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RESEARCH ARTICLE

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A study on indicators and evaluation stages of aluminum tolerance in soybean

Abstract To determine an appropriate indicator and a suitable stage for evaluating tolerance of soybeans to aluminum (Al) toxin is one of the keys to effective breeding for the trait. Seventeen accessions selected as tolerant from a previous test program by using average membership index (F_{Ai}) as indicator, plus one tolerant (PI.416937) and one sensitive (NN1138-2) check, were assayed in sand culture pot experiments, totaling four experiments, each for evaluation at V3, V5, V7 and V9 stage, respectively, each in a randomized complete block design with three replications, and each genotype exposed to two Al levels (0 and 480 μM). The relative values of shoot dry weight (RSDW), root dry weight (RRDW), total plant dry weight (RTDW), total root length (RTRL) and total root surface area (RRSA) as the tolerance indicators as well as F_{Ai} were compared. All the indicators showed significant variation in Al tolerance among genotypes over and across the leaf stages, but Genotype × Stage interactions were significant only for RTRL and RRSA, indicating that they were less stable among stages than RTDW, RSDW and RRDW. Among the latter three, RTDW was chosen as the major indicator of Al tolerance due to its relatively better stability, higher correlation with other indicators and easier measuring procedure than the others. The seedling age applicable for screening was not definitive, but V5 appeared to compromise between time spent resulting from screening the relatively older seedlings at later stages and low variation among genotypes at a younger stage. The differences of Al tolerance among the tested accessions were further detected by using RTDW, and superior Al tolerant accessions identified were PI.509080 (South Korea), N23533 and N24282 (Northeast China) and PI.159322 (USA), comparable to the putative tolerant check PI.416937 (Japan) at all vegetative stages.

Keywords soybean (*Glycine max* (L.) Merr.), aluminum toxin tolerance, indicator, leaf stage, acidic soil

1 Introduction

Soybean (*Glycine max* (L.) Merrill) is the world's most important modern-day food legume crop, supplying about 40% of dietary proteins (Kauffman, 1987) but is one of the crops affected by aluminum (Al) toxicity. Al toxicity occurs when soil pH is less than 5.0 when Al dissolves from non-toxic Al oxides and silicates into the phytotoxic Al^{3+} ions (Kochian, 1995). The critical Al concentration for Al toxicity in soil solutions depends on pH, nutrient status in the soil and plant genotype. A wide range from 2 mg $Al^{3+}\cdot L^{-1}$ to 30 mg $Al^{3+}\cdot L^{-1}$ (75 μM–1110 μM) has been reported as a critical concentration for Al toxicity to different crop genotypes (Wu et al., 1997). It has been estimated that 40%–50% of world's arable soils have pH below 5.0 and therefore, possess Al toxicity problems (Aniol et al, 1980). Some measures taken to reduce this acidity include liming and irrigation, but they are expensive to small-scale farmers (Kochian et al., 2004).

Overall health of soybean plants and seed yield is affected by aluminum toxin. Aluminum damage in soybean results from absorption and accumulation of Al ions in the roots, stems and leaves, which cause root injury, leaf yellowing and stunted shoot growth, and, finally, reduced yields (Foy et al., 1992; Jayawardane et al., 1995). Root systems are anchored in soil and are important parts for water and nutrient uptake and are target organs for Al toxicity. Selection among soybean genotypes for Al tolerance on the basis of root characteristics may be an effective and valuable approach for improving crop production in acid soils. Genetic variation exists for acid soil adaptation among soybean germplasm (Foy et al.,

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1992), and an opportunity to identify germplasm for cultivar improvement, therefore, exists.

Appropriate screening techniques with suitable indicators are important for successful identification of soybean cultivars tolerant to Al stress. Another factor is the optimum growth stage for screening in terms of cost involved and timeliness of the results. Three screening methodologies have been used in evaluating soybean genotypes for Al tolerance. They are evaluation in field with high aluminum in soil (Bushamuka and Zobel, 1998), evaluation in pot sand culture (Villagarcia et al., 2001; Qi et al., 2008) and evaluation in hydroponics (Bianchi-Hall et al., 2000).

