Hydroponic Screening for Selection of Aluminium Tolerant Rice (*Oryza sativa* L.) Genotypes at Seedlings Stage Using Different Indices

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In acid soil, Al is solubilised into a phytotoxic form, $Al(H_2O)_6^{3+}$ which is known as Al^{3+}. Al toxicity is the primary growth-limiting factor for plants in acid soils. Breeding of rice for Al tolerance are important approach for increasing grain yield in acid soils. In the present endeavour, rice genotypes were screened at seedling stage based on vigour index, root tolerance index and hematoxylin staining in stressed nutrient solutions to select the tolerant genotype(s) against Al toxicity. It was observed that use of different screening indices for Al toxicity tolerant genotypes of rice have given different results. Thus, screening of tolerant genotypes using one index may lead to inappropriate conclusion. Comparing all the selection indices it was found that Radhunipagal and UBKVR-16 were the common genotypes which fallen into tolerant class for every index. Finally genotypes were grouped into different clusters using D^2 statistic to find out whether the tolerant genotypes fall into one cluster. Those two Al toxicity tolerance genotypes were grouped into one cluster, which strengthens our findings.

Keywords: rice, Al-toxicity, seedling vigour, root tolerance index, hematoxylin staining, D^2 statistics

Introduction

Al is one of the most abundant mineral in the soil, comprising approximately 7%. At neutral or weakly acidic pH, Al exists in the form of insoluble aluminosilicate or oxide. It has been estimated that over 50% of the world's potentially arable lands are acidic (Von Uexküll and Mutert 1995). Although the poor fertility of acid soils is due to a combination of mineral toxicities (Al and Mn) and deficiencies (P, Ca, Mg and Mo), Al toxicity is the most important factor, being a major constraint for crop production on 67% of the total acid soil area (Eswaran et al. 1997).

$Al(H_2O)_6^{3+}$ which is known as Al^{3+} is dominant in acid soil below pH 5.0 and is the most toxic form. Al toxicity is the primary growth-limiting factor for plants in acid soils (Foy 1992) and is most severe in soils with low base saturation, poor in Ca and Mg. It af-

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fects the productivity and limits the agricultural expansion, mainly on acid uplands and low land with acid sulphate soils throughout the tropics. A severe inhibition of root growth is the major direct effect of Al-toxicity on plants. Such reduction in root growth generally restricts water and nutrient uptake, which leads to poor growth by disturbing plant metabolism. Furthermore, Al-toxicity inhibits shoot growth, too, by inducing nutrient (Mg, Ca and P) deficiencies, drought stress, and hormonal imbalances. At the cellular level, Al affects cell walls, membrane lipids and nucleic acids and induces cell autolysis, as well as, calcium deficiency.

A large area of rice cultivation falls under problem soils whose pH is < 5.0 and it is the growth limiting factor leading to reduction in yield of rice. Amendment of soils sometimes helps to avoiding Al-stress, but often it is either insufficient or too expensive. Since tolerance is genetically determined, selection is possible for better Al toxicity tolerance. In the present study, an attempt was made to screen rice genotypes against Al toxicity at seedling stage using different indices, namely vigour index, root tolerance index and hematoxylin staining.

Materials and Methods

Materials

Forty rice genotypes were used in this study. The study materials consists of fourteen released varieties, one hybrid, eight aromatic local cultivars, one high yielding scented variety, eleven advanced lines developed at Uttar Banga Krishi Viswavidyalaya, four advanced lines of IET received from DRR, Hyderabad and one maintainer line-IR58025B.

Seedling vigour

For the germination experiments, seeds were placed in 9.0 cm Petri dishes on two layers of filter paper moistened with test solution. Each treatment consisted of two replicates of 100 seeds. Germination counts were made daily for 16 days. Germination was considered when the radicle protruded 2 mm long.

