Evaluation of Aluminum Sensitivity in Barrel Medic Germplasm

Yawadee Srimake

Math and Science, Leeward Community College, University of Hawaii System, 96-045 Ala'Ike, Pearl City, HI 96782

Susan C. Miyasaka¹

Department of Tropical Plant and Soil Sciences, University of Hawaii, Manoa, 875 Komohana Street, Hilo, HI 96720

ADDITIONAL INDEX WORDS. *Medicago truncatula*, screening method, agarose, hematoxylin, root growth

ABSTRACT. Aluminum (Al) toxicity in acid soils is a major constraint to global agricultural production, affecting ≈30% of the world's arable land area. To study Al tolerance in barrel medic (*Medicago truncatula*), we assessed responses to excess Al in 91 accessions collected from different geographic regions. Root elongations were used to characterize the sensitivity of each accession. Seedlings were grown in an agarose medium that contained three levels of Al (50, 100, and 200 μM), and root elongation was measured at 72 hours after exposure to Al. The ratio of root elongation in the presence and absence of Al [relative root growth (RRG)] differed among accessions. At 50 μM Al, we observed the greatest range of intraspecific variation. Aluminum sensitivity of 30 accessions was tested further by hematoxylin staining. Relative root growth was regressed linearly against the visual staining score, and a significant, negative, linear relationship was found between RRG at 50 or 100 μM Al and the intensity of staining scores. Twelve selected accessions differing in their resistance were grown in Al-toxic soil to confirm their Al response. Such information could be useful in breeding or selecting for improved Al tolerance in barrel medic, as well as other crop species.

Aluminum toxicity has been considered the most important limiting factor of plant growth on acid soils, which affects one-third of the world's arable land area. In acid soils, crop productivity is affected by multiple stresses, such as deficiencies of phosphorus (P), calcium (Ca), and magnesium (Mg), and toxicities of manganese (Mn), iron (Fe), and Al (von Uexkull and Mutert, 1995). Aluminum species that are present in the environment, such as Al oxides, gibbsite, Al silicates, and organic-Al complexes are nontoxic. However, under acidic conditions, Al^{3+} is solubilized into the soil solution where it rapidly inhibits root growth (Kinraide, 1991; Kochian, 1995; Miyasaka et al., 2006).

Although soil acidity problems can be overcome by fertilization and liming practices, it is often economically impractical to correct in many parts of the world. Aluminum tolerance has been reported in many plant species, and using Al-tolerant cultivars provides the most effective strategy for production of economically important crops in acid soils (Foy, 1988; Ishitani et al., 2004).

Barrel medic originated in the Mediterranean basin. It was widely naturalized to New South Wales, Australia, and became an important forage crop in the low-input farming system typical of Western Australia, alternating with other cover crop species (Crawford et al., 1989). In the north central United States, barrel medic was used as a short-season annual pasture legume to increase soil nitrogen (N) (Zhu et al., 1998). It has also been chosen as a model legume for genomic studies because of its small diploid genome, fast generation time, self-pollination, and high transformation efficiency (Cook, 1999).

Aluminum tolerance in the barrel medic germplasm has been reported based on a hydroponic screening method (Sledge et al., 2005), an Al-toxic soil assay, and a lumogallion root staining method (Narasimhamoorthy et al., 2007). Results from the hydroponic screening indicated that sufficient variation of Al tolerance exists within this collection of barrel medic accessions to select for Al-sensitive or Al-tolerant accessions (Chandran et al., 2008; Sledge et al., 2005). However, the reported Al sensitivity of particular accessions also varied, depending on the screening method (Narasimhamoorthy et al., 2007).

Little is known about physiological mechanisms that are responsible for Al tolerance in *M. truncatula*. It is important to remember that each evaluation method could result in difference stresses, and genetic determinants underlying tolerance of each stress (e.g., Al toxicity or proton toxicity) may differ (Khu et al., 2012).

A consistent, quick evaluation method is key to selecting or breeding an Al-tolerant cultivar (Samac and Tesfaye, 2003). Agar and agarose gels have been used often in rhizosphere studies. In particular, agarose is a suitable substrate for studies of Al interaction in the rhizosphere, because it contains very low levels of P and other Al-complexing substances that could interfere with plant responses to Al toxicity (Calba et al., 1996). Agarose culture has an advantage over the hydroponic system, because solution culture can result in hypoxia particularly for plants that are sensitive to low oxygen conditions (Tamas et al., 2006). In addition, solution culture could remove border cells and mucilages that possibly protect root tips from Al toxicity (Miyasaka and Hawes, 2001).

Relative root growth, which is a measure of root elongation inhibition, can be used as an indicator for response to Al toxicity. This parameter has been shown to be suitable for estimating Al tolerance of accessions of various plants such as *Arabidopsis thaliana* (Kobayashi et al., 2005)],

Received for publication 8 Oct. 2015. Accepted for publication 26 Feb. 2016. We thank Douglas R. Cook and Varma Penmetsa (University of California, Davis) for their generous help in providing laboratory space and research guidance.

