root exudation of carboxylic acids.

Phospho*enol*pyruvate carboxylase (PEPC) is a cytosolic enzyme widely distributed in most plant tissues, green algae and microorganisms but not in animal cells. PEPC is an important enzyme for the carbon economy of the cell, playing a central role in CO_2 fixation of C_4 and crassulacean acid metabolism (CAM) plants. PEPC activity produces OA (oxaloacetic acid) and malate that replenish the citric acid cycle, the so-called anaplerotic function, providing carbon skeletons for nitrogen assimilation. Recent findings suggest that PEPC may play an important role in the adaptation of plants to environmental stress factors.

Inherent differences in efficiencies of P uptake and utilization exist between tropical forage grass and legume species. Previous research showed that phosphorus use efficiency (PUE) in grass (*e.g., Brachiaria* spp.) is much higher than that of the legumes (*e.g., Stylosanthes* spp.) regardless of P supply. Widespread adoption of forage cultivars depends on efficiently acquiring P from the soil and using them for growth. Plant attributes appear to be linked to different strategies to acquire and use phosphorus. Understanding these linkages is

fundamental in integrating plant attributes in a selection index. It is essential to elucidate the mechanisms of plant species conferring superior adaptation to P deficient acidic soils. Although knowledge about the principal mechanisms involved in efficient P acquisition by plants has evolved substantially during recent years, the detailed mechanisms of internal P requirements among different genotypes of crop and forage plants are still not fully understood. In collaboration with the group at Hokkaido University, we have evaluated the role of phospho*enol*pyruvate carboxylase in improving PUE under P deficiency and soil acidity in *Brachiaria* hybrid cv. Mulato comparing with wheat (low P-sensetive) and rice (low P-tolerant).

Materials and Methods

Experiment 1. Effect of low pH and low P in soil. Experiment was conducted in a glasshouse (43° 3' N, 141° 2' E, altitude 17 m; maximum temperature 32° C; minimum temperature 16° C; average photoperiod during experiment = 14.8 h light and 9.2 h darkness; maximum photon flux density = 1550 µmol m^{-2} s^{-1}) of Graduate School of Agriculture of Hokkaido University, Sapporo, Japan. Seeds of *Brachiaria* hybrid cv. Mulato CIAT 36061 (developed from parents of *Brachiaria ruziziensis* clone 44-06 and *Brachiaria brizantha* cv. Marandú and identified as FM9201/1873), wheat (*Triticum aestivum* L.) and rice (*Oryza sativa* L. cv. Kitaake) were surface sterilized with 1% of sodium hypochlorite for 10 min. Rice seeds were germinated on a petri dish for 3 days. Seeds (germinated in case of rice) (2-6) were sown in small plastic pots (160 ml) containing soils with two levels of P (+P and -P) and three levels of pH (4.0, 4.5 and 5.0), of which soil were collected from a long-term (25 years-old) experimental field without P fertilizer input, with pH (H_2O) 4.0. Initially, soil was fertilized with 1.87 g N kg^{-1} and 0.8 g K kg^{-1} soils as $(NH_4)_2SO_4$ and K_2SO_4, respectively. In case of +P treatments, $Ca(H_2PO_4)_2$ was added at the rate of 2.22 g kg^{-1} of soil while no P was added in -P treatments. Available P concentration (Bray II, mg/100g soil) was 5.5-5.9 in –P treatment, and 14.0-15.6 in +P treatment, which was higher in lower pH soil. The pH levels of soil were adjusted by adding appropriate amounts of calcium carbonate or 0.1 N H_2SO_4 to the soil. Each treatment was replicated for 6 times arranged in complete randomized block design.

Plants were harvested at 28-50 days of cultivation. Their roots were washed with tap water and then gently washed with deionized water, and plants were separated into root, stem and leaf. Half of each sample was dried in air-forced oven at 80°C for 72 h, then weighed, and ground for nutrient analysis. Another half of each sample was chilled by liquid nitrogen and then stored at -80°C for PEPC and organic acid analyses. Leaves of *Brachiaria* hybrid, wheat and rice were analyzed for PEPC and organic acid analysis. Dried sample was digested with sulfuric acid and hydrogen peroxide. Total nitrogen and P were measured by semi-micro Kjeldahl-method and vanado-molybdate yellow method, respectively.

Lyophilized leaves were homogenized in cold 0.01N HCl sample:HCl = 1:10 in leaves) to determine the organic acid concentrations in the plant. The extract was filtered with a membrane filter (pore size = 0.45 ìm). The organic acid anions were analyzed by a Capillary Ion Analyzer under the following measurement conditions: electrolyte, 2.5% CIA-PAK™ OFM Anion BT in 120 mM $Na_2B_4O_7$; capillary fused silica; and detection, 185 nm. Identification and detection of organic acid anions were done by comparing their retention time and absorption spectra with those of known standards.

For the measurement of PEPC activity under malate inhibition conditions, 200 ìl of the supernatant of the protein extraction solution was mixed with 300 ìl of saturated Na_2CO_3, and set on ice for 10 min. The precipitate was obtained by centrifugation at 17,000 g for 10 min at 4°C, then resuspended with 20 ìl of 50 mM Hepes-NaOH (pH 7.5), 5% (v/v) glycerol, 5 mM $MgCl_2$, 1 mM EDTA, 14 mM 2-mercaptoethanol, 1 mM PMSF, and 10 ìg mL^{-1} chymostatin. After

centrifugation at 17,000 g for 5 min at 4^{0}C, 20 ìl of the supernatant was mixed with 913 ìl of pre-reaction mixture containing 100 mM Hepes-NaOH (pH 7.3), 10 mM $MgCl_2$, 1 mM Na_2CO_3, and 0.2 mM NADH (dissolved in 50 mM Tris-HCl (pH 7.4), and 20 ìl of 50 mM malate and 2 units of MDH. The change in the amount of NADH was monitored after the addition of 40 ìl of 50 mM PEP. To measure the maximum activity of PEPC, 0.2 g of leaf was homogenized with 50 mM Hepes-KOH (pH 7.4), 10 % (v/v) glycerol, 1 mM EDTA, 10 mM $MgCl_2$, 5 mM DTT, 1 mM PMSF, 10 ìM leupeptin, and 5 % (w/v) polyvinyl-polypyrrolidone. 25 ìl of the supernatant was mixed with 908 ìl of pre-mixture, containing 100 mM Hepes-NaOH (pH 7.5), 10 mM $MgCl_2$, 1 mM $NAHCO_3$, 0.2 mM NADH (in 50 mM Tris-HCl), 2 ìl of MDH (diluted to 2 units with 50 % (v/v) glycerol). The amount of NADH was monitored after the addition of 40 ìl of 100 mM PEP. The amount of soluble protein was determined by the Bradford method, using BSA as a standard. This method was applied also in experiment 2.

Experiment 2. PEPC response to low phosphorus in hydroponic solution. *Brachiaria* hybrid cv. Mulato and rice (*Oryza sativa* L. cv. Kitaake) were grown hydroponically under greenhouse conditions. Seedlings were pre-cultured in a 56 L vessel containing 2.12 mM N, 0.77 mM K, 1.25 mM Ca, 0.82 mM Mg, 35.8 µM Fe, 9.1 µM Mn, 46.3 µM B, 3.1 µM Zn, 0.16 µM Cu, 0.05 µM Mo, with 6 µM P. After one week pre-culture, plants were transplanted to 56 L vessels with three levels of P concentration (0 µM, 6 µM and 32 µM, respectively) for two weeks. Phosphorus concentration was measured and adjusted to the respective levels of treatment everyday. The pH of the nutrient solution was adjusted to 5.2 ± 0.1 everyday. The nutrient solution was completely renewed once a week. Three plants were pooled for one replication and each experiment was conducted with three replications. A total of three experiments were conducted. Half of the collected plants was dried in 80ˆC oven for 3 days and weighed. The remaining half was frozen in liquid nitrogen and stored at –80 °C until the analysis of Pi and enzyme activities. All the statistical analyses were done using the SPSS (Windows 10.0) computer program.

