Indian J. Genet., 73(2): 194-198 (2013)
DOI : 10.5958/j.0975-6906.73.2.028

Short Communication

Hematoxylin staining of seedling roots is a potential phenotypic index for screening of aluminium tolerance in rice (*Oryza sativa* L.)

Bidhan Roy* and Sanjib Bhadra

Department of Genetics and Plant Breeding, Regional Research Station, Terai Zone, Pundibari, Cooch Behar 736 165

(Received: December 2012; Revised: April 2013; Accepted: April 2013)

Abstract

Hematoxylin staining is an early indicator of Aluminium (Al) toxicity effects on the apices of young, developing roots and has also been used as a precocious, non-destructive way of studying Al-sensitivity in plant species. $Al(H_2O)_6^{3+}$ which is known as Al^{3+} is dominant in acid soil below pH 5.0 and is the most toxic form. Presence of Al in the rhizosphere of rice in acid soil restricts root growth and significantly reduces the crop productivity. In this present study, 40 rice genotypes were screened for Al-toxicity tolerance based on the hematoxylin staining of root apex of rice seedlings. The genotypes were classified into tolerant, moderately tolerant and susceptible. Based on staining intensity the genotypes IET 22838, Gobindabhog, Kalabhog, Khasha, Badshahbhog, Radhunipagal, Mohanbhog, Pusa Basmati-1, UBKVR-18, UBKVR-16, UBKVR-6 were found tolerant against Al-toxicity in the nutrient solution at 30 ppm.

Key words: Rice root, hematoxylin staining, aluminium tolerance

Acid soils are base-unsaturated. The extent of base-unsaturation within pH range 5.0-6.0 generally varies from 16% to 67%. The active H^+ and Al^{3+} are bound to the clay. The $Al(OH)^{2+}$ is of minor significance and exists over a narrow pH range. The Al^{3+} is predominant below pH 5.0, $Al(OH)^{2+}$ between pH 4.7 and 6.5, $Al(OH)_3$ between 6.5 and 8.0 and $Al(OH)_4^-$ above pH 8.0. Acid soils are phytotoxic as a result of nutritional disorders, deficiencies, or unavailability of essential nutrients such as calcium, magnesium, molybdenum, and phosphorus, and toxicity of aluminum (Al), manganese, and hydrogen activity [1, 2] The solubility of soil compounds and, therefore, nutrient availability to plants is related to soil pH. In India, the acid soils are found in the Himalayan region, the eastern and north-eastern plains, peninsular India and the coastal plains under different agro-climatic situations. The soils occupy about 90 million hectares, constituting over one fourth of total geographical area of the country (NBSSLUP, Nagpur, India).

Plant genetic resources are a rich source of valuable traits that could be used to improve crop species. Species have genotypic differences in growth response to aluminum toxicity [3]. Yamamoto *et al*. [4], and Ishikawa and Wagatsuma [5] found that some ecotypes can be more tolerant to Al than others. Selection and breeding of Al resistant genotypes are important for increasing grain yield in acidic soil. The available screening methods for assessing Al tolerance in crops are based on the inhibition of root elongation in hydroponic culture and visual detection of Al tolerance levels by staining of seedlings root with hematoxylin. Laboratory and greenhouse based techniques to screen for Al tolerance are widely used because they are quick, highly accurate, non-destructive, and can be applied at early developmental plant stages. Considering the importance of the cultivation of tolerant genotypes of rice in acid soil to increase yield, an attempt was made to screen rice genotypes against Al toxicity at seedling stage using hematoxylin staining.

The grains of each genotype were surface

*Corresponding author's e-mail: bcroy10@yahoo.com
Published by Indian Society of Genetics & Plant Breeding, F2, First Floor, NASC Complex, PB#11312, IARI, New Delhi 110 012
Online management by indianjournals.com

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. The roots of seedlings grown for seven days, in the presence or absence of Al, were gently shaken in 200 ml distilled water for 15 minutes. The water was then replaced by 200 ml of aqueous hematoxylin solution [0.2% hematoxylin (Merck) and 0.02% potassium iodide, w/v] and left at the same slow agitation for 20 minutes. Finally, the solution was replaced again by 200 ml water, thereby repeating the first step. The root apices were excised and observed under a stereomicroscope to evaluate their staining pattern. Four root tips were examined for each treatment and photographed using digital camera.