¹Corresponding author. E-mail: miyasaka@hawaii.edu.

maize [*Zea mays* (Llugany et al., 1995)], rye [*Secale cereal* (Hede et al., 2002)], bermudagrass [*Cynodon dactylon* (Liu, 2005)], and wheat [*Triticum aestivum* (Tang et al., 2002)].

Hematoxylin is a natural dye extracted from the heartwood of a logwood tree (*Hematoxylin campechianum*). It has been reported as a sensitive method for the evaluation of Al accumulation in a number of plants, because it has the property of turning blue when it forms a complex with Al (Delhaize et al., 1993). The staining pattern of Al accumulation in roots was similar to lipid peroxidation and callose production, which are other symptoms of Al toxicity in pea (*Pisum sativum*) roots (Yamamoto et al., 2001). Both the hematoxylin method and RRG have been used for Al-tolerance screening in many crop plants (Poschenrieder et al., 2008), such as chickpea [*Cicer arietinum* (Singh and Raje, 2011)], barley [*Hordeum vulgare* (Echart et al., 2002)], and wheat (Tang et al., 2002; Zhou et al., 2007).

Soil-based screening is considered the most realistic method of evaluating plants for Al tolerance; however, soil assays are time consuming and only a few ecotypes or genotypes can be evaluated using this method. Short-term screening systems such as hydroponics or sand cultures have been developed that correlated well to soil-based assays for certain crop species (Brauer and Staley, 2005; Hossain et al., 2005; Villagarcia et al., 2001; Voigt and Staley, 2004).

The objectives of this study were to 1) identify barrel medic accessions that differed in response to Al toxicity and 2) determine the best method(s) of screening barrel medic for Al tolerance. The identification of Al-tolerant accessions in this model legume can be used in the future to determine molecular mechanisms underlying Al responses, with the overall goal of improving Al tolerance in barrel medic and other crop species.

Materials and Methods

PLANT MATERIALS. Seeds of 91 accessions and cultivars were obtained from the U.S. Department of Agriculture Germplasm Resources Information Network (GRIN) and the Institut National de la Recherche Agronomique (INRA). Accessions from GRIN are identified as starting with “PI” or “W.” Most of these accessions (47) from INRA had not been characterized previously for Al tolerance.

SEED GERMINATION. Seed coats were individually nicked on the opposite side of the root radical. Five volumes of 10% commercial grade bleach ($\approx 5\%$ NaClO) were added. Seeds were immersed in bleach for 2 min, rinsed with sterile water five times, soaked in sterile water overnight, then transferred to the top surface of two sheets of moistened filter paper (Whatman No. 42; GE Healthcare Life Sciences, Marlborough, MA) and placed either in a petri dish (100 mm diameter, polystyrene; Thermo Fisher Scientific, Waltham, MA), or one side of 50-mL test tubes. Petri dishes or test tubes were sealed with parafilm (Bemis Co., Oshkosh, WI) and inverted so that seeds were on the top side of horizontally placed containers, allowing roots to grow downward into moist air.

They were placed in the dark at 4 °C for 36–48 h to synchronize germination. Following this cold treatment, seeds were incubated at room temperature (25 °C) in the dark for 16–24 h. Seedlings with roots that were 1–1.5 cm in length were used in subsequent experiments.

SCREENING METHODS: INHIBITION OF ROOT ELONGATION. Six seedlings of each accession were grown on agarose gel (1% agarose and 0.5 mM CaCl₂) that contained varying Al levels

(0, 50, 100, and 200 μM of AlCl₃ at pH 4.5). These Al and pH levels were selected based on results from a preliminary study on genotype A17 (Srimake, 2012).

Agarose medium was autoclaved, then filter-sterilized AlCl₃ stock solution, 0.5 mM CaCl₂, and either 0.5 HCl or 1 M NaOH were added to achieve the desired Al levels and final pH. Thirty milliliters of medium was poured into a 100- × 100- × 15-mm² disposable petri dish with grid (Fisherbrand; Thermo Fisher Scientific). A 9- × 6-cm filter paper (Whatman No. 42) was placed on top of roots, and 1 mL of the same treatment solution was poured over the filter paper in each plate. Initial length of each root was marked.

The plates were placed at a 60° angle at room temperature (25 °C) under fluorescence light at 16/8 h (light/dark). Root elongation of seedlings was measured at 72 h after transfer and RRG (%) was calculated as the root length at particular Al levels divided by root length at the control level (0 Al) × 100. The experiments were repeated three times. One-way analysis of variance was conducted on RRG [PROC GLM (SAS version 9.1 for Windows; SAS Institute, Cary, NC)]. A probability value of or below 0.05 ($P \leq 0.05$) was considered to be statistically significant. Means between accessions were compared using Tukey’s range test.