Results and Discussion

Experiment 1. Effect of low pH and low P in soil. Dry weights of three crops were lower at -P treatment than those at +P treatment (Table 24). Dry weights of wheat and rice decreased with decrease of soil pH regardless of P treatments.

Table 24. Effect of phosphorus and pH treatments on dry matter production (g/plant).

		-P			+P		
Plant	Organ	pH 4.0	pH 4.5	pH 5.0	pH 4.0	pH 4.5	pH 5.0
Brachiaria	Shoot (S)	0.19±0.03	0.27±0.02	0.20±0.02	0.27±0.03	0.33±0.02	0.32±0.02
	Root (R)	0.06±0.02	0.09±0.01	0.06±0.01	0.08±0.02	0.10±0.01	0.09±0.02
	Total	0.25±0.07c	0.36±0.03b	0.26±0.04c	0.35±0.04b	0.43±0.04a	0.41±0.04a
	R/S ratio	0.32	0.33	0.30	0.30	0.30	0.28
Wheat	Shoot	0.03±0.01	0.03±0.00	0.04±0.01	0.02±0.00	0.04±0.00	0.07±0.01
	Root	0.01±0.00	0.02±0.01	0.03±0.01	0.01±0.00	0.03±0.01	0.05±0.02
	Total	0.04±0.01d	0.05±0.01c	0.07±0.02b	0.03±0.01c	0.07±0.01b	0.12±0.03a
	R/S ratio	0.33	0.66	0.75	0.50	0.75	0.71
Rice	Shoot	2.14±0.25	2.11±0.24	2.44±0.28	2.39±0.27	2.25±0.26	2.81±0.32
	Root	0.41±0.09	0.41±0.09	0.45±0.10	0.40±0.09	0.47±0.11	0.49±0.11
	Total	2.55±0.12c	0.52±0.05c	2.89±0.13b	2.79±0.05bc	2.72±0.14bc	3.30±0.11a
	R/S ratio	0.19	0.19	0.20	0.17	0.21	0.17

Note:Values are the mean of three replicates. Treatment means are different (P<0.05) if denoted by different letters (Duncan's multiple range test).

However, dry weight of *Brachiaria* hybrid at -P treatment were higher at pH 4.5 than those of pH 4.0 and 5.0, adapting well to low pH (4.0) even at low levels of soil P, which was more adaptable than the higher pH (5.0). At +P treatment, dry weight of *Brachiaria* hybrid was not much affected by soil pH. Root-to-shoot ratio of *Brachiaria* hybrid increased by P-deficiency, whereas that of wheat and rice was stable or decreased by P-deficiency (Table 24). This ratio remained constant in *Brachiaria* hybrid and rice regardless of decreasing soil pH, however decreased in wheat by a decrease of soil pH. Thus, relative root growth was vigorous in *Brachiaria* hybrid and rice under low pH, also vigorous in rice under low pH, and weak in wheat under low P and low pH. Consequently, root function of Brachiaria hybrid was maintained or stimulated under low pH and low P soils.

Amount of P absorbed by plants was quite similar to dry matter production, indicating that P accumulation and tolerance to low pH were key factors that contributed to plant production in this experiment (Table 25). When relative P absorption ability under acidic low P soil was estimated as (amount of P at -P. pH 4.0/ amount of P at +P. pH 5.0), this ratio was 0.52 in *Brachiaria,* 0.33 in wheat, 0.47 in rice, suggesting that *Brachiaria* hybrid had greater ability to absorb P from acidic low P soil than other crops. Amount of nitrogen absorbed by *Brachiaria* hybrid was less affected by P and pH treatments, whereas response of amount of N absorbed to P and pH treatments was almost similar to those of dry matter production and absorption of P by rice and wheat. It appeared that root activity of *Brachiaria* hybrid was less affected because of constant ability of nitrogen uptake. As expected, available P in soil was affected by P treatment and pH and therefore plant growth was affected by P availability. Root activity was severely depressed in wheat by low P and low soil pH and in rice by low pH.

Phosphorus use efficiency (PUE) was higher in *Brachiaria* hybrid than that of wheat and rice (Table 26). PUE of *Brachiaria* hybrid increased tremendously by P deficient treatment, however, that of rice and wheat was remained constant or slightly increased. In *Brachiaria* hybrid, P use efficiencies were remained constant, or decrease slightly with a decrease of soil pH. Thus *Brachiaria* hybrid seems to utilize absorbed P more efficiently compared to wheat and rice especially under low pH condition.

Fumarate was found as a major organic acid in leaves of *Brachiaria* hybrid regardless of P and pH levels followed by oxalate (Table 27). Oxalate concentrations in leaves of *Brachiaria* hybrid were decreased under P deficient condition. Fumarate was also a major organic acid in wheat leaves followed by malate. A trace amount of oxalate was detected in leaves of wheat. On the other hand, oxalate, a-ketogluterate, malate and

Table 25. Effect of phosphorus and pH treatments on total amount of phosphorus (mg P/plant) accumulated.

		-P			+P		
Plant	Organ	pH 4.0	pH 4.5	pH 5.0	pH 4.0	pH 4.5	pH 5.0
Brachiaria	Shoot	0.19±0.03	0.27±0.02	0.20±0.02	0.27±0.03	0.33±0.02	0.32±0.02
	Root	0.06±0.02	0.09±0.01	0.06±0.01	0.08±0.02	0.10±0.01	0.09±0.02
	Total	0.25±0.07c	0.36±0.03b	0.26±0.04c	0.35±0.04b	0.43±0.04a	0.41±0.04a
Wheat	Shoot	0.03±0.01	0.03±0.00	0.04±0.01	0.02±0.00	0.04±0.00	0.07±0.01
	Root	0.01±0.00	0.02±0.01	0.03±0.01	0.01±0.00	0.03±0.01	0.05±0.02
	Total	0.04±0.01d	0.05±0.01c	0.07±0.02b	0.03±0.01c	0.07±0.01b	0.12±0.03a
Rice	Shoot	2.14±0.25	2.11±0.24	2.44±0.28	2.39±0.27	2.25±0.26	2.81±0.32
	Root	0.41±0.09	0.41±0.09	0.45±0.10	0.40±0.09	0.47±0.11	0.49±0.11
	Total	2.55±0.12c	0.52±0.05c	2.89±0.13b	2.79±0.05bc	2.72±0.14bc	3.30±0.11[a]

Note:Values are the mean of three replicates. Treatment means are different (P<0.05) if denoted by different letters (Duncan's multiple range test).

Table 26. Phosphorus use efficiency*of whole plants grown in soil with phosphorus and pH treatments.

Plant	-P			+P		
	pH 4.0	pH 4.5	pH 5.0	pH 4.0	pH 4.5	pH 5.0
Brachiaria	0.79±0.04ab	0.88±0.05a	0.79±0.01ab	0.53±0.01c	0.52±0.01c	0.72±0.07b
Wheat	0.36±0.01b	0.49±0.01a	0.49±0.05a	0.30±0.02c	0.40±0.01	0.39±0.01b
Rice	0.48±0.01a	0.47±0.01a	0.48±0.01a	0.31±0.01b	0.29±0.01c	0.30±0.01bc

Note: *Phosphorus use efficiency = Total dry weight (g/plant)/Total amount of phosphorus uptake (mg/plant). Values are the means of three replications ±SE. Different letters in each species indicate statistical significance (P<0.05) by Duncan`s multiple range test .

Table 27. Organic acid concentration (m mol/g leaf dry weight) in leaves of plants grown in soil with phosphorus and pH treatments.