The vigour index (VI) was calculated using the following equation (Dhindwal et al. 1991; Sparg et al. 2005):

$$VI = (shoot\ length + root\ length) \times germination\ percentage.$$

Values for different classes of tolerance have been decided as follows:
– The difference between highest and lowest values of vigour index, i.e. 2421.94 – 1216.14 = 1205.78
– Then, the difference has been divided by 3, i.e. 1205.78/3 = 401.92
– One part of the difference, i.e. 401.92 has been added to the minimum value of vigour index, i.e. 1216.14 + 401.92 = 1618.06 ≅ 1618.00
  The genotypes bearing values of vigour index below 1618.00 were considered as susceptible.

– Again, 401.92 has been added to 1618.00 to find out the moderately tolerant class, i.e. $1618.06 + 401.92 = 2019.98 \cong 2020.00$
The genotypes bearing the values of vigour index ≥ 1618.00 and < 2020.00 were considered as moderately tolerant and the genotypes bearing the values of vigour index ≥ 2020.00 were considered as tolerant toward Al toxicity at 30 ppm.

Solution culture

A liquid hydroponic solution was prepared following Yoshida's solution (Yoshida et al. 1976). The pH was adjusted to 3.5 ± 0.15 with 1N H_2SO_4 before addition of Al to prevent precipitation. Each genotype was subjected to three different concentrations (30, 60 and 90 ppm) of Al in the form of $AlCl_3.6H_2O$ along with a control.

We have modified the method of hydroponic screening for Al toxicity. It was performed in culture tubes of 25×150 mm size. For floating of germinated seeds, a 2.0 mm thick thermocol piece of 23 mm diameter was placed so that the thermocol along with the seeds (and then seedling) can freely move up and down as level of the liquid nutrient in the culture tube changes. Three holes were made in the thermocol piece for inoculation of germinated seeds. The culture tubes were place on the culture stands with a holding capacity of 18 culture tubes per culture stand.

The culture tubes were filled with Yoshida's nutrient solution leaving 1.0 cm from top. The seeds of each genotype were surface sterilized by dipping the seeds in 2% (w/v) Bavistin solution for 10 minutes. Treated seeds were rinsed with autoclaved distilled water and incubated on autoclave-sterilized tissue paper for germination. On 5th day after soaking, the seeds with 0.5–0.8 cm long coleoptiles were chosen for inoculation on the nutrient solution having different concentrations of Al. Three germinated seeds of each genotype were placed on the thermocol in two replications. The radical of each seed has passed through the hole in the thermocol in such a way that it touched the nutrient medium. Cultures were kept in control environment at 25 ± 2ºC under 16/8h light/dark cycle for 21 days. The culture medium was replaced every week with freshly prepared Yoshida's nutrient solution in respective treatment. The data were collected on 21st day after inoculation. The parameters considered for data collection were root length (cm) and shoot length (cm).

RTI was calculated as the maximum root length in Al stressed culture divided by maximum root length in control. Based on RTI values, the genotypes have been classified into three groups – i) tolerant (≥ 0.65), ii) moderately tolerant (0.35–0.64) and iii) susceptible (< 0.35).

Hematoxylin staining

Hematoxylin staining and scoring of stain intensity was done as described by Singh et al. (2009) and Roy and Bhadra (2013).

Statistical analysis

The experimental plan used was complete randomized blocks with 40 treatments (geno-types) per replication and two replications, in a total of 80 experimental units; each unit consisted of three seedlings. Statistical analyses of data were conducted with absolute val-ues, using Al concentration and cultivar as variables. The data were subjected to standard statistical methods of analysis of variance (ANOVA) using AgRes Statistical Software, (c) 1994 Pascal Intl Software Solutions, Version 3.01 and significant differences were compared by LSD. The analysis of data was used to interpret the results and draw conclu-sions.