To categorize Al-sensitive, intermediate, or Al-tolerant accessions or cultivars, the method of Sledge et al. (2005) was modified as follows. Student’s *t* tests (PROC TTEST, SAS version 9.1 for Windows) were used to compare differences between mean root lengths of control seedlings with those of seedlings grown at various Al concentrations for each accession or cultivar. A probability value of ≤ 0.05 in all levels of Al was considered to be Al sensitive; one with a probability value of ≥ 0.50 in all levels of Al was considered to be Al tolerant; and ones with intermediate probability values were considered to be intermediate in Al response. In addition, accessions or cultivars with RRG above 100% at 50 μM Al were considered Al tolerant.

SCREENING METHODS: ACCUMULATION OF AL. Aluminum was detected with hematoxylin using the method of Tamas et al. (2005). Briefly, seedlings of 30 accessions were grown for 72 or 120 h in various Al-containing agarose medium. Treated roots were washed in distilled water for 15 min and stained with a 0.2% hematoxylin and 0.02% potassium iodide (KIO₃) solution for 10 min at room temperature. After washing the entire root system with distilled water for 15 min, root tips (0.5 cm in length) were excised and washed with deionized water for an additional 15 min.

Root tips (5 mm) were observed under a stereomicroscope (model SZX16; Olympus, Tokyo, Japan) and digital images were captured. To score the intensity of staining, we selected the darkest colored root, determined its color balance as RGB 40/14/56 using Photoshop (version CS; Adobe Systems, San Jose, CA) and set this intensity to a value of 5 in a scale of 0 to 5. The 5-rated image was imported into PowerPoint (version 2007; Microsoft, Redmond, WA), and image transparency was increased in 20% steps to establish references for intensity ratings of 4, 3, 2, and 1. The absence of color was assigned a zero intensity rating. Intensity of color of each root tip was compared visually to this scale and scored. Color intensity of root tips for each accession or cultivar was regressed linearly against RRG in the agarose assay, using Excel software (version 2007; Microsoft).

SCREENING METHODS: SCREENING IN AL-TOXIC SOIL. Soil experiments were conducted with 12 selected accessions using Leilehua soil series (Order: Ultisol; Very-fine, ferruginous, and

Fig. 1. Relative root growth [RRG (ratio of root elongation in the presence and absence of Al)] of 91 barrel medic accessions or cultivars grown in agarose medium at 50, 100, or 200 µM Al.

isothermic Ustic Kanhaplohumults), pH 4.6. For the limed treatment, calcium carbonate (CaCO_3) was added to achieve pH 5.5. The soil was analyzed at the Agricultural Diagnostic Service Center, College of Tropical Agriculture and Human

Resources, University of Hawaii at Manoa. Soil was extracted with 1 M KCl and analyzed with an inductively coupled plasma emission spectrometer (Optima 7000 DV; PerkinElmer, Waltham, MA).

Polyvinyl chloride pipes were cut in half vertically, resealed with tape, and used as pots (10 cm diameter $\times$ 17.5 cm height). Bottom of pots were enclosed with a fiberglass screen (mesh 18/14; Phifer, Tuscaloosa, AL).

The pots were filled with 900 g dry weight soil. The following fertilizers were mixed into the surface soil to a depth of 2.5 cm: P and Ca, added as $\text{Ca}(\text{H}_2\text{PO}_4)_2$ at 0.33 and 0.21 g·kg⁻¹ soil, respectively; potassium and N added as KNO_3 at 0.27 and 0.1 g·kg⁻¹ soil, respectively; Mg added as MgSO_4 at 0.05 g·kg⁻¹; and micronutrient fertilizer (Granusol; Mivena Specialty Fertilizer Co., Sprangseweg, The Netherlands) at 0.2 g·kg⁻¹ soil (0.01 g·kg⁻¹ Mg, 0.001 g·kg⁻¹ B, 0.01 g·kg⁻¹ Fe, 0.01 g·kg⁻¹ Mn, 0.003 g·kg⁻¹ Copper, 0.01 g·kg⁻¹ Zinc).

Twelve accessions or cultivars that represented putative Al-tolerant lines (PI 577633, PRT 179, and W6 6037), intermediate lines (A 17, A 20, DZA 058, PI 535648, PI 577609, and W6 6092), and Al-sensitive lines (PI 535614, PI 535622, and PI 566889) were selected for the Al-toxic soil study. Two seedlings of each accession were planted in a pot. Pots were arranged as a randomized complete block design with three replications per treatment. Experiments were conducted in the Magoon Greenhouse facility, University of Hawaii at Manoa (lat. 21°17'47.8''N, long. 157°49'0.26''W). The average air temperature was 23 °C (maximum 28 °C, minimum 20 °C), and the average photoperiod was 12 h.