Plant	Treatments		organic acids					
	P	pH	Oxalate	Fumarate	a-ketoglutarate	Malate	Citrate	Total
Brachiaria	-P	4.0	12±0.1b	159±7.2b	n.d.	n.d.	n.d.	171±3.7
		4.5	9±0.1d	106±0.7d	n.d.	n.d.	n.d.	115±0.4
		5.0	10±0.3cd	121±0.9c	n.d.	n.d.	n.d.	131±1.1b
	+P	4.0	20±1.2a	156±2.1b	n.d.	n.d.	n.d.	176±1.6
		4.5	11±0.2bc	146±0.5b	n.d.	n.d.	n.d.	157±0.4
		5.0	11±0.1bc	248±2a	n.d.	n.d.	n.d.	259±1.4
Wheat	-P	4.0	2±0.0c	35±0.2e	n.d.	3±0.1e	n.d.	40±0.2
		4.5	3±0.1b	69±0.1c	n.d.	8±0.7ab	n.d.	80±0.3
		5.0	4±0.2a	109±2.1a	n.d.	8±0.3ab	n.d.	121±0.9
	+P	4.0	0.0±0.1d	28±0.9f	n.d.	5±0.9d	n.d.	34±0.6
		4.5	3±0.1b	48±0.2d	n.d.	9±0.2a	n.d.	60±0.2
		5.0	3±0.1b	94±0.7b	n.d.	6±0.3cd	n.d.	103±0.4
Rice	-P	4.5	39±2.9bc	n.d.	6±0.1c	24±5.3ab	7±1.3b	76±0.4
		5.0	42±1.5ab	n.d.	5±0.3c	28±5.6a	13±1.1c	88±1.1c
	+P	4.0	42±1.0cb	n.d.	5±1.3c	17±2.1bc	13±4.0c	77±1.6
		4.5	44±1.3a	n.d.	12±0.5b	11±0.4c	16±0.1a	83±0.4
		5.0	39±0.6bc	n.d.	4±0.1c	20±1.3abc	7±0.4b	70±1.4

Note : n.d. = not detected. Values are the means of three replications ±SE. Different letters in each organic acid in each crop species under various P and pH treatments differ significantly. Treatment means are different (P<0.05) if denoted by different letters (Duncan's multiple range test).

citrate were detected in leaves of rice plant. It appeared that phosphorus and pH treatments had less effect on amount of total organic acid anions in rice plants. Total organic acid level was higher in *Brachiaria* hybrid than in rice and wheat, indicating that in *Brachiaria* hybrid organic acid metabolism and its pool was active and large. PEPC maximum activity in leaves was extremely high in *Brachiaria* hybrid because of its C_4 photosynthetic pathway (Table 28). PEPC maximum activity was higher in –P treatment than in +P treatment, and did not respond to soil

Table 28. PEPC activity and malate inhibition ratio of PEPC in leaves of plants grown in soil with phosphorus and pH treatment.

Plant	Treatments		Malate inhibition ratio*	PEPC activity
	Phosphorus	pH		(μmol/mg protein/min)
Brachiaria	-P	4.0	0.33±0.02	8.46±0.09b
		4.5	0.24±0.06	9.15±0.15a
		5.0	0.58±0.01	9.51±0.23a
	+P	4.0	0.54±0.07	4.40±0.07c
		4.5	0.49±0.04	5.32±0.11d
		5.0	0.43±0.06	5.80±0.11c
Wheat	-P	4.0	0.59±0.02	0.13±0.01b
		4.5	0.61±0.04	0.12±0.02c
		5.0	0.65±0.02	0.15±0.01a
	+P	4.0	0.48±0.05	0.09±0.01d
		4.5	0.68±0.01	0.04±0.00f
		5.0	0.54±0.02	0.05±0.00e
Rice	-P	4.0	0.72±0.09	0.09±0.01d
		4.5	0.69±0.01	0.12±0.02b
		5.0	0.49±0.06	0.10±0.01c
	+P	4.0	0.76±0.01	0.09±0.01d
		4.5	0.69±0.06	0.12±0.02b
		5.0	0.49±0.06	0.10±0.01c

Note: Values are the means of three replications ±SE. Different letters in each species indicate statistically significant (P<0.05) by Duncan's test.
*Malate inhibition ratio = [(- malate PEPC activity)-(+ malate PEPC activity)] / [(- malate PEPC activity)].

pH. PEPC maximum activity of rice and wheat was slightly higher in -P treatment than in +P treatment. Malate inhibition ratio of PEPC in *Brachiaria* hybrid was lower in –P treatment than in +P treatment, especially at lower soil pH (Table 28). This ratio in wheat did not respond to P and pH treatment, and in rice did not respond to P treatment, but increased with decrease of soil pH.

Experiment 2. PEPC response to low phosphorus hydroponic medium. Phosphorus concentration in leaf (mg/g) and root of both *Brachiaria* hybrid and rice was extremely low with -P treatment (Table 29). Phosphorus concentration in *Brachiaria* hybrid leaf was 0.67 at –P treatment and 8.33 at +P treatment. Thus in nutrient culture, P treatment was extremely low

Table 29. Phosphorus concentrations (mg g^{-1} DW) in plants grown in hydroponics with different phosphorus treatment.

Plant	Phosphorus		Phosphorus concentration (mg/g DW)	
	Treatments	Leaf	Root	Whole plant
Brachiaria	-P	0.67±0.01b	1.09±0.06b	0.84±0.03b
	+P	8.33±0.12a	7.7±0.06a	8.20±0.08a
Rice	-P	0.37±0.01b	0.70±0.01b	0.72±0.00b
	+P	6.95±0.15a	6.34±0.09a	6.83±0.14[a]

Note : Values are the means of three replications ±SE. Different letters in each organ in each crop species under P treatments differ significantly. Treatment means are different (P<0.05) if denoted by different letters (Duncan's multiple range test).

at –P treatment and high at +P treatment, comparing with soil culture experiment. In *Brachiaria* hybrid, oxalate and fumarate were dominant organic acids, and these two decreased by –P treatment (Table 30). In rice, oxalate, a-ketogluterate, malate, and citrate were main organic acids. However, P response was different between oxalate, a-ketogluterate (decreasing by -P treatment) and malate, citrate (increasing by –P treatment).

By -P treatment, PEPC activity of *Brachiaria* hybrid decreased in leaf, and increased in roots (Table 31). Also by –P treatment, PEPC activity of rice remained almost constant in both leaf and roots. Malate inhibition ratio of PEPC in leaf decreased in *Brachiaria* hybrid, but remained constant in rice under –P treatment (Table 31). This ratio was higher in rice than in *Brachiaria* hybrid, indicating that PEPC of rice was mostly inactive.

Brachiaria species are adapted to low-fertility acid soil of the tropics because they are highly tolerant to high Al and low P and Ca. In P deficient condition, they may improve P acquisition by enhancing its root growth, uptake efficiency and ability to utilize poorly available P to plants. It was expected that *Brachiaria* species adapted to low P medium by two strategies; 1) efficient P-uptake, and 2) efficient P utilization in tissue. From acidic low P soil, *Brachiaria* hybrid had relatively high ability to absorb P from soil compared to rice and wheat. This was owing to high root activity in *Brachiaria* than the other two crops. It appeared that in *Brachiaria* hybrid under low P conditions (both soil and hydroponics culture), 1) PEPC activity increased in both leaves and roots, 2) malate inhibition ratio in leaves decreased (results not shown), and 3) organic acid levels decreased. Therefore, it is highly probable that high P uptake depends on high PEPC activity and high rate in organic acid

Table 30. Concentrations of (mmol/g DW) of organic acids in leaves of plants grown in hydroponics with different phosphorus treatments.

Plant	Treatment	---------- organic acids ----------					
	P treatment	Oxalate	Fumarate	a-ketoglutarate	Malate	Citrate	Total
Brachiaria	-P	10.5±1.1b	90.4±8.0b	n.d.	n.d.	n.d.	100.9
	+P	27.4±5.6a	134.0±16.3a	n.d.	n.d.	n.d.	161.4
Rice	-P	14.1±0.30b	n.d.	9.5.±1.20b	15.3±0.10a	3.98±0.40a	42.9
	+P	69.6±6.80a	n.d.	18.7±1.20a	3.97±0.9b	2.94±0.80b	95.2

Note : n.d. : not detected. Values are the means of three replications ±SE. Different letters in each organic acid in each crop species under P treatments differ significantly. Treatment means are different (P<0.05) if denoted by different letters (Duncan's multiple range test).