Field screening methodology is less precise, less efficient and, therefore, less attractive to breeders since this method is challenged by many factors, including soil heterogeneity in physical and chemical characteristics, tedious excavations of roots, and low screening rate. In addition, ranking genotypes for Al tolerance may be soil type-dependent and, therefore, not easily reproducible across large geographical areas. Hydroponics, on the other hand, is more attractive to soil-based screening because it allows rapid evaluation of a large number of genotypes in a shorter time. Besides, accurate phenotype data are expected from hydroponics because of the tight control of the screening conditions. Despite these advantages, breeders have not fully adopted results from pure hydroponics because it is usually limited to young seedlings, and there is doubt whether rankings of seedling Al tolerance apply to the field (Bianchi-Hall et al., 1998; Ferrufino et al., 2000). In comparison to hydroponics, the sand culture screening method deals with relatively older seedlings, and being a solid medium, sand is physically more similar to field conditions. Sand is almost inert, and since nutrients and Al are applied in solution form, the concentration of Al can be regulated and reproduced with consistency. Therefore, this method is a potential viable alternative between field and hydroponics screenings. However, a potential challenge to adopting sand culture method is in finding an appropriate indicator for Al tolerance that is easy to score, representative, stable and reproducible across other screening methodologies and over environments.

Genotypes are frequently evaluated for Al tolerance under sand culture at young vegetative stages from three-leaf stage (V3) onwards using various indicators. For example, relative dry weights of shoots and roots (Villagarcia et al., 2001), taproot length, surface area and lateral root (Gai et al., 2007) and an average membership index F_{Ai} of several growth traits (Qi et al., 2008) have been used in soybean. However, some indicators are very burdensome to score, while others are unstable, unreliable and incomparable among experiments. For example, relative taproot length growth, which is an appropriate indicator in hydroponics, is not easy to evaluate under sand culture because it is fragile and easily breaks in the solid medium. For roots and shoots, evaluations involve first separating the two components from the plants and dealing with each one separately. Determination of surface area, volume, and total root length is labor-intensive and requires specific equipments to accomplish. From these experiences, an idea of having a single indicator that includes majority of the possible individual indicators and yet reflects whole-plant response to Al stress was suggested. Such an indicator would not only be representative but also economical. Qi et al. (2008) used an F_{Ai} index (average membership index) from four growth-related traits, i.e. number of leaves, plant height, shoot dry weight and root dry weight, and used it to assess Al tolerance among soybean genotypes. However, the approach was costly in view of the substantial labor and time needed to acquire data for the individual indicators and synthesizing into the single aggregate F_{Ai} index.

Therefore, there is a compelling need to identify an easy, inexpensive, environmentally stable and reliable indicator that soybean breeders would routinely use to screen germplasm for Al tolerance in sand culture. The objectives of this study were thus (1) to identify an indicator for replacing membership index (F_{Ai}) and (2) to evaluate for a suitable growth stage for screening Al tolerance in soybean seedlings.

2 Materials and methods

2.1 Plant materials

Seventeen accessions were used in this study. The accessions comprised Chinese released cultivars (CRC), Chinese landraces (CLR), Southeast Asia introductions (SEA) and America cultivars (AMC) screened and classified as tolerant materials in our preliminary screening experiment of 2007, in which a total of 450 accessions were screened for Al tolerance in sand culture by measuring the relative growth of four traits (number of leaves, plant height, dry weight of shoot and dry weight of root) that were used to calculate the average membership index as follows: For each trait of each genotype in each replication, the observation was transformed into a membership index $F = (X-X_{\min})/(X_{\max}-X_{\min})$, where $X_{\max}$ and $X_{\min}$ are the maximum and minimum values in the replication, respectively. Then the average membership index of each genotype F_{Ai} was obtained from averaging over the F values of the replications and traits. The detailed procedure can be referred to Qi et al. (2008). Based on the frequency distribution of F_{Ai}, the accessions were classified into categories of tolerance levels according to the procedure of Gai et al (2007). Seventy-eight accessions demonstrated at least moderate tolerance to Al toxicity. From this group, the seventeen accessions comprising the top statistical group of F_{Ai} were selected for this experiment. Two accessions, PI.416937 and NN1138-2, were included as controls in the current study. PI.416937 has consistently been demonstrated to be tolerant in hydroponics screening (Campbell and Carter, 1990; Bianchi-Hall et al., 1998; Villagarcia et al., 2001) and also by our previous evaluation in sand culture (Gai et al., 2007), though some reports have indicated a moderate tolerance in field or large pot (Bushamuka and Zobel, 1998; Ferrufino et al. 2000). NN1138-2, on the other hand, is sensitive to Al regardless of the screening system (Qi et al., 2008). All materials were obtained from the Soybean Research Institute, Nanjing Agricultural University, Jiangsu, China.