At 28 d after transplanting, pots were cut open, and whole roots were carefully removed. Plants were rinsed in distilled water. Images of whole plants and roots were taken using a digital camera (model A620; Canon, Tokyo, Japan). Roots were separated from shoots and lengths of

longest roots were measured. Shoots and roots were weighed immediately for fresh weights, then dried at 80 °C for 48 h to achieve constant dry weight. Relative root growth was calculated as the ratio of root length in unlimed soil to that in limed

soil. Relative root growth of each accession or cultivar grown in Al-containing agarose was regressed linearly against that of the same accession grown in Al-toxic soil using Excel (version 2007).

Results

INHIBITION OF ROOT ELONGATION. Increasing Al levels in agarose medium significantly reduced root lengths ($P < 0.01$), and there were significant differences among accessions in response to Al toxicity [$P < 0.01$ (Fig. 1)]. Relative root growth differed significantly among 91 accessions or cultivars grown in Al-containing agarose medium [$P < 0.01$ within each Al level (Fig. 1)].

Root response to Al of accessions or cultivars was significantly different ($P < 0.05$) at varying Al levels. In other words, some accessions appeared to be Al sensitive or Al tolerant at 50 μM Al relative to genotype A17, but showed no significant difference relative to A17 at higher Al levels.

Aluminum response was categorized based on RRG of accessions or cultivars at both 50 and 100 μM Al, using the

modified method of Sledge et al. (2005). Results for selected accessions or cultivars are shown in Table 1. When grown at 50 μM Al, root growth of eight accessions (F 20047, PI 577633, PRT 179, W6 6048, W6 6049, F 20025, F 20015, and F 20031) was greater in the presence of Al than under control conditions [RRG $> 100\%$ (Fig. 1)], and were considered to be putative Al tolerant. To be categorized as putative Al tolerant or sensitive, accessions or cultivars must have had similar results at both 50 and 100 μM Al. For example, accessions PI 535614, PI 535622, PI 577605, PI 566889, PI 566890, and W6 6047 had the lowest RRG across all Al levels, and were considered to be Al sensitive.

Distribution of RRG of accessions was plotted according to Al level (Fig. 2). Across all Al levels, RRG ranged from 0% to 148%, with an average of 70%. At 50 μM Al, RRG ranged from 14% to 148%, the greatest range of RRG among the three Al levels (Figs. 1 and 2). At 100 μM Al, RRG ranged from 5% to 81% with an average of 33% (Figs. 1 and 2). As Al levels increased, the range of RRG among accessions was reduced, and at 200 μM Al, RRG of all accessions was lower than 30%, suggesting that the assay was saturated at this level.

Table 1. Aluminum (Al) response of barrel medic accessions or genotypes evaluated with three different screening methods (Al-containing agarose assay, hematoxylin assay, and Al-toxic soil assay) and compared with results of three earlier researchers (Chandran et al., 2008; Narasimhamoorthy et al., 2007; Sledge et al., 2005).

Accession	Country of origin	Al response based on agarose assay ^z	Al response based on hematoxylin assay ^y	Al response based on soil assay ^x	Al response based on hydroponic assay (Sledge et al., 2005)	Al response based on hydroponic/soil/lumogallion assays (Narasimhamoorthy et al., 2007)	Al response based on hydroponic assay (Chandran et al., 2008)
A 17 (Jemalong)	Australia	S	S	N	S	S/S/S	N
A 20	Australia	S	S	N	—	—	S
PI 535614	Tunisia	S	S	S	T	T/S/S	—
PI 535622	Tunisia	S	S	S	T	—	—
PI 537168	Cyprus	S	N	—	T	T/N/T	—
PI 566889	Turkey	S	S	N	S	—	—
PI 566890	Greece	S	S	—	T	T/N/T	—
PI 577605	Cyprus	S	S	—	S	—	—
PI 577609	Sweden	T	N	N	S	—	—
PI 577626	Algeria	T	T	—	S	S/N/T	—
PI 577633	Greece	T	N	T	S	—	—
PI 190089	Australia	S	—	—	—	S/S/S	S
W6 6037	Spain	T	T	T	S	S/T/T	—
W6 6050	Tunisia	S	T	—	—	—	S
W6 6092	Tunisia	S	N	N	S	—	—
W6 6099	Portugal	N	—	—	—	—	T
W6 6110	Italy, Sicily	S	T	—	—	—	S

^zS = sensitive; N = intermediate; T = tolerant. In the agarose assay, Student's *t* tests were used to compare root elongation in plants grown in control (0 Al) or Al-containing agarose (50 or 100 μM Al) for each accession or cultivar, using a method modified from Sledge et al. (2005). Aluminum-sensitive accessions or cultivars had *P* values ≤ 0.05 in all levels of Al, Al-tolerant ones had *P* values of ≥ 0.50 in all levels of Al, and intermediate ones had intermediate *P* values.