Table 31. PEPC activity (m mol/min/mg protein) and malate inhibition ratio in leaves of plants grown hydroponical in different phosphorus treatment.

Plant	P treatment	Malate inhibition ratio* in leaves	PEPC activity	
			Leaves	Roots
Brachiaria hybrid	-P	0.49±0.03	2.01±0.20b	0.31±0.02a
	+P	0.62±0.01	3.11±0.27[a]	0.18±0.02b
Rice	-P	0.84±0.02	0.06±0.01[a]	0.21±0.02a
	+P	0.89±0.01	0.05±0.00b	0.18±0.01b

Note: Values are the means of three replications ±SE. Different letters in each organ in each crop species under P treatments differ significantly. Treatment means are different (P<0.05) if denoted by different letters (Duncan`s multiple range test).
*Malate inhibition ratio = [(- malate PEPC activity)-(+ malate PEPC activity)] / [(- malate PEPC activity)].

metabolism resulting in exudation of organic acids from roots to solubilize relatively less available P in soil.

On the other hand, P use efficiency (PUE) of *Brachiaria* hybrid was extremely higher in -P treatment than +P treatment, comparing with wheat and rice. The PUE of *Brachiaria* hybrid was significantly augmented in response to P-deficiency, which was maximum (0.88) at pH 4.5.

Therefore, *Brachiaria* hybrid has an excellent P utilization mechanism in tissue once P is absorbed. A high P use efficiency is advantageous in low P acid soils, because the plant can then maximize the amount of biomass produced per unit P and thus dominate use of the resources available.

To explain one of the mechanisms of high P use efficiency, it has been suggested that P deficiency induces some glycolytic enzymes. Phospho*enol*pyruvate carboxylase (PEPC) and phospho*enol*pyruvate phosphatase (PEPP) that catalyze bypass reaction of pyruvate kinase (PK), responsible for the regulation of carbon flow from glycolysis to TCA cycle, are induced under P deficiency. This induction is supposed to play an important role in organic acid metabolism and Pi recycling under P deficient condition. In P deficient bean leaves, the increased rate of malate synthesis and enhanced accumulation of aspartate and alanine, the products of PEP metabolism, were observed. It was suggested that the increased activity of PEPC and the utilization of PEP to amino acids synthesis might be the most important response for phosphate recycling in bean leaves at the early stage of P deficiency.

Similar increases in PEPC activity in response to P-deficiency have been noted in the cluster roots of white lupin, in chickpea (*Cicer arietinum*), oilseed rape, and *Sesbania rostrata*. Also, enhanced expression and activity PEPC has been linked with P-deficiency-induced biosynthesis and root exudation of carboxylic acids.

Under P-deficient conditions, the PEPC reaction, which liberates oxaloacetate and Pi, may have a function for Pi recycling in PEP catabolism as a bypass for the ADP- and Pi-depended pyruvate kinase. In general, however, C_4-PEPC in C_4 cycle should not contribute to Pi recycling and large amounts of organic acid production because in C_4 cycle substrates are recycled. In the present experiment, PEPC activity in leaves of *Brachiaria* hybrid, a C_4 plant, was increased up to 3 folds in response to P-deficiency.

It appeared that higher PEPC activity might be related to higher PUE in *Brachiaria* hybrid. In current study, we could not estimate whether C_4- or C_3-PEPC of *Brachiaria* hybrid had a function in responding to P deficiency, suggesting the need for further research to define the precise role of PEPC of *Brachiaria* in adaptation to low P acid soils.

In summary, in *Brachiaria* hybrid under acidic low P soil: 1) P uptake remained high because of high relative P absorption ability and high root activity (estimated from high nitrogen absorption rate), and 2) PUE was significantly high which was appeared to be associated with higher PEPC activity and lower malate inhibition ratio in leaves. Thus, PEPC activity contributed greatly to P uptake/and PUE of *Brachiaria* hybrid and contributed less to rice and wheat (low P use efficient crops) under low P and pH conditions. Consequently, total organic acid level in *Brachiaria* leaf was lower in -P treatment than in +P treatment indicating that organic acids were metabolized actively by increased PEPC activity and decreased malate inhibition ratio in leaves.

Taken together, these results suggest that PEPC activated in *Brachiaria* hybrid under low P supply and low pH conditions could contribute to its greater adaptation to tropical acid soils with low P availability.

3.1.2 Development of a greenhouse method to screen *Brachiaria* genotypes for aluminum resistance and root vigor

Contributors: P. Wenzl (DArT P/L, Australia), A. Arango, A. Chaves, M. Buitrago, G. Patiño, J. Miles and I. M. Rao (CIAT)

Rationale

Some *Brachiaria* grasses such as *B. decumbens* cv. Basilisk are well-adapted to the soils of Neotropical savannas. *Brachiaria ruziziensis* pastures, by contrast, tend to degrade within a few years after establishment. 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). Edaphic adaptation presumably is an aggregate trait expressed in mature plants that comprises physiological components conferring adaptation to these stress components.

We are seeking to develop apomictic 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*, which lacks both agronomic traits. Efficient screening methodologies are required to recover the desired traits through stepwise accumulation of favorable alleles in subsequent cycles of recombination and selection. Our aim was to develop a greenhouse-based method to assess edaphic adaptation of large segregating populations.

The main objective of this study was to establish and validate a high-throughput 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. The vigor of root growth under nutrient deprivation is likely to influence a plant's adaptation to a range of nutrient deficiencies because nutrient acquisition relies on soil foraging, particularly in the case of immobile nutrients such as P. Aluminum toxicity was incorporated because previous experiments had confirmed that *Brachiaria* genotypes differ for this trait.

We initially tested the procedure by monitoring, during up to three weeks, the growth of adventitious roots of stem cuttings from the three parental genotypes (*B. decumbens* cv. Basilisk, *B. brizantha* cv. Marandu, and *B. ruziziensis* 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*.

Materials and Methods

The three main parents of the *Brachiaria* breeding program (*B. decumbens* cv. Basilisk, *B. brizantha* cv. Marandú, tetraploid *B. ruziziensis* clone 44-02) and a group of 38 *B. ruziziensis* × *B. decumbens* hybrids were propagated in a 40 kg of a 1:3 mixture of sand and soil from an experimental station in Santander de Quilichao (990 m above see level; Oxisol – Plinthic Kandiudox; Cauca Department, Colombia). The sand-soil mixture was fertilized with (milligram of element per kilogram of soil): 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 ($Na_2MoO_4 \cdot 2\ H_2O$). Soil chemical characteristics before mixing with sand and application of fertilizer 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.

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 two centimeters 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 23-25 days, 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 sand-soil mixture that had received a full fertilization. This re-potting cycle was repeated four times in the course of this study.

All growth experiments with nutrient solutions were performed in the greenhouse at CIAT headquarters (3° 30' N, 76° 21' W; 965 m above see level). Typical conditions in the greenhouse were: 19 to 36°C; 48 to 96 % relative humidity and 1,100 μmol m^2 s^{-1} maximum photon-flux density during the day.

The effectiveness of two treatments designed to evaluate adaptation to infertile, acid soils was initially tested using the three parental genotypes. The bases of the stem cuttings produced from tillers of potted plants were inserted into 1.5 cm-thick polyurethane foam discs (diameter: 4 cm) and transplanted to racks floating on a large volume of aerated, low-ionic-strength nutrient solution (Photo 17). This solution, known to support close-to-maximum growth of *Brachiaria* seedlings, contained (in µM): 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.

After nine days, twelve pairs of rooted stem cuttings from each clone were selected for within-pair homogeneity (Photo 17). One propagule of each pair was transferred to solution 1 (200 ìM $CaCl_2$, pH 4.20), the other to solution 2 (200 ìM $CaCl_2$, 200 ìM $AlCl_3$, pH 4.2). The pH of both solutions was adjusted by adding calculated quantities of HCl (64.9 ìM for solution 1; 39.2 ìM for solution 2) and measured with a pH electrode designed for low-ionic-strength solutions. The two groups of 36 stem cuttings (twelve per parent) were grown in two plastic trays wrapped in black polyethylene bags, which held 20 liters of the two solutions (Photo 17). The solutions were continuously aerated and renewed every second day to minimize pH changes (typically, the pH increased by up to 0.15 units when roots were bigger). Root growth was monitored by measuring the length of the longest root every third day, for up to 21 days. The whole experiment was performed twice.