2.2 Experiment design

From the preliminary experiment using the sand culture protocol of Villagarcia et al. (2001), the concentration 480 μM Al^{3+} $(SO_4)_3 \cdot 18H_2O$ was identified to detect a wide continuum of variation in traits tested for six soybean materials, including PI.416937 and NN1138-2. This concentration approximated to 450 μM Al^{3+} used by Villagarcia et al., (2001), therefore, was used in the present study. Six seedlings per genotype growing in Φ25 × h28 cm plastic pots were thinned to three per pot 7 d after planting. In the first week, all pots were supplied with 800 mL of complete nutrient solutions (Villagarcia et al., 2001) to promote vigorous plant growth. From the second week, seedlings were supplied with 800 mL modified 1/5 strength Steinburg nutrient solution (Foy et al., 1967) with Al (480 μM) or without Al (0 μM) adjusted to pH 4.1 with 1 M HCl at 3-day interval throughout the experimental period of 8 weeks (up to V9 stage). The intent of this 3d interval was to minimize the accumulation of excess Al in the sand media to mimic practical field conditions having mild Al stress.

The experimental design was a randomized complete block in three replications for each of the four vegetative growth stage (Vstage) experiments, i.e. V3 (3-fully expanded leaf stage, about 21 days after planting), V5 (5-leaf stage, about 35 days after planting), V7 (7-leaf stage, about 49 days after planting) and V9 (9-leaf stage, about 63 days after planting). There were two pots per plot per genotype in each block, one for control (0 μM) and the other for treatment (480 μM). The control and treatment pots were paired to calculate relative trait values for each of the 19 genotypes so that there were a total of 3 observations per genotype in each experiment of each Vstage. The pots were placed under a plastic shelter with mean 26°C–28°C day temperatures at Jiangpu Experimental Station, Nanjing Agricultural University, China, from 15 September to 18 November, 2008.

2.3 Measurements and data analysis

Because the emergence of leaves in the seedlings was somewhat faster in control pots than in Al treatment, measurements were taken according to the Vstage of the latter. At each Vstage, the three seedlings of each genotype in each pot were harvested by cutting the shoot above the cotyledon node and separating the two parts. Individual root systems were then floated in water and spread to untangle overlapping root parts. The roots were digitalized with an EPSON scanner to acquire root images before drying. Shoots and roots were separately dried to constant weight at 65°C for 48 h and then weighed. Five sets of data for indicators were generated, i.e. relative values of shoot dry weight (RSDW), root dry weight (RRDW), total plant dry weight (RTDW), total root length (RTRL) and total root surface area (RRSA). The total root length and total root surface area were determined using the root analysis software WinRHIZO (Version 4.0b, Regent Instruments Inc., Quebec, Canada). The relative value of each indicator was obtained by dividing the treatment of each line by its control. The joint analyses of variance of four dates for each of the indicators were conducted, and correlations among the indicators were calculated by using SAS procedures (SAS Institute, 2004).

3 Results

3.1 Selecting suitable Al indicator

The five indicators tested in this study are basically growth traits which were previously observed sensitive to Al toxin (Gai et al., 2007; Qi et al., 2008). Each plot was composed of two paired pots with and without Al stress, and the experiment was designed as a single factor study with environment controlled uniformly. Therefore, the indicators were comparable in the present study.

The ANOVA results in Table 1 show that genotypic means over and across all leaf stages were significantly ($P = 0.001$) different for all indicators, indicating that they could detect differences in Al tolerance among the genotypes at any of the four leaf stages. The Vstage × Genotype interactions were significant only for RTRL and RRSA, showing that they were less stable among stages than RTDW, RSDW and RRDW. The evaluation of Vstages in this study coincided with an interval of approximately 14 d, and the duration between V3 and V9 was nearly 40 d. This period was reasonably long for a potential significant Genotype by Vstage interaction to occur. However, of the five traits, RTDW, RSDW and RRDW remained comparably steady up to V9.