^yDarker-staining root tips were characteristic of Al-injured roots and correlated significantly with lower relative root growth [RRG (ratio of root elongation in the presence and absence of Al)] in the agarose assay. Accessions or cultivars were characterized as Al sensitive or Al tolerant, based on intensity rating of color in root tips and correlation with RRG in the agarose assay. At 50 μM Al, plants with root tips with a mean staining intensity of ≤ 0.3 were considered to be Al tolerant, those with a mean staining intensity of ≥ 1.3 were considered to be Al sensitive, and those with intermediate staining intensity were considered to be intermediate in Al response. At 100 μM Al, plants with root tips with a mean staining intensity of ≤ 3.2 were considered to be Al tolerant, those with a mean staining intensity of ≥ 3.6 were considered to be Al sensitive, and those with intermediate staining intensity were considered to be intermediate in Al response. Also, those lines with inconsistent groupings between 50 and 100 μM Al were considered to be intermediate.

^xRRG in unlimed and limed Al-toxic soil was used to characterize Al response. Accessions or cultivars with mean RRG $\geq 60\%$ were considered to be Al tolerant, those with mean RRG $\leq 40\%$ were considered to be Al sensitive, and those with intermediate values were considered as intermediate in Al response.

Fig. 2. Distribution of relative root growth [RRG (ratio of root elongation in the presence and absence of Al)] of barrel medic accessions or cultivars at three Al levels (50, 100, or 200 μM Al) in agarose medium.

ACCUMULATION OF AL. Thirty barrel medic accessions, including putative Al-tolerant, intermediate, and Al-sensitive lines were tested using the hematoxylin dye, and color intensity of root tips. Stained roots were given a score (0 to 5) according to their color intensity (Cançado et al., 1999). No significant difference in color intensity was found among all accessions grown in the control condition (zero intensity rating). Increasing Al levels in agarose resulted in increased intensity of staining [1–5 (Fig. 3)]. All accessions except three [PI 535648, PI 353739, and W6 6037 (4.83 score)] had an intensity rating of five when grown in 200 μM Al. By contrast, the responses of accessions differed substantially when grown in 50 or 100 μM Al containing medium.

In general, the accumulation of Al was higher in Al-sensitive accessions, which exhibited more intense staining in 50 or 100 μM Al containing medium (Fig. 3B). The results indicated that greater amounts of Al accumulated in root tips of Al-sensitive accessions exposed to Al compared with Al-tolerant accessions.

Relative root growth was regressed linearly against the quantitative staining score, and a significant ($P < 0.05$), negative, linear relationship was found between RRG at 50 or 100 μM and the intensity of staining scores (Fig. 4). At the highest Al level of 200 μM , there was no significant correlation, probably due to the limitation of the assay as the color intensity was saturated.

Accessions or cultivars were characterized as Al sensitive or Al tolerant, based on intensity rating of color in root tips and correlation with RRG in the agarose assay. Darker-staining roots were indicative of greater Al injury. At 50 μM Al, plants with root tips with a mean staining intensity of ≤ 0.3 were considered to be Al tolerant, those with a mean staining intensity of ≥ 1.3 were considered to be Al sensitive, and those with intermediate staining intensity were considered to be intermediate in Al response. At 100 μM Al, plants with root tips with a mean staining intensity of ≤ 3.2 were considered to be Al tolerant, those with a mean staining intensity of ≥ 3.6 were considered to be Al sensitive, and those with intermediate staining intensity were considered to be intermediate in Al response. Also, those lines with inconsistent groupings between 50 and 100 μM Al were considered to be intermediate.

Results for selected cultivars or accessions are shown in Table 1.

SCREENING IN AL-TOXIC SOIL. Soluble Al concentrations were 271 and 64 $\text{mg}\cdot\text{kg}^{-1}$ before and after lime addition, respectively. Root lengths of all accessions were reduced significantly when grown in unlimed soil ($P < 0.05$) except W6 6037 (Fig. 5). This accession (W6 6037) appeared to be exceptionally Al tolerant, because its root lengths were quite similar in limed or unlimed soil.

Relative root growth in unlimed and limed Al-toxic soil was used to characterize Al response. Accessions or cultivars with mean RRG $\geq 60\%$ were considered to be Al tolerant, those with mean RRG $\leq 40\%$ were considered to be Al sensitive, and those with intermediate values were considered as intermediate in Al response. Categories of Al response based on Al-toxic soil assay for selected accessions or cultivars are shown in Table 1.