Brachiaria ruziziensis 44-02, *B. decumbens* cv. Basilisk and the 38 *B. ruziziensis* × *B. decumbens* hybrids were included in ten 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 section. At harvest, after 21 days of growth, roots were separated from aerial parts. The roots were stained for 24 hours 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 (Photo 17). The images were analyzed with WinRHIZO software (Régent Instruments Inc., Québec, Canada) to measure total root length (RL) and the average root diameter (RD) for each individual root system. The aerial parts were dried at 60°C for 48 hours and their dry weights record.

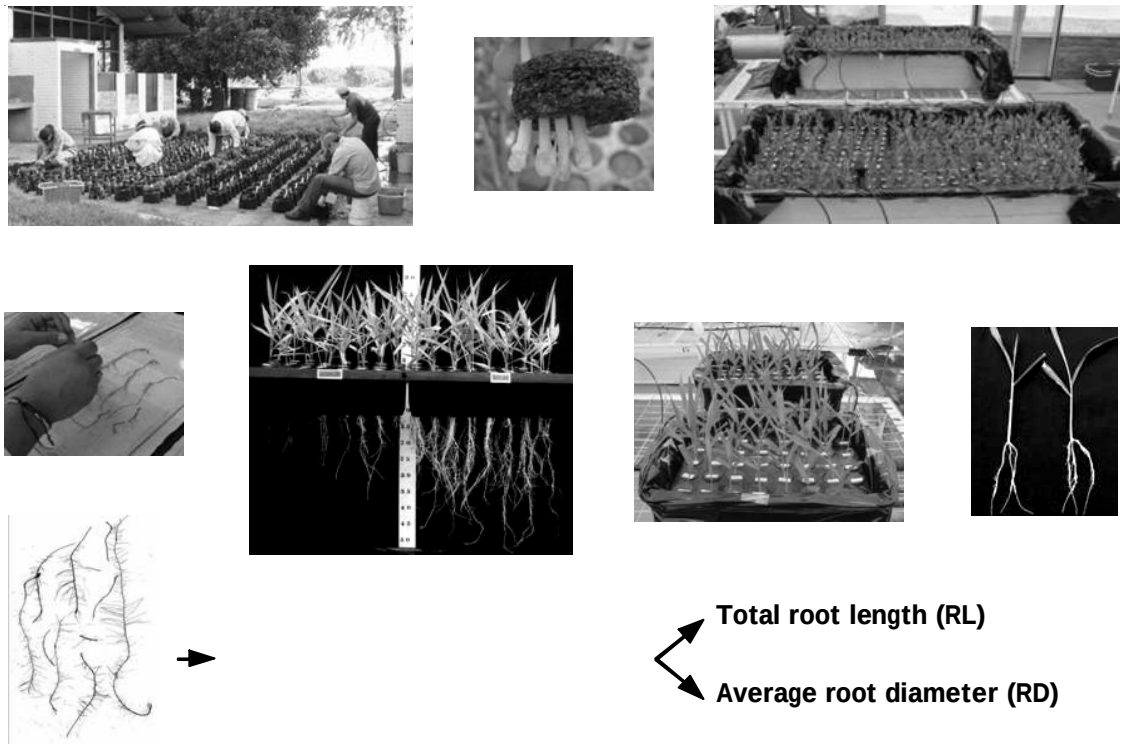

Photo 17. Procedure to identify acid soil-adapted *Brachiaria* genotypes. Plants were propagated in a mixture soil and sand (3:1). Vegetative propagules (stem cuttings), excised from these plants, were floated at the surface of a low-ionic-strength nutrient solution to produce adventitious roots. After nine days, pairs of rooted stem cuttings were selected for homogeneity. One propagule of each pair was transferred to solution 1 (200 ìM $CaCl_2$, pH 4.20), the other to solution 2 (200 ìM $CaCl_2$, 200 ìM $AlCl_3$ 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 (RD). 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.

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 days). The apices were stained with hematoxylin. Zones of Al-induced damage were visualized by fixing apices in a 1:1 mixture of 3.7 % phormol (pH 7.4) and glutaraldehyde, and cutting 70-µm thick longitudinal sections.

The pooled RL and RD data from the ten experiments designed to test the refined screening procedure were log-transformed nd adjusted, by linear regression, for harvest mean and the dry weight of stem cuttings. This procedure was designed to remove the variance components caused by 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 (or RD) 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 virtually uncorrelated ($r^2 = 0.02$).

Results and Discussion

The commercial *Brachiaria* grass cultivars are widely propagated by stem cuttings, a feature that enables breeders to generate genetically identical clonal propagules for evaluation of phenotypic characters. When the basal node of stem cuttings was incubated in a low-ionic-strength nutrient solution for nine days, all tested *Brachiaria* genotypes typically produced two to four adventitious roots (Photo 17). 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 ìM $CaCl_2$, pH 4.20). Solution 2 was identical to solution 1, but also contained 200 ìM $AlCl_3$. 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 towards the end of the experiment. Roots of *B. decumbens* and *B. brizantha* continued to elongate in solution 1 for the entire period of evaluation (three weeks). Those of *B. ruziziensis*, by contrast, ceased to elongate after approximately one week and were considerably shorter (Figure 27, 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 (Figure 27, right panel).

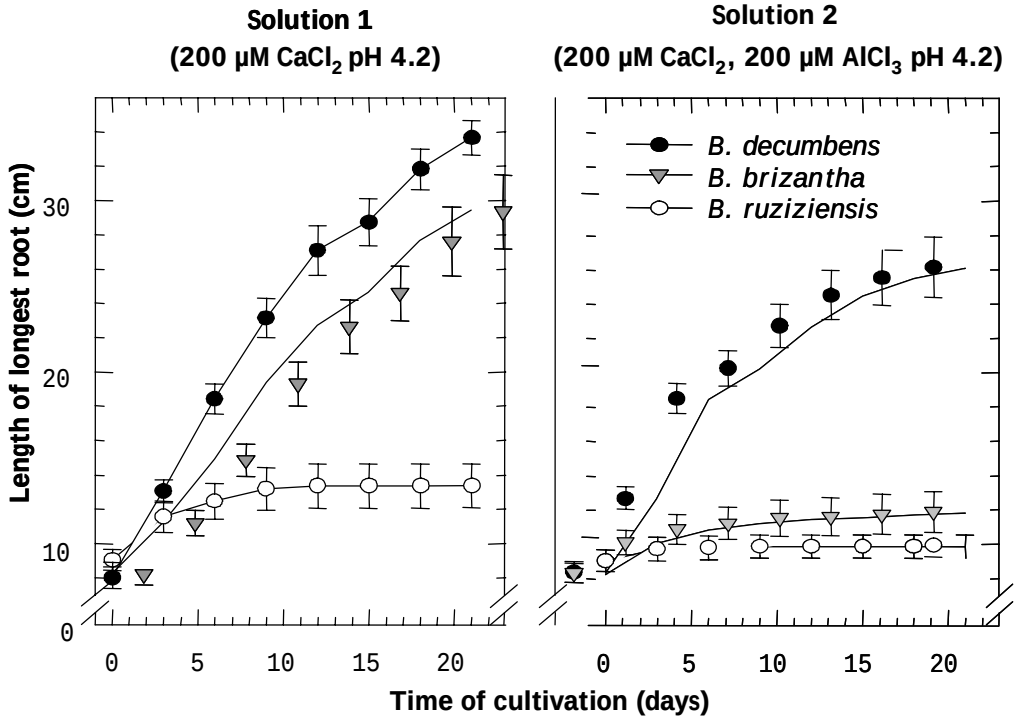

Figure 27. Initial test of the two treatments (without and with Al) using the three parental genotypes of the *Brachiaria* breeding program. The length of the longest root was recorded for up to 21 days of growth in the basal treatment (left panel) and the Al treatment (right panel).