Data were also subjected to multivariate analysis following Mahalanobis' D^2 statistics (Mahalanobis 1928, 1936) to measure the genetic divergence followed by the clustering of genotypes. D^2 statistics were performed using GenRes Statistical Software, (c) 1994 Pascal Intl Software Solutions, Version 3.01.

Results

Optimum Al concentration for screening

The optimal Al concentration for screening genotypes depends on the plant species being evaluated. However, optimum Al concentration also depends on the purpose of screening. If it is part of an on-going breeding program and the aim is simply to identify the most Al tolerant plants, higher Al concentrations can be applied. However, if the purpose is to quantitatively characterize the Al tolerance of genotypes, a lower Al concentration has to be applied to better separate the germplasm.
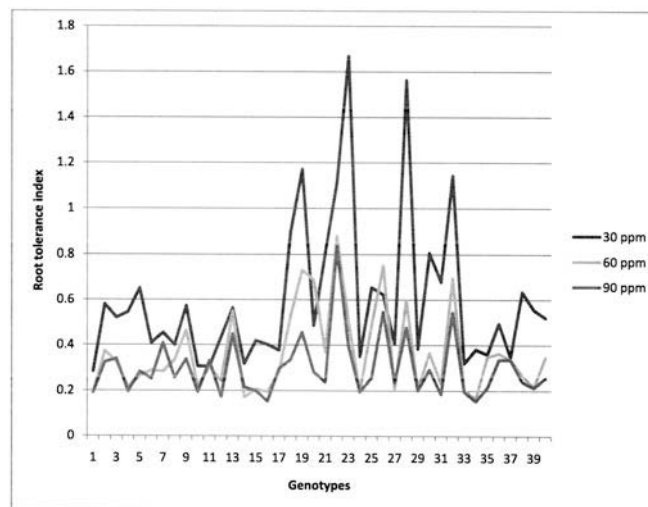

Figure 1. Relative tolerance index for forty rice genotypes at different concentrations of Al stresses.
Y-axis = RTI values, X-axis = genotypes

Table 1. ANOVA of vigour index, RTI and hematoxylin staining at 30 ppm of Al in nutrient solution

Source/treatment	d.f.	Mean sum of square		
		Vigour index	RTI	Hematoxylin staining
Total	79	120107.99	0.134	1.053
Treatments	39	169570.83**	0.229**	2.118**
Error	39	71881.72	0.042	0.014

** denote significance $P = 0.01$

In this present endeavour, a good degree of separation in rice genotypic responses could be observed only at 30 ppm of Al concentrations (Fig. 1). The higher concentrations, 60 and 90 ppm are highly toxic to the rice genotypes. Thus, 30 ppm of Al in nutrient solution has been considered for classification of the genotypes into three classes, namely tolerant, moderately tolerant and susceptible.

Vigour index

Vigour index showed significant variations among the varieties (Table 1). Vigour index varied from 1216.14 (Pusa Basmati-1) to 2421.94 (UBKVR-3) in 30 ppm, in nutrient solution (Table 2). Hede et al. (2002) used root vigour to classify the rye genotypes into different classes of tolerance to Al toxicity. In this endeavour, we used seedling vigour for classification of rice genotypes into three classes – tolerant, moderately tolerant and susceptible.

Root tolerance index

Significant difference was observed for RTI among the 40 genotypes (Table 1). Root length severely decreased with increased Al concentration in medium (Fig. 2). The RTI values at 30 ppm ranged from 0.284 to 1.668 (Table 2). As per the RTI values at 30 ppm of Al concentration, the local cultivar Radhunipagal seems to be the most tolerant genotypes. Other tolerant genotypes are Gobindobhog, Badshabhog, Kalobhog, UBKVR-11, UBKVR-16, UBKVR-18, Khasha and IET 22838 (Table 3). Rout and Das (2002) also suggested in their review article that the rice genotypes having RTI value more than 0.90, and may be classified as tolerant.