Significant, positive correlations were found between soil-based RRG and agarose-based RRG at 100 μM Al ($R^2 = 0.62$) and 200 μM Al ($R^2 = 0.71$). Although the range of RRG at 200 μM Al containing agarose was small (Fig. 2), rankings of accessions were similar to those in Al-toxic soil. In contrast, RRG of accessions grown at 50 μM Al containing agarose were not significantly correlated with soil-based RRG ($R^2 = 0.35$).

No stimulatory effects of Al on RRG were found in the soil experiment, in contrast to results obtained in the 50 μM Al containing agarose assay. No correlation between relative root dry weight, relative shoot dry weight, and genotypic ranking of Al sensitivity was found (data not shown).

COMPARISON OF ASSAYS. Forty-four accessions that we studied had been tested previously for their response to Al toxicity. Our current results were similar in some cases, but different in others (Table 1). For example, accession W6 6037, which was consistently Al tolerant in the current study, was reported as Al tolerant in a soil study and lumogallion staining assay (Narasimhamoorthy et al., 2007), but as highly Al sensitive in hydroponic studies (Sledge et al., 2005). Several accessions, for example PI 535614 and PI 535622, which were rated as Al sensitive in the current study, were evaluated as tolerant in earlier hydroponic experiments (Narasimhamoorthy et al., 2007; Sledge et al., 2005). In other cases, results were consistent among studies: e.g., accessions PI 577605 and PI 190089 were scored as Al sensitive in this study and previous studies (Chandran et al., 2008; Narasimhamoorthy et al., 2007; Sledge et al., 2005).

Discussion

Variation in sensitivity to Al toxicity exists among the barrel medic germplasm. In this study, we reported Al responses for 47 accessions that not been tested previously. In addition, we compared our results for Al sensitivity of 44 accessions that had been tested previously.

Concentration of Al is a key factor to screening methods that are based on direct root-ion interaction in a growing medium

Fig. 3. Hematoxylin staining in three barrel medic accessions or cultivars that differed in response to Al toxicity based on relative root growth (ratio of root elongation in the presence and absence of Al) of seedlings grown at 50 or 100 μM Al in agarose assay: (A) W6 6037 (Al-tolerant), (B) PI 535622 (Al-sensitive), (C) A17 (intermediate), and (D) visual intensity score. Darker staining is considered as indicative of greater Al injury. At 50 μM Al, plants with root tips with a mean staining intensity of ≤ 0.3 were considered to be Al tolerant, those with a mean staining intensity of ≥ 1.3 were considered to be Al sensitive, and those with intermediate staining intensity were considered to be intermediate in Al response. At 100 μM Al, plants with root tips with a mean staining intensity of ≤ 3.2 were considered to be Al tolerant, those with a mean staining intensity of ≥ 3.5 were considered to be Al sensitive, and those with intermediate staining intensity were considered to be intermediate in Al response. Also, those lines with inconsistent groupings between 50 and 100 μM Al were considered to be intermediate.

such as hydroponic or sand culture (Narasimhamoorthy et al., 2007; Villagarcia et al., 2001). An interaction between accessions and Al concentration was observed in the agarose experiment, in

Fig. 4. Correlation between relative root growth [RRG (ratio of root elongation in the presence and absence of Al)] of 30 barrel medic accessions or cultivars grown in agarose medium at 50, 100, or 200 μM Al, and color intensity of hematoxylin staining of root tips. Color intensity was scored visually between 0 and 5, with 0 as the absence of staining and 5 as the darkest color. Darker staining is considered as indicative of greater Al injury.

Fig. 5. Root length and relative root growth [RRG (ratio of root elongation in the presence and absence of Al)] of 12 selected barrel medic accessions grown in limed or unlimed acid soil (Leilehua soil series; very-fine, ferruginous, isothermic ustic Kanhaplohumults) for 28 d.

which RRG of certain accessions differed at varying Al concentrations. Based on the distribution of RRG, 50 or 100 μM Al containing agarose medium appeared to be a reliable method to test for Al tolerance or Al sensitivity of barrel medic accessions.

Hematoxylin staining appears to be another suitable procedure to determine Al tolerance in barrel medic. The intensity of staining in Al-treated roots was correlated significantly and negatively with RRG measured in 50 or 100 μM Al containing agarose medium. However, the color intensity was limited at 200 μM Al due to color saturation. In this study, a range of color intensity (0–5) among 30 Al-sensitive and Al-tolerant accessions indicated the usefulness of this staining procedure to characterize variation of Al sensitivity among accessions of barrel medic. Similarly, a color difference was reported between an Al-sensitive and an Al-tolerant accession of barrel medic (Chandran et al., 2008). Also, in chickpea, Singh and Raje (2011) concluded that simple genetic control of Al tolerance could be identified on the basis of either hematoxylin staining score or root regrowth soon after Al treatment. By contrast, in lentil, Singh et al. (2012) concluded that hematoxylin staining could not be used to identify Al sensitivity.