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. 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*.

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) and average root diameter (RD).

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 ìM $CaCl_2$. Not surprisingly, roots of *Brachiaria* grasses in neotropical savannas represent a carbon sink of world-wide significance. The RL values showed a considerable degree of positive transgressive segregation, perhaps as a result of hybrid vigor (Figure 28, 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. The performance of individual genotypes was consistent across harvests, as can be deduced from the mean standard error (0.034 log units) compared to the range of RL values measured for the group of 38 hybrids (0.933 log units) (Figure 28, 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 mature plants, such as the frequency of initiation of adventitious roots, the tendency of nutrient-deprived plants to allocate carbon to roots rather than shoots, and the efficiency with which nutrient reserves in stem cuttings are remobilized to sustain root growth. Any of these components is likely to influence a plant's adaptation to infertile and acid soils.

Aluminum resistance was less straightforward to measure because the inherent differences in root vigor had to be taken into account to accurately quantify the effect of Al toxicity on root development for different genotypes. A similar problem has been encountered previously with rye seedlings, 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 Figure 28 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 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. decumbens* was significantly superior to Al resistance of *B. ruziziensis*. In contrast to root vigor, the two parents were close to the two extremes of the Al-resistance distribution (Figure 28, right panel). Though based on a limited number of segregants, this segregation pattern would be consistent with multiple genes contributing to Al resistance, a similar situation as in species such as rice and probably maize. The absence of positive

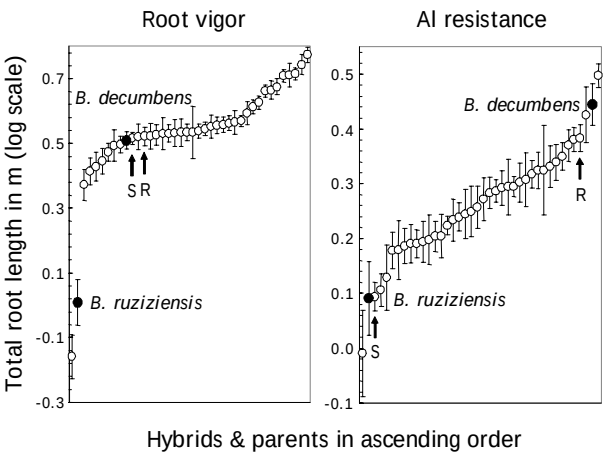

Figure 28. Segregation of root vigor (left panel) and Al resistance (right panel) in a group of 38 *B. ruziziensis* × *B. decumbens* F1 hybrids. Root vigor is the total root length (RL) in solution 1 (200 ìM $CaCl_2$, pH 4.20), adjusted for the effects of stem-cutting biomass and harvest. Aluminum resistance is the stem-cutting-biomass and harvest-adjusted RL in solution 2 (200 ìM $CaCl_2$, 200 ìM $AlCl_3$ 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 (Photo 18) are designated as "R" for Al-resistant and "S" for Al-sensitive.

transgressive segregation suggests that *B. decumbens* contains most of the alleles that contribute importantly to Al resistance.

Aluminum toxicity not only inhibits root elongation, but also induces lateral swelling of roots. Aluminum-sensitive genotypes, therefore, should not only be characterized by a decrease in RL, but also an increase in RD. We found that the RL-based Al-resistance index was indeed negatively correlated with a similar index based on RD ($r^2 = -0.75$). 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 Figure 28). In agreement with our classification, root apices of Al-sensitive genotypes stained strongly; while those from Al-resistant genotypes remained clear (Photo 18).

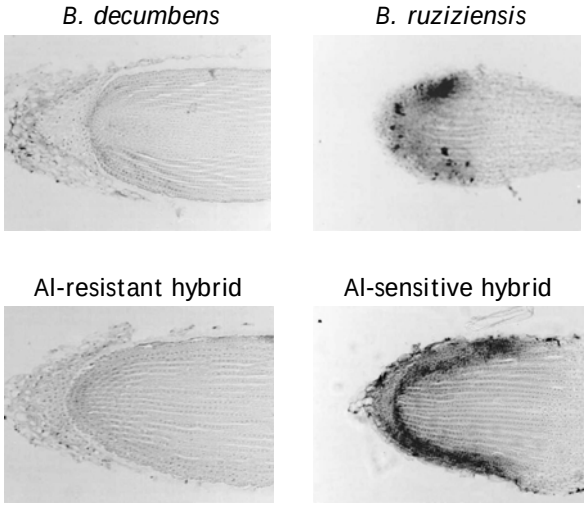

Photo 18. Hematoxylin-staining patterns for root apices of genotypes identified by the screen as Al-resistant (left side) and Al-sensitive (right side). The two *B. ruziziensis* × *B. decumbens* hybrids used for this test are also highlighted in Figure 28.

From the combined results of this study we conclude that our screening procedure quantifies component traits expressed in mature plants which are likely to influence the adaptation of *Brachiaria* genotypes to infertile, acid soils (root vigor, Al resistance). This conclusion was further verified by testing *Brachiaria* hybrid "Mulato", a recently released cultivar. The glasshouse screen revealed good root growth and intermediate Al resistance, consistent with its good vigor and responsiveness to applied nutrients on acid soils.

We characterized root growth and root-system morphology in detail to test the effectiveness of the two experimental treatments (solution1 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. In order 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 separately assess 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 (Photo 18, right panel).

The latter approach may exclude potentially useful segregants for component traits. However, this approach has significantly increased the efficiency of the *Brachiaria* breeding program at CIAT by enabling breeders to quickly discard a large number of non-adapted genotypes. In a more recent breeding cycle, 745 sexual segregants were screened for both edaphic adaptation and spittlebug resistance in the course of six months, 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.

Root vigor of mature plants does not appear to be a widely-used selection criterion in breeding programs targeting edaphic adaptation. Root vigor of seedlings has received some breeding attention yet is unlikely to bear much relevance for edaphic adaptation of *Brachiaria*, which is manifest in the persistence of pastures over 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.

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. Although seedling-based assays have been successfully applied to *Brachiaria* grasses, poor germination of *Brachiaria* seeds at the surface of nutrient solutions and the poor viability of hydroponically-grown seedlings upon 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.

In summary, we developed and validated a hydroponic screening procedure that uses vegetative propagules (stem cuttings) of *Brachiaria h*ybrids to rapidly evaluate root vigor and aluminum resistance, two key components of edaphic adaptation. A simplified version of this procedure is being used to select for aluminum resistant apomictic and sexual hybrids of *Brachiaria*. Adaptation to infertile, acid soils is almost certainly a trait under complex genetic control. Ultimately, well-adapted genotypes will be confirmed only when plants are subjected to the stress syndrome *in situ*. However, the *Brachiaria* breeding progress achieved during the last four breeding cycles, indicates that the screening procedure outlined enables breeders to recover some key genetic components of acid-soil adaptation.

3.1.3 Mapping of QTLs associated with aluminum resistance in *Brachiaria* species

Contributors: C. Rosero, G. Gallego, J. Vargas, C. Quintero, M.C. Duque, M.E. Buitrago, P. Wenzl, I.M. Rao, J.W. Miles and J. Tohme (CIAT)

Rationale

Acid soils have been estimated to occur on about 40% of the arable land (3.95 billions of ha). Plant growth on these soils is constrained mainly by aluminum (Al) toxicity and deficiencies of nutrients such as phosphorus (P), nitrogen (N), and calcium (Ca). There is considerable variation

within and between plant species in their ability to resist Al, and this variation within some species has allowed breeders to develop genotypes that are able to grow on acid soils. Within the *Brachiaria* genus, Al resistance of signalgrass (*B. decumbens* Stapf cv Basilisk), a widely sown tropical forage grass, is outstanding compared with the closely related ruzigrass (*B. ruziziensis* Germain and Evrard cv Common). Our main objective was to identify specific genes that are related with high level of Al resistance in signalgrass. Our research approach was to combine traditional genetic improvement tools with those that are available on structural and functional genomics. For this purpose, a molecular genetic map has been developed, using a F1 population (previously charaterized under Al stress conditions) formed by 180 individuals from *Brachiaria ruziziensis* x *Brachiaria decumbens* cross. This molecular map was built by means of 169 AFLPs and SSR markers that were distributed in 19 linkage groups. The map was the base for QTLs analysis procedures, establishing a relation between phenotypic data with molecular data.