The correlation coefficients among all the indicators were significant ($P < 0.001$; $r > 0.6$) over the 9-leaf period of stress (Table 2). Among the indicators, RTDW was highly significant and stably correlated ($r > 0.71$, $P < 0.001$) with all the other traits. Additionally, RTDW was better in detecting difference among the 19 accessions, even if they were all ranked as tolerant

Table 1 Joint ANOVA for indicators of 19 soybean genotypes exposed to Al stress in 4 experiments each for Vstage V3, V5, V7 and V9

sources	DF	mean square				
		RSDW	RRDW	RTDW	RTRL	RRSA
genotype	18	0.08***	0.09***	0.07***	0.09***	0.11***
Vstage	3	0.36***	0.76***	0.86**	0.67***	0.65***
Rep (Vstage)	8	0.00	0.02**	0.01	0.04**	0.01**
Vstage × Genotype	54	0.004	0.003	0.003	0.008**	0.008***
error	144	0.003	0.002	0.003	0.002	0.001
total	227					

Note: RSDW = relative value of shoot dry weight; RRDW = relative value of root dry weight; RTDW = relative value of total plant dry weight; RTRL = relative value of total root length; RRSA = relative value of root surface area. Vstage indicates vegetative leaf stage. *, ** and *** indicate significance at $P = 0.05$, 0.01 and 0.001, respectively.

Table 2 Pearson correlation coefficients for Al tolerance indicators over growth leaf stage (up to 9-leaf stage) in soybean seedling

indicators	$F_{Ai}^{\#}$	RSDW	RRDW	RTDW	RTRL
RSDW	0.84***				
RRDW	0.63**	0.68**			
RTDW	0.71***	0.71***	0.78***		
RTRL	0.79***	0.75***	0.74***	0.79***	
RRSA	0.67**	0.72***	0.75***	0.76***	0.82***

Note: *, ** and *** mean significance at $P < 0.05$, 0.01, and 0.001 respectively. # means values for correlation analysis are the cultivars' F_{Ai} from preliminary experiment.

when F_{Ai} was used in a previous testing program for a large sample of accessions (Table 3). Although the difference of effectiveness in detecting Al tolerance among the five indicators was not very large, RTDW was preferred due to its relatively better stability, higher correlation with other indicators and easier measuring procedure than the others. Compared to RSDW and RRDW that are measured separately, RTDW is measured on the whole plant after simply removing it from the sand in pots and is thus an easier measuring procedure. Therefore, RTDW was chosen and further reported in the following sections.

3.2 Selecting suitable stage for screening using RTDW

RTDW was significantly reduced under chronic Al stress from a mean of 0.699 at V3 to 0.424 at V9 (Table 3). Differences in RTDW among the 19 soybean genotypes were found as early as V3; however, differences were more effective at later stages (V5–V9), especially at V5–V7 which showed a tendency for higher coefficient of variation (CV). The optimum stage for screening genotypes was selected on the basis of CV since the CVs indicated extend of spread in RTDW among genotypes, and the higher the value, the better the separation among genotypes. On this basis, comparably high CVs were at V5–V9 and were accordingly optimum stages. However, among the stages, V5 was chosen as the preferred stage for RTDW because seedlings were reasonably old compared to younger stage V3. At V3, the seedlings might be too young to allow sufficient genetic expression, while screening at V7–V9 (42–56 days after planting) might not add much value to results; instead, it would be wasteful of resources. Similar results were obtained for RSDW and RRDW (data omitted here).

3.3 Selecting elite materials for Al tolerance

Adaptive tolerance to Al confers genotype superior ability to withstand long-term exposures, and such materials are likely more often ranked as Al-tolerant at most stages of growth. Based on the ranking of mean RTDW across the leaf stages of this study, the 19 genotypes were further divided into Al-tolerant (the first 5 higher mean RTDW accessions), less Al-tolerant (the last 5 lower mean RTDW accessions) and medium Al-tolerant (the rest 9 varieties) groups. The five accessions, PI.509080, N24282, N23533, PI.159322 and PI.416937, were identified as Al-tolerant accessions at all stages of growth (Fig. 1). Of these, N23533, N24282 and PI.509080 tended to perform as good as or better than the putative tolerant check PI.416937 between V3 and V5 stages. On the other hand, the accessions M076, N24190, N25511 and N25366 were consistently grouped as the less tolerant ones, with N25366 even close to the sensitive check NN1138-2 at all stages of growth and N24190 attaining values as low as for the sensitive check at V7 and V9. It was observed that significant genotype × Vstage interactions resulted in some inconsistent rankings within all the three groups. It should be mentioned that, here, we call this group as the