Hematoxylin staining

Quantitative measurement of hematoxylin staining showed significant variations among the genotypes (Table 1). Most of the aromatic local cultivars showed hematoxylin scoring near to one except Kalojeera and Chinikamani at 30 ppm of Al stress in nutrient solution (Table 2).

Based on staining the genotypes were classified into three groups – (i) tolerant (no staining or partial staining), (ii) moderately tolerant (moderate staining) and (iii) susceptible (deep staining). Tolerant genotypes were IET 22838, Gobindobhog, Kalobhog, Khasha, Badshabhog, Radhunipagal, Mohanbhog, Pusa Basmati-1, UBKVR-18, UBKVR-16 and UBKVR-6 (Table 3).

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Table 2. Mean values of vigour index, RTI and hematoxylin staining of 40 genotypes
under 30 ppm of Al concentration in nutrient solution

Genotypes	Treatments					
	Vigour index		RTI		Hematoxylin staining	
					Scoring	Intensity
Annada	1786.69	cdefghijk	0.284	j	3.38 k	Deep
Satabdi	1988.63	abcdefghi	0.580	efghij	3.88 m	Deep
MTU 1010	1993.03	abcdefghi	0.521	ghij	3.13 ij	Deep
MTU 1075	2130.81	abcdef	0.545	fghij	3.00 hi	Deep
Parijat	2129.76	abcdef	0.652	defghij	1.88 d	Moderate
Gontr-Bidhan-1	2042.00	abcdefgh	0.409	ghij	3.00 hi	Deep
MTU 7029	1634.30	fghijkl	0.454	ghij	3.25 jk	Deep
IR 64	1941.25	abcdefghi	0.401	bcde	3.63 l	Deep
IET 5656	1794.30	cdefghijk	0.573	efghij	3.00 hi	Deep
Pratikha	1386.11	jkl	0.307	ij	3.88 m	Deep
Aiswarya	1329.24	kl	0.306	ij	3.25 jk	Deep
Masuri	1537.42	ghijkl	0.435	ghij	3.13 ij	Deep
Krishna Hamsa	1540.48	ghijkl	0.564	fghij	2.63 g	Moderate
IR58025B	1487.25	ijkl	0.318	ij	3.25 jk	Deep
IET 21255	1839.57	bcdefghijk	0.419	ghij	3.00 hi	Deep
Heera-2	1591.27	fghijkl	0.403	hij	3.88 m	Deep
BRI-dhan-29	1504.41	hijkl	0.378	hj	4.00 m	Deep
IET 22838	1609.71	fghijkl	0.898	bcdef	1.13 ab	Partial
Gobindobhog	1607.00	fghijkl	1.172	b	1.00 a	Partial
Kalobhog	1755.00	defghijkl	0.487	bcd	1.00 a	Partial
Khasha	1605.87	fghijkl	0.809	bcdefg	1.13 ab	Partial
Badshabhog	1783.31	cdefghijk	1.116	bc	1.00 a	Partial
Radhunipagal	2117.50	abcdef	1.668	a	1.25 b	Partial
Kalojeera	2079.25	abcdefg	0.350	ij	2.88 h	Deep
Mohanbhog	1822.11	bcdefghijk	0.655	defghij	1.63 c	Partial
Chinikamani	1731.52	defghijkl	0.624	defghij	2.63 g	Moderate
Pusa Basmati-1	1216.14	l	0.407	ij	1.00 a	Partial
UBKVR-11	1920.37	abcdefghij	1.562	a	2.13 e	Moderate
UBKVR-15	1677.80	efghijkl	0.384	hij	3.00 hi	Deep
UBKVR-18	1979.25	abcdefghi	0.805	bcdefgh	1.13 ab	Partial
UBKVR-19	2361.31	ab	0.679	cdefghi	2.38 f	Deep
UBKVR-16	2025.18	abcdefghi	1.144	b	1.00 a	Partial
UBKVR-4	1629.93	fghijkl	0.320	ij	4.00 m	Deep
UBKVR-8	2226.04	abcd	0.381	ij	4.00 m	Deep
UBKVR-3	2421.94	a	0.360	ij	3.63 l	Deep
UBKVR-9	2309.52	abc	0.496	ghij	3.00 hi	Deep
UBKVR-1	1711.30	defghijkl	0.346	ij	3.88 m	Deep
UBKVR-6	2195.13	abcde	0.635	defghi	1.50 bc	Partial
KMR-3	1962.82	abcdefghi	0.556	fghij	3.25 jk	Deep
IVT4007-B	1555.22	ghijkl	0.521	ghij	3.00 hi	Deep
Range	1216.14–2421.94		0.284–1.668		1.00–4.00	
Mean	1823.99		0.634		2.66	