Materials and Methods

An F1 hybrid population (263 plants) from the interspecífic cross between *B. decumbens (*CIAT 606, Al resistant) and *B. ruziziensis* (BRUZ 44-02, susceptible), was used for this study. SSRs primers developed from *B. decumbens* and AFLPs primer combinations (EcoRI/MseI), previously tested in the parents and determined as polymorphic were evaluated in the population. Additionally, SSRs primers from rice were evaluated and few SCARs markers that were developed from AFLPs alleles were tested in this population. Polymorphic markers of each parental were subset, and a X^2 analysis was made (global α=0.05%) for each group tor test the goodness of fit to 1:1 and 3:1 or 5:1 ratios, for single dosage markers (SDM) and double dosage markers (DDM). A linkage map was constructed (only with SDMs) with the Mac computer program MapMaker/EXP v3.0b (LOD min=12.0 and r= 0.3), calculating the distances (in cM) by Kosambi's mapping function. Five quantitative variables (root length, root diameter, abundance of root tips, root length/shoot biomass and specific root length) that are indicative of Al resistance were employed for QTLs analysis. The aluminum effect (eal) over each genotype of the population was calculated by doing a LS mean over each data variable. Genotypes without replications were excluded of the posterior phase of the analysis, as well as the data with total root length of less than 90 cm, because of its great response variability to Al stress conditions. QTLs analysis was preformatted in WinQTLCartographer v2.5 program (9). Three kind of analysis was made: Single point analysis (SPA), Interval mapping (IM) and Compositive interval mapping (CIM). The threshold to report a QTLs (LOD) was calculated by permutations methodology with 1 000 permutations ($\propto$= 0.05).

Results and Discussion

From 73 SSRs primers sets of *Brachiaria*, only 36 were tested in the population due to their polymorphism, these primers amplified 66 alleles for Al resistant parent CIAT 606 and 33 for Al sensitive parent BRUZ 44-02. Because of amplification problems, we were not able to evaluate 27 SSRs. Five primers were monomorphic, and the other five were not yet standarizathed. Three SSRs primers from rice chromosome one, have been evaluated in the population producing five alleles for the parent 606, and it was also possible to score two SCAR markers (ScC6b2 and ScC6b2).

Twelve AFLP's primer combinations were evaluated for their high polymorphism, scoring 454 markers allelic loci due to their complex banding pattern. Of these, 229 bands were shared, the rest were polymorphic, but from these ones almost 3/4 are bands from the Al resistant parent 606 (179). This amount of molecular information sharing shows a strong relationship between the genomes of the two contrasting parents, 606 and 44-02.

A low percentage from AFLP's polymorphic alleles of each parent (14.5% for 606 and 15.2%

to 44-02) were not evaluated, because of lack of segregation in the population. This in fact depends on the marker dosage, being one of the biggest limitations of using polyploid species such as *Brachiaria*. The SSRs are more effective markers for SDMs detection. This separation is very important when working with polyploids species because the other configurations used could endanger the map precision, affecting the distance as well as the position of markers.

A linkage map with 19 linkage groups was constructed for the tetraploid species *B. decumben*s, the map has 180 SDMs from which 114 are AFLPs, 64 SSRs and 2 SCAR markers, covering 1362.9 cM (Figure 29). The 19 linkage groups obtained are closer to the haploid number of this specie (18 chromosomes). Perhaps, the small size of *B. decumbens* chromosomes explain the fact that with only 180 markers we achieve a linkage group number closer to the search, and point out to a considerable genome coverage in the present linkage map.

Five phenotypic and quantitative variables including root length (*rl*), root diameter (*rd*), number of root tips (*t*), root length to shoot dry weight (*rlsdw*) and specific root length (*srl*) that are indicative of Al resistance (*eal_rl, eal_rd, eal_t, eal_rlsdw, eal_srl*) were employed for QTLs analysis. These pheotypic variables showed transgresive segregation that could be due to polyploid condition thereby contributing to heterosis or hybrid vigor.

With the three QTLs analysis methods (SPA, IM and CIM) it was possible to establish putative associations between moleculars markers, finding six putative QTLs that are associated with Al resistance. Three were localizated in LG5, the others in LG3, LG4 and LG15. The associations found by the mapping analysis are compiled in the Table 32 (see also Figure 29).

The small percentage of variation explained for each QTL, could be due to complexity that involves the aluminium resistance trait, and may be the six putative QTLs found point out some genes of small efect that could interact and produce the observated phenotype. However, more saturation level and/or fine mapping is hended to make further progress. Finally, it is also necessary to confirm the putative QTLs identified so far. This is because the phenotipic evaluation was made only under hydroponic conditions.

Further Work is in Progress to; (i) saturate the linkage map of *B. decumbens* (CIAT 606) with SSRs of genetically related species and with some *Brachiairia* SSRs primers that are not yet standarizathed, and (ii) conduct QTLs analysis for Al resistance with additional phenotypic data.

Table 32. Mapping analysis results. LG, position of the first marker, and the flanking markers associated with aluminum-resistance variable are indicated. The most closer markers with correlation are indicated in bold and with underline.

Method	Variable	LG	Flanking Markers	QTL Position	Markers Position	LOD	Aditive	R2
IM	eal_rd	15	GM_24 - **C5b10**	36,68	21,7 - **36,6**	3,014	0,052	0,053
IM	eal_slr	3	**rGM_72a** - rRM_46b	112,16	**88,3** - 113,5	3,008	0,110	0,058
IM	eal_slr	5	**GM_11a** - rGM_11b	104,22	**104,1** - 110,4	2,297	-0,089	0,041
CIM	eal_lr	5	**GM_12a** - GM_11a	103,45	**103,3** - 104,1	3,260	-0,072	0,052
CIM	eal_lr	5	**GM_11a** - rGM_11b	108,22	**104,1** - 110,4	3,098	-0,072	0,053
CIM	eal_rd	5	**GM_11a** - rGM_11b	108,22	**104,1** - 110,4	2,993	0,051	0,050
CIM	eal_rd	15	GM_24 - **C5b10**	35,83	21,7 - **36,6**	3,317	0,052	0,054
CIM	eal_lrsdw	4	**C1b12** - rC10b8	52,34	**52,3** - 63,3	3,024	-0,081	0,050
CIM	eal_slr	3	**rGM_72a** - rRM_46b	112,16	**88,3** - 113,5	4,111	0,120	0,068
CIM	eal_slr	5	**rRM_151** - rRM_466a	0,01	**0,0** - 14,1	2,801	-0,091	0,043
CIM	eal_slr	5	**GM_11a** - rGM_11b	104,22	**104,1** - 110,4	3,010	-0,094	0,045

LG Linkage group, IM Interval mapping, CIM Compost interval mapping.