Here, we found a significant, positive correlation between RRG measured in 100 or 200 μM Al containing agarose, and that in soil-based screening. Narasimhamoorthy et al. (2007) suggested that a soil-based assay was a more rigorous test than other methods, because barrel medic genotypes that appeared tolerant to Al in hydroponics or root staining were sensitive when

screened in Al-toxic soil. The whole-plant assay in soil provides a system to evaluate plant growth with the complete root system in contact with substrate. However, one limitation of soil-based assays is that each Al-toxic soil differs in pH, mineral nutrient concentrations, and other characteristics, making comparisons of germplasm between researchers more difficult.

Enhancement of root growth of barrel medic was found at 50 μM Al containing agarose. Similar results were reported earlier in maize (Mariano and Keltjens, 2004). This phenomenon might be the result of displacement of excess protons from the plasmalemma by Al cations (Poschenrieder et al., 2008).

In our studies, we did not find any significant differences among root and shoot dry weights of barrel medic grown in Al-toxic soil. Similar results were reported in soybean [*Glycine max* (Villagarcia et al., 2001)] and alfalfa [*Medicago sativa* (Dall'Agnol et al., 1996)].

In summary, we recommend measurement of RRG of barrel medic in an Al-containing agarose assay at 100 μM Al. This method appears as a simple, quick test of Al sensitivity, based on correlation with a hematoxylin staining assay and with an Al-toxic soil study.