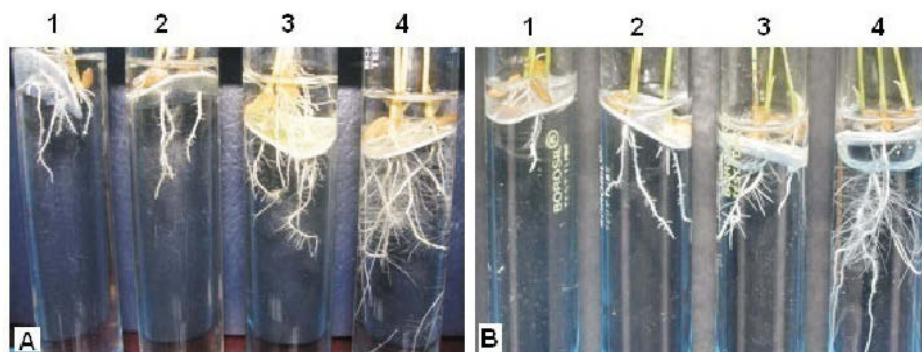Values bearing same letter in the column are not significantly different at P = 0.01 of LSD

Figure 2. Effects of Al on root growth. A) UBKVR-3, 1. 90 ppm of Al, 2. at 60 ppm of Al, 3. at 30 ppm of Al and 4. Control. B) IET 5656, 1. 90 ppm of Al, 2. at 60 ppm of Al, 3. at 30 ppm of Al and 4. Control. There is notable reduction in root length in stressed environments as compared to control environment

Table 3. Common tolerant genotypes of three different indices

Particular	Screening indices		
	1. Seedling vigour index	2. Root tolerance index	3. Hematoxylin staining
Tolerant genotypes	MTU 1075, Radhunipagal, Kalojeera, UBKVR-3, UBKVR-6, UBKVR-8, UBKVR-9, UBKVR-16, UBKVR-19	Radhunipagal, Gobindobhog, Badshabhog, Kalobhog, UBKVR-11, UBKVR-16, UBKVR-18, Khasha, IET 22838	IET 22838, Gobindobhog, Kalobhog, Khasha, Badshabhog, Radhunipagal, Mohanbhog, Pusa Basmati-1, UBKVR-18, UBKVR-16, UBKVR-6
Comparing 1&2	*Radhunipagal, UBKVR-16*		
Comparing 1&3	*Radhunipagal,* UBKVR-6, *UBKVR-16*		
Comparing 2&3	*Radhunipagal,* Gobindobhog, Badshabhog, Kalobhog, Khasha, *UBKVR-16,* UBKVR-18		
Comparing all	*Radhunipagal, UBKVR-16*		

Clustering of genotypes based on D^2 statistics

The significant difference signifies the appropriateness of the use of D^2 statistics for clustering of the genotypes into different groups. Data were subjected to multivariate analysis following Mahalanobis' D^2 statistics (Mahalanobis 1928, 1936) to measure the genetic divergence followed by the clustering of genotypes.