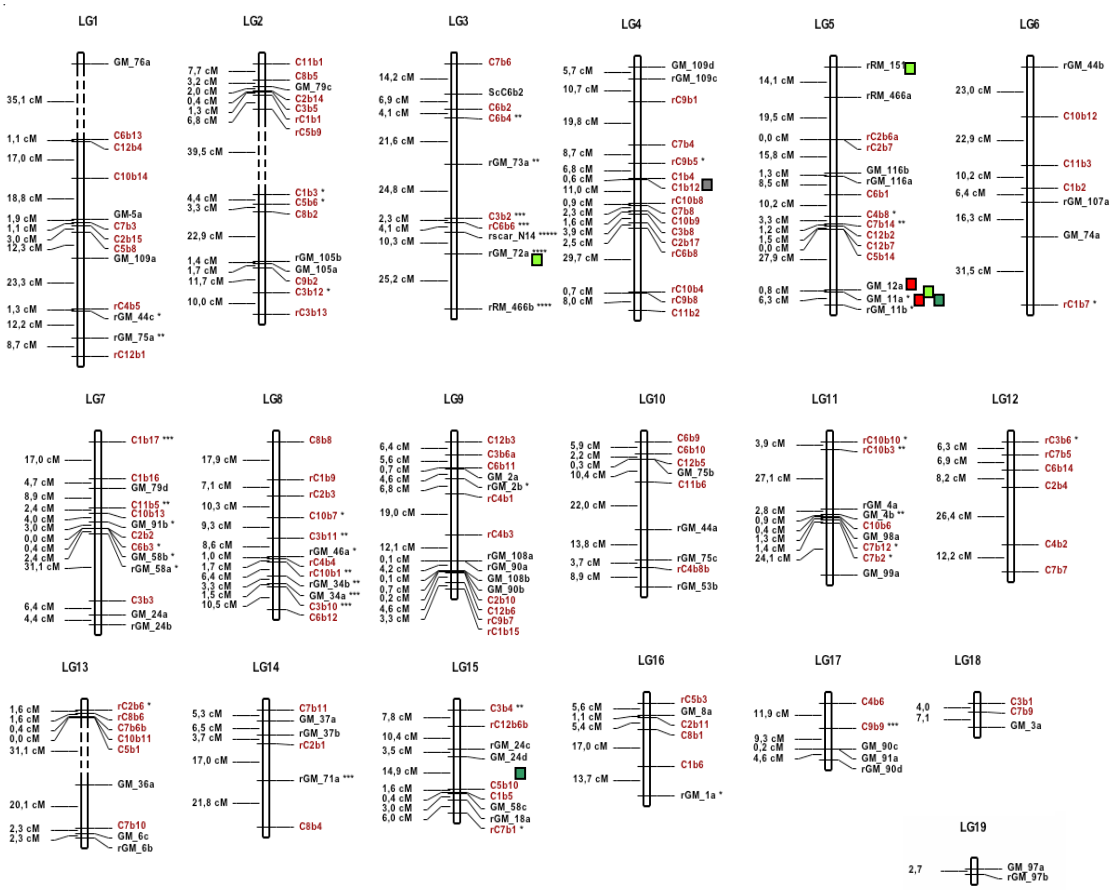

Figure 29. Genetic Linkage Map of *B. decumbens*, with localization of QTLs for Al resistance. SDMs of AFLPs, SSRs, and SCARs were used in this map. Distances between markers more than 30 cM, are shown with broken line. The boxes show the most closer marker associated to the region with the trait of interest: (■) eal_rl; (■); eal_rd, (■); eal_rlsdw and (■) eal_srl.

3.1.4 Isolation and characterization of candidate genes for Al tolerance in *Brachiaria*

Contributors: A. F. Salcedo, M. E. Recio, A. L. Chavez, J. Thome, I. M. Rao, and M. Ishitani (CIAT)

Rationale

Previous results demonstrated that there is pronounced difference in aluminium (Al) resistance between *B. decumbens* (resistant) and *B. ruzisiensis* (susceptible). The objective of this work is to identify candidate genes responsible for high level of Al resistance in *B. decumbens* using PCR-based technology. Identification of these candidate genes would enhance our understanding of molecular mechanisms of Al resistance, thereby contributing to genetic improvement of both forage and field crops for Al resistance.

Materials and Methods

Seeds of *B. decumbens* and *B. ruziziensis* were germinated in 200 M $CaCl_2$ (pH 4.2) for 4 - 5 days in the greenhouse. Homogeneous seedlings with root lengths between 4 and 5 cm were transferred to continuously aerated solutions, of 200 µM CaCl2 (pH 4.2) with and without $AlCl_3$. Seedlings were left to grow in the greenhouse. Root length was measured at 0 h, 3 h, 6 h, 24 h, and 72 h after Al treatment. Root tips (1 cm length) from *B. decumbens* and *B. ruzisiensis* were collected at 0 h, 3 h, 6 h, 24 h, and 72 h after Al treatment. Total RNA was isolated from the root tissue using Trizol® reagent by following manufacture's protocol (Invitrogen, USA). The

RNA samples from each time point were pooled to isolate poly(A) RNA using PolyATtract® mRNA Isolation Systems (Promega, USA). mRNAs (300 ηg each) were used for cDNAs synthesis using SMART cDNA synthesis kit (Takara-Clontech, USA). Differential expressed genes between *B. decumbens* and *B. ruzisiensis* were selected by PCR-Select cDNA subtraction kit (Takara-Clontech, USA). The cDNA fragments obtained from a forward subtractive library (cDNAs from *B. decumbens* and *B. ruziziensis* as tester and driver, respectively) were directly cloned into a T/A cloning vector pGEMT-easy (Promega, USA) in Escherichia coli DH5á.

In order to obtain candidates for differentially expressed genes in *B. decumbens* the subtracted library was screened using a PCR-Select Differential Screening Kit (Takara-Clontech, USA). Individual colonies that showed the presence of inserted DNA were picked, and grown in a 384 microplate with LB-ampicillin freezing solution, (yeast extract 5 g, tryptone 10 g. NaCl 10 g, $K_2HPO_4.3H_2O$ 63 g, KH_2PO_4 18 g, $MgSO_4$ 0.4 3g sodium citrate 4.4 g, $(NH_4)_2SO_4$ 9 g and 349 ml of glycerol autoclaved until 1L). The colony array was blotted onto nylon membranes (Amersham, USA) resting on LB-agar and then cultured overnight at 37 °C. Cloned DNA then was denatured, neutralized and affixed to the membranes by using UV (120 mJ).

Probes for hybridization were derived from the forward- and reverse-subtractive libraries as described in the PCR-Select Differential Screening Kit (Takara- Clontech, USA). Radio labeled probes with (á-^{32}P)-dATP were purified by spin filtration (Takara-Clontech, Chroma-spin 400 columns). Hybridization was carried out by using a Hibridizer HB-1(Techne). Membranes were pre-hybridized for 6 hours at 70 °C in a solution of 1% BSA, 1 mM EDTA, 7% SDS, and 0.25 M sodium phosphate. Denatured probes were added and were hybridized to the cloned DNA's overnight at 70 °C. After hybridization, membranes were washed two times for 20 minutes in a low-stringency washing buffer [2× standard saline citrate (SSC)/0.5% SDS] and one time for 20 minutes in a high-stringency washing buffer (0.2× SSC/0.5% SDS). Membranes were exposed to film (Kodak MXG-1) for 16-72 hours. Positive clones were sequenced and homology analysis was undertaken using the BLAST-X algorithm of GeneBank NCBI, (http://www.ncbi.nlm.nih.gov/) and Gramene (www.gramene.org) databases.

Results and Discussion

We tested root growth of two *Brachiaria* genotypes, *B. decumbens* and *B. ruziziensis* under Al stress conditions used in this work to understand phenotypic responses to Al toxicity of the genotypes. As shown in Figure 30, root growth of both genotypes was not significantly different under control conditions (-Al). Under Al treatment (+Al) conditions root growth was inhibited for both genotypes. However, the Al resistant genotype, *B. decumbens* had less inhibition of root elongation compared with the Al sensitive genotype, *B. ruziziensis*. The small difference in root elongation with Al treatment between the two genotypes appeared at 24 hours after treatment. Since the root phenotype was present at the measured time point, gene expression related to that phenotype must occur prior to the expression of the phenotype.

To identify genes involved for Al resistance in roots, differentially expressed genes were isolated using a Clontech PCR-Select cDNA subtraction kit followed by PCR-Select Differential Screening Kit. Photo 19 shows clones derived from this process. 35 clones were identified as differentially expressed genes and were sequenced. 20 of the 35 were found to have sequence homology in the GenBank and Gramene protein databases as shown in Figure 31 and Table 33.

Among 35 clones, 8 clones of differentially expressed genes had the same sequence which

(Continued)