Literature Cited

- Brauer, D. and T. Staley. 2005. Early developmental responses of white clover root hair lengths to calcium, protons, and aluminum in solution and soil cultures. *Crop Sci.* 45:1216–1222.
- Calba, H., B. Jaillard, P. Fallavier, and J.D. Arvieu. 1996. Agarose as a suitable substrate for use in the study of Al dynamics in rhizosphere. *Plant Soil* 178:67–74.
- Cançado, G.A.M., L.L. Loguercio, P.R. Martins, S.N. Parentoni, E. Paiva, A. Borém, and M.A. Lopes. 1999. Hematoxylin staining as a phenotypic index for aluminum tolerance selection in tropical maize (*Zea mays* L.). *Theor. Appl. Genet.* 99:747–754.
- Chandran, D., N. Sharopova, K.A. Vanden Bosch, D.F. Garvin, and D.A. Samac. 2008. Physiological and molecular characterization of aluminum resistance in *Medicago truncatula*. *BMC Plant Biol.* 8:89.
- Cook, D.R. 1999. *Medicago truncatula*: A model in the making! *Curr. Opin. Plant Biol.* 2:301–304.
- Crawford, E.J., A.W.H. Lake, and K.G. Boyce. 1989. Breeding annual *Medicago* species for semiarid conditions in southern Australia. *Adv. Agron.* 42:399–437.
- Dall'Agnol, M., J.H. Bouton, and W.A. Parrott. 1996. Screening methods to develop alfalfa germplasms tolerant of acid, aluminum toxic soils. *Crop Sci.* 36:64–70.
- Delhaize, E., S. Craig, C.D. Beaton, R.J. Bennet, V.C. Jagdish, and P.J. Randall. 1993. Aluminium tolerance in wheat (*Triticum aestivum* L.). Uptake and distribution of aluminium in root apices. *Plant Physiol.* 103:685–693.
- Echart, C.L., J.F. Barbosa-Neto, D.F. Garvin, and S. Cavalli-Molina. 2002. Aluminum tolerance in barley: Methods for screening and genetic analysis. *Euphytica* 126:309–313.
- Foy, C.D. 1988. Plant adaptation to acid, aluminum-toxic soil. *Commun. Soil Sci. Plant Anal.* 19:959–987.
- Hede, A.R., B. Skovmand, J.-M. Ribaut, D. Gonzalez-de-Leon, and O. Stolen. 2002. Evaluation of aluminium tolerance in a spring rye collection by hydroponic screening. *Plant Breed.* 121:241–248.
- Hossain, M., M. Zhou, and N. Mendham. 2005. A reliable screening system for aluminium tolerance in barley cultivars. *Austral. J. Agr. Res.* 56:475–482.
- Ishitani, M., I. Rao, P. Wenzl, S. Beebe, and J. Tohme. 2004. Integration of genomics approach with traditional breeding towards improving abiotic stress adaptation: Drought and aluminum toxicity as case studies. *Field Crops Res.* 90:35–45.
- Khu, D.M., R. Reyno, E.C. Brummer, and M.J. Monteros. 2012. Screening methods for aluminum tolerance in alfalfa. *Crop Sci.* 52:161–167.
- Kinraide, T.B. 1991. Identity of the rhizotoxic aluminum species. *Plant Soil* 134:167–178.
- Kobayashi, Y., Y. Furuta, T. Ohno, T. Hara, and H. Koyama. 2005. Quantitative trait loci controlling aluminium tolerance in two accessions of *Arabidopsis thaliana* (Landsberg erecta and Cape Verde Islands). *Plant Cell Environ.* 28:1516–1524.
- Kochian, L.V. 1995. Cellular mechanisms of aluminum toxicity and resistance in plants. *Annu. Rev. Plant Physiol. Mol. Biol.* 46:237–260.
- Liu, H. 2005. Aluminum resistance among seeded bermudagrasses. *HortScience* 40:221–223.
- Llugany, M., C. Poschenrieder, and J. Barceló. 1995. Monitoring of aluminium-induced inhibition of root elongation in four maize cultivars differing in tolerance to aluminium and proton toxicity. *Physiol. Plant.* 93:265–271.
- Mariano, E.D. and W.G. Keltjens. 2004. Variation for aluminum resistance among maize genotypes evaluated with three screening methods. *Commun. Soil Sci. Plant Anal.* 35:2617–2637.
- Miyasaka, S.C. and M.C. Hawes. 2001. Possible role of root border cells in detection and avoidance of aluminum toxicity. *Plant Physiol.* 125:1978–1987.
- Miyasaka, S.C., N.V. Hue, and M.A. Dunn. 2006. Aluminum, p. 439–497. In: A.V. Barker and D.J. Pilbeam (eds.). *Handbook of plant nutrition*. CRC Press, New York, NY.
- Narasimhamoorthy, B., E.B. Blancaflor, M.E. Payton, and M.K. Sledge. 2007. A comparison of hydroponics, soil, and root staining methods for evaluation of aluminum tolerance in *Medicago truncatula* (barrel medic) germplasm. *Crop Sci.* 47:321–328.
- Poschenrieder, C., B. Gunse, I. Corrales, and J. Barceló. 2008. A glance into aluminum toxicity and resistance in plants. *Sci. Total Environ.* 400:356–368.
- Samac, D.A. and M. Tesfaye. 2003. Plant improvement for tolerance to aluminum in acid soils: A review. *Plant Cell Tissue Organ Cult.* 75:189–207.
- Singh, D. and R.S. Raje. 2011. Genetics of aluminium tolerance in chickpea (*Cicer arietinum*). *Plant Breed.* 130:563–568.
- Singh, D., H.K. Dikshit, and R. Singh. 2012. Variation of aluminium tolerance in lentil (*Lens culinaris* Medik.). *Plant Breed.* 131:751–761.
- Sledge, M.K., P. Pechter, and M.E. Payton. 2005. Aluminum tolerance in *Medicago truncatula* germplasm. *Crop Sci.* 45:2001–2004.
- Srimake, Y. 2012. Aluminum tolerance in *Medicago truncatula* Gaertn. PhD Diss., Univ. Hawaii at Manoa, Honolulu, HI.
- Tamas, L., S. Budicova, J. Huttova, I. Mistrik, M. Šimonovicova, and B. Siroka. 2005. Aluminum-induced cell death of barley-root border cells is correlated with peroxidase- and oxalate oxidase-mediated hydrogen peroxide production. *Plant Cell Rpt.* 24:189–194.
- Tamas, L., S. Budicova, M. Šimonovicova, J. Huttava, B. Siroka, and I. Mistrik. 2006. Rapid and simple method for Al-toxicity analysis in emerging barley roots during germination. *Biol. Plant.* 50:87–93.
- Tang, Y., D.F. Garvin, L.V. Kochian, M.E. Sorrells, and B.F. Carver. 2002. Physiological genetics of aluminum tolerance in the wheat cultivar Atlas 66. *Crop Sci.* 42:1541–1546.
- Villagarcia, M.R., T.E. Carter, Jr., T.W. Rufty, A.S. Niewoehner, M.W. Jennette, and C. Arrellano. 2001. Genotypic rankings for aluminum tolerance of soybean roots grown in hydroponics and sand culture. *Crop Sci.* 51:1499–1507.
- Voigt, P.W. and T.E. Staley. 2004. Selection for aluminum and acid-soil resistance in white clover. *Crop Sci.* 44:38–48.
- von Uexkull, H.R. and E. Mutert. 1995. Global extent, development and economic impact of acid soils. *Plant Soil* 171:1–15.
- Yamamoto, Y., Y. Kobayashi, and H. Matsumoto. 2001. Lipid peroxidation is an early symptom triggered by aluminum, but not the primary cause of elongation inhibition in pea roots. *Plant Physiol.* 125:199–208.
- Zhou, L.-L., G.-H. Bai, B.F. Carver, and D.-D. Zhang. 2007. Identification of new sources of aluminum resistance in wheat. *Plant Soil* 297:105–118.
- Zhu, Y.P., C.C. Sheaffer, M.P. Russelle, and C.P. Vance. 1998. Dry matter accumulation and dinitrogen fixation of annual *Medicago* species. *Agron. J.* 90:103–108.