   Based on relative magnitude of D^2 values, 40 genotypes were grouped into five clusters (Table 4). Eighteen genotypes were accommodated in Cluster I, while Cluster II consisted of 12 genotypes, Cluster III had four genotypes, Cluster IV had five, and finally the Cluster V retained only one genotype. The pattern of distribution of genotypes from diverse geographical region into different clusters is random except the Clusters III and V. The members of those clusters are all advanced lines developed at UBKV, Pundibari. However, remaining three clusters accommodated their members from different groups of rice, such as traditional cultivars, high yielding varieties and advanced lines. Cluster I consisted of high yielding varieties from different geographical origin and only one advanced line of

Table 4. Clustering pattern, size and constituents of clusters involving 40 genotypes
of rice through D^2 statistics

Cluster	Genotypes	No. of genotypes
I	Annada, Satabdi, Parijat, Gontr-Bidhan-1, Pratikha, Masuri, Krishna Hamsa, IR58025B, IR 64, IET 5656, MTU 1010, MTU 1075, MTU 7029, Aiswarya, Heera-2, IET 21255, BRI-dhan-29, UBKVR-1	18
II	IET 22838, Gobindobhog, Kalobhog, Khasha, Badshabhog, *Radhunipagal,* Kalojeera, Mohanbhog, Chinikamani, Pusa Basmati-1, UBKVR-11, *UBKVR-16*	12
III	UBKVR-15, UBKVR-18, UBKVR-19, UBKVR-9	4
IV	UBKVR-4, UBKVR-8, UBKVR-3, KRM-3, IVT4007-B	5
V	UBKVR-6	1

UBKVR-1, Pundibari. Cluster II accommodated all local aromatic cultivars from different parts of West Bengal along with one advanced line of IVT-B (DRR, Hyderabad), two advanced lines of UBKVR, Pundibari.

Discussion

Most of the genotypes showed high seedling vigour at control. Remarkable reduction in seedling vigour has been reported in stressed environments. Low seedling vigour has been identified as the factor mostly responsible for poor germination and uneven seedling establishment (Okelola et al. 2007). They observed distinct characteristics in seedling vigour and seed yield and identified seed germination, speed of germination, seedling vigour index, seedling emergence and seedling establishment as the most desirable seed vigour traits in rice. This shows that a satisfactory selection programme for improvement of these seed quality characters is possible in rice. Based on the above criteria of classification, nine genotypes were classified as tolerant, 19 genotypes as moderately tolerant and 12 genotypes as susceptible (Table 3). Hede et al. (2002) used root vigour to classify the rye genotypes into different classes of tolerance to Al toxicity.

Till date, there were no clear divisions for categorization of vigour index. In literature, simply it is available as high and low vigour without any distinct boundaries based on the magnitude of vigour index. In this endeavour, we have elaborated the method of classification considering the value of vigour index, which has been already elaborated in materials and methods.

The genotypes possessing the RTI value more than 0.80 were considered as tolerant to Al toxicity. Wu et al. (1997) also suggested similar classification for Al toxicity tolerance in rice. It is notable that the RTI values of six genotypes, Gobindobhog, Kalobhog, Badshabhog, Radhunipagal, UBKVR-11 and UBKVR-16 were greater than 1.0, indicating that Al concentration which is cytotoxic to susceptible genotypes could stimulate root growth of tolerant genotypes. This phenomenon has also been reported earlier in rice (Wu et al. 1997; Sivaguru and Paliwal 1993) and in other crops (Dinev and Stancheva 1993).

Root length is often used as a selection criterion for Al-toxicity tolerance. Relative root length has been used to asses Al-tolerance of rice (Zhang et al. 2007), sorghum (Furlani

and Clark 1981; Ohki 1987), and wheat (Kerridge et al. 1971). Wenzl et al. (2006) also used relative root length technique to screen Brachiariagrass Genotypes for Al toxicity tolerance ability. Relative root length and root length also used at International Rice Research Institute, Philippines (IRRI 1977) for screening of rice varieties.