Table 3 Variations among accessions for Al tolerance at different vegetative stages for RTDW

accessions	sources	$F_{Ai}^{\#}$	leaf stages				
			V3	V5	V7	V9	mean
N25511	CRC	0.780 abcd	0.522 hi	0.459 i	0.334 e	0.332 fgh	0.412 g
N25366	CRC	0.774 bcd	0.458 j	0.449 i	0.365 e	0.308 gh	0.395 g
N25267	CRC	0.756 d	0.751 cde	0.657 fg	0.582 cd	0.457 bcd	0.612 cd
N24615	CRC	0.767 cd	0.717 ef	0.708 de	0.54 d	0.503 abc	0.617 cde
PI.159322	**AMC**	**0.815 abcd**	**0.784 bc**	**0.700 de**	**0.670 ab**	**0.475 bcd**	**0.665 abcd**
M076	AMC	0.811 abcd	0.583 g	0.583 h	0.425 e	0.401 defg	0.498 ef
M042	AMC	0.841 abcd	0.716 ef	0.698 def	0.611 bcd	0.451 bcd	0.619 bcd
PI.509080	**SEA**	**0.748 abcd**	**0.878 a**	**0.863 a**	**0.662 abc**	**0.514 ab**	**0.729 a**
N24760	SEA	0.791 cde	0.697 ef	0.739 bcd	0.584 cd	0.431 cde	0.613 cd
N24750	SEA	0.771 bcd	0.688 f	0.656 g	0.610 bcd	0.453 bcd	0.602 cd
N24690	SEA	0.793 abcd	0.670 f	0.669 efg	0.566 cd	0.440 bcde	0.586 de
N24190	CLR	0.844 abcd	0.528 fg	0.461 gh	0.420 i	0.284 fe	0.423 h
N4570.1	CLR	0.861 ab	0.793 bc	0.767 b	0.620 abcd	0.447 bcd	0.657 abcd
N23533	**CLR**	**0.849 abc**	**0.855 a**	**0.759 bc**	**0.607 bcd**	**0.469 bcd**	**0.673 abcd**
N24282	**CLR**	**0.846 abcd**	**0.835 ab**	**0.779 b**	**0.658 abc**	**0.476 bcd**	**0.687 abc**
N4671.2	CLR	0.851 abc	0.780 bcd	0.769 b	0.597 bcd	0.364 efg	0.628 abcd
N1126	CLR	0.867 a	0.724 def	0.720 cd	0.614 bcd	0.422 de	0.620 bcd
PI.416937	**Ck1**	**0.854 abc**	**0.835 ab**	**0.780 b**	**0.724 a**	**0.560 a**	**0.725 ab**
NN1138-2	Ck2	0.276 e	0.467 ij	0.367 j	0.325 e	0.269 h	0.357 g
	Mean	0.784	0.699	0.662	0.555	0.424	0.585
	CV (%)	15.03	18.81	20.46	23.50	20.64	19.27
	$LSD_{0.05}$	0.090	0.059	0.042	0.106	0.080	0.098
	range	0.867–0.276	0.458–0.878	0.367–0.863	0.325–0.724	0.269–0.560	0.357–0.729

Note: For each Vstage, down the mean column, means followed by the same letter are not significantly different using Fisher's LSD (SAS Institute, 2004); Ck1 and Ck2 refer to check cultivars. For source column, CRC = Chinese released cultivars; AMC = America cultivar; SEA = South East Asia; CLR = Chinese landraces. Boldface indicates the five accessions with highest RTDW values average over Vstages. Underlined refers to the five accessions with lowest values average over Vstages. $^{\#}$ means values are the cultivars F_{Ai} from preliminary experiment.

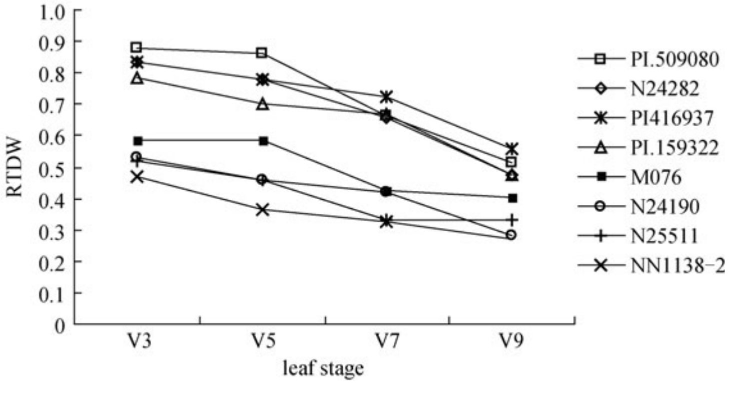

Fig. 1 Decline in RTDW (relative value of total plant dry weight) of 8 representative soybean accessions selected among 19 ones to represent Al-tolerant (upper four lines) and Al-sensitive categories (lower four lines).

less-tolerant group rather than the sensitive group because the 17 accessions were chosen as tolerant accessions different from a large number of sensitive ones in the preliminary test and that these less-tolerant ones need to be further verified.