Root growth study of rice (Roy and Mandal 2005), var. IR72 somaclones at R3 generation showed normal root growth and distribution in response to Al-toxicity at seedling stage in Al-toxicity tolerant lines. Notably, root length of tolerant somaclones remains almost unaltered across stress gradients. Inhibition of root growth was a typical effect of Al, and the extent of the inhibition depended on both cultivar and Al concentration. Assessment of Al tolerance based on root growth and RTI has been used extensively in genetic and molecular studies (Somers et al. 1996).

Hematoxylin, a dye commonly used in cytogenetic studies, has also been used as a precocious, non-destructive way of studying Al sensitivity in plant species (Wagatsuma et al. 1995). Wenzl et al. (2006) also used hematoxylin staining to screen Brachiariagrass genotypes for Al toxicity tolerance ability.

An important aspect of this technique is that the reaction between hematoxylin and Al is specific, such that other stressing factors would exert a minimal effect, if any, on the evaluation processes of the Al effects. This technique proved conducive in identifying tolerant and sensitive genotypes after a very short exposure time of seedlings to Al, well before differences in the seminal root length become detectable.

Due to its quick, inexpensive, and easy screening protocol, the hematoxylin method is a very efficient way to evaluate large numbers of seedlings from segregating populations derived from elite germplasm. The root growth method (including the RTI parameter), in which the root length of every single plant has to be measured after Al treatment, is more laborious than the hematoxylin method. The extra cost of the root growth method is justified when screening new or exotic germplasm, such as gene bank accessions. The RTI parameter will identify genotypes that may have superior alleles for Al tolerance, even though their genetic background may lack desirable agronomic characters such as plant and root vigour. Similarly, the vigour index also time taking process, laborious and additionally it requires seedling shoot length.

All the three screening methods were compared in all possible ways for selecting Al toxicity tolerant genotypes (Table 3). Radhunipagal and UBKVR-16 were found to be tolerant comparing seedling vigour index and root tolerance index. When the seedling vigour index and hematoxylin staining methods were compared, Radhunipagal, UBKVR-6 and UBKVR-16 were found to be tolerant. Comparing the root tolerance index and hematoxylin staining seven genotypes were found to be tolerant, namely Radhunipagal, Gobindobhog, Badshabhog, Kalobhog, Khasha, UBKVR-16 and UBKVR-18. The most important is the consideration of all the screening indices to find out the best tolerant genotypes. Radhunipagal and UBKVR-16 were the common Al toxicity tolerant genotypes under all screening methods. Thus these two genotypes may be considered as the Al toxicity tolerant. The above comparison showed that the use of one index for screening of tolerant rice genotypes using hydroponic culture may produce misleading results. Thus it is suggested to use more than one index for screening of tolerant genotypes of rice using hydro-

ponic culture. Use of different screening indices at a time on a group of rice genotype for selection of Al toxicity tolerant genotype(s) will minimize the weakness of one method by the others and strengthen the selection potentiality.

Finally our intension was to see whether the tolerant genotypes identified through different indices are being grouped into single cluster. From the Table 3, it was found that Radhunipagal and UBKVR-16 are the common genotypes which fallen into tolerant class for every index used for screening tolerant genotypes against Al toxicity. These two genotypes were grouped into one cluster, i.e. Cluster II, which further strengthens our findings.

The study materials may be classified into three groups, namely high yielding varieties, traditional local cultivars and newly developed advanced lines. The traditional local varieties were grouped either in tolerant or moderately tolerant classes based on RTI and haematoxylin staining. Only two genotypes – Gobindobhog and Khasha were grouped under susceptible class when vigour index was used for classification. Howeler and Cadavid (1976) also suggested that the traditional tall cultivars of rice have higher Al tolerance ability. Use of D^2 statistics for strengthening the correctness of screening of genotypes for abiotic stress tolerance was done for the first time.

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