From our study, PI.509080, N24282, N23533, PI.159322 and PI.416937 were further confirmed as the Al-tolerant elite accessions based on mean RTDW where PI.509080 is a plant introduction from South Korea (Gai, et al., 2001), N24282 and N23533 are both landraces from

Northeast China, the former from Inner Mongolia and the latter from Heilongjiang province, and PI.159322 is a plant introduction from the USA, while the tolerant check PI.416937 is a plant introduction from Japan. It is interesting that from the present study, the tolerant check PI.416937 is still the best one with second highest mean RTDW but smallest variation among stages.

4 Discussion

There appeared somewhat inconsistencies between the preliminary test and the present test that the 17 tolerant accessions in the former performed large variation in the latter with some accessions even close to the sensitive check. The reason for this might be due to the different indicators used and the multiple stages tested. In the former, F_{Ai}, composed of relative number of leaves, plant height, shoot dry weight and root dry weight, was used as Al tolerance indicator, while in the latter, it was RTDW. Here, RTDW as a whole plant indicator might be better than the separated parts in F_{Ai} as dry matter accumulation is concerned. On the other hand, in the former, measurements were taken only at V3 stage, while in the latter, four stages were considered. To combine the results from both studies, it seems that RTDW can recognize more variation and perform relatively stable among stages, along with its simple measuring procedure, and, therefore, might be better than F_{Ai} for evaluation of Al tolerance of germplasm accessions in sand culture.

In fact, choosing an appropriate indicator for Al-tolerance screening in soybean seedlings is an essential step toward achieving quicker and more accurate results. In sand culture, a trait that is simple, easy, fast and economical to undertake is desirable. In this study, five single indicators were compared, and among them, RTDW was chosen due to its relatively better stability, higher correlation with other indicators and easier measuring procedure than the others. Further, RTDW was preferred, though the difference of effectiveness in detecting Al tolerance among the five indicators was not very large because RTDW was likely to be representative of the other traits. Actually, RTDW is a reflection of various growth and developmental processes and is virtually the indicator for valuating whole plant tolerance to stress (Foy, 1996). A strong positive phenotypic correlation between several traits suggests an opportunity for indirect selection (Falconer, 1989). For example, in this study, selection for RTDW might result in a concurrent increase of other traits. In addition, the other indicators of this study were effective measures of Al tolerance as well but were considered subsidiary to RTDW. Subsidiary indicators such as RTRL and RRSA in this study might be related to constitutive or adaptive tolerance (Bernardo, 2008). Use of RTRL and RSA requires specific equipments and more labor or time to accomplish; nevertheless, the joint use of RTDW and subsidiary indicators can assure an incorporative, accurate and efficient screening.

Stage of seedling growth also determines speed and accuracy of screening. Too old seedlings may prolong screening period and yet cost ineffective, while too young seedlings may not have sufficient variability. Importance of seedling age for screening for abiotic stresses has been documented in other crops, such as drought (Zaidi et al., 2004) and low K in cotton (Tian et al., 2008). In this study, appropriate stage was decided as V5 (about 42 days after planting) which coincided with screening at about 28 days after treated with Al stress. At this stage, it was suggested that genotypes were neither too young to compromise for genetic variability expression nor too old to be wasteful of resources. Moreover, at V5, RTDW better separated materials into more discernable ranks of superiority in Al tolerance (Fig. 1). Although there were inconsistencies in rankings of genotypes due to genotype by leaf stage interactions, there was no essential difference in the members of the Al tolerance categories.

In summary, the present study suggests to use RTDW at V5 stage as a major Al tolerance indicator to replace F_{Ai} and, based on it subsidiary indicators, can be used to assure the accuracy as it is needed.

5 Conclusion

Comparisons among indicators at various stages of seedling growth identified RTDW as simple, easy and relatively stable indicator with better detection of difference in Al tolerance among genotypes under sand culture. RSDW and RRDW could as well be used, but RTRL and RRSA require specific equipments. The seedling age applicable for screening was not definitive, but V5 appeared to compromise between time wasted resulting from screening relatively older seedlings at later stages and low genetic variability at younger stages (V3). Superior Al-tolerant cultivars identified were PI.509080 (from North Korea), N24282, N23533 (Northeast China), PI.159322 (USA) and the check PI.416937 (Japan).

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