Scoring was done based on the intensity of hematoxylin staining. Four seedlings per genotype per replication were scored arbitrarily with some modification of scoring of Cancado *et al*. [6] and Singh *et al*. [7]. Singh *et al*. [7] used 0 to 3 and Cancado *et al*. [6] used 0 to 5 scale as scoring system. Whereas, we have used 0 to scale as 4 scoring system. The 'no staining' and 'partial staining' seedlings were classified as tolerant, the 'moderate staining', seedlings as moderately tolerant, and those deeply stained as sensitive (Table 1). The average score of the four referees was considered for analysis.

The experimental plan used was complete randomized blocks with 40 treatments (genotypes) 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 average values, 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.

Hematoxylin staining is an early indicator of Al toxicity effects on the apices of young, developing roots grown in nutrient solution. This method also suggests the existence of different Al resistance mechanisms in rice, since the two resistant cultivars behaved differently. In the absence of Al, root tips remained unstained. In contrast, after a treatment with Al, a staining pattern consisting of three groups: (i) partial staining, (ii) an Moderate staining and (iii) deep staining.

A simple visual inspection of stained root apices from contrasting phenotypes revealed that hematoxylin was sufficient to discriminate between Al tolerant and Al sensitive genotypes. Hematoxylin staining showed significant variations across the genotypes (Table 2). Staining intensity showed almost similar trend across the Al concentrations in the nutrient solution for corresponding rice genotype (Table 3). The susceptible genotypes showed deep blue staining of root accompanied with short, thicker and abnormal root growth. The roots of the susceptible cultivars are also brittle in nature. These results corroborate with the findings of Roy and Mandal [8] and van Sint *et al*. [9]. The sensitive genotypes showed an intense blue color in the roots coupled with a severe epidermal degradation that extended from the elongation zone up to the root tip. This pattern of injury was in clear agreement with previous observations in pea-root apices [10]. Symptoms of Al injury appeared first on the root. The roots in the susceptible genotypes were short, thick, wrinkled, stubby and with zigzag bending along the length. This is in agreement with the reports of Singh *et al.* [7] in pea.

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 a part of an on going breeding program and the aim is simply to

Table 1. Average score based on hematoxylin staining

Staining	Average score	Class
No staining	0.00	Tolerant
Partial staining	> 0 to 1.75	Tolerant
Moderate staining	> 1.75 to 2.75	Moderately tolerant
Deep staining	> 2.75 to 4.0	Susceptible

Table 2. ANOVA of seedling characters for Hematoxylin staining

Source/ treatment	d.f.	Al concentrations		
		30 ppm	60 ppm	90 ppm
Total	79	1.053	1.044	1.048
Replication	1	0.078	0.153	0.094
Treatments	39	2.118**	2.091**	2.098**
Error	39	0.014	0.020	0.022

**Denotes significance P = 0.01, respectively

Table 3. Hematoxylin staining based on score mean

S.No.	Genotypes	30 ppm**		60 ppm**		90 ppm**	
		Score	Staining	Score	Staining	Score	Staining
1.	Annada	3.38 k	Deep	3.75 jk	Deep	4.00 k	Deep
2.	Satabdi	3.88 m	Deep	4.00 k	Deep	4.00 k	Deep
3.	MTU 1010	3.13 ij	Deep	3.00 fg	Deep	3.25 fgh	Deep
4.	MTU 1075	3.00 hi	Deep	3.00 fg	Deep	3.13 efg	Deep
5.	Parijat	1.88 d	Moderate	2.00 cd	Moderate	2.13 b	Deep
6.	Gontr-Bidhan-1	3.00 hi	Deep	3.13 h	Deep	3.38 hi	Deep
7.	MTU 7029	3.25 jk	Deep	3.38 i	Deep	3.50 hi	Deep
8.	IR 64	3.63 l	Deep	3.88 jk	Deep	4.00 k	Deep
9.	IET 5656	3.00 hi	Deep	3.25 gh	Deep	3.38 ghi	Deep
10.	Pratikha	3.88 m	Deep	3.88 jk	Deep	3.88 jk	Deep
11.	Aiswarya	3.25 jk	Deep	3.38 hi	Deep	3.50 hi	Deep
12.	Masuri	3.13 ij	Deep	3.25 gh	Deep	3.25 fgh	Deep
13.	Krishna Hamsa	2.63 g	Moderate	2.75 ef	Moderate	2.88 cde	Deep
14.	IR58025B	3.25 jk	Deep	3.38 hi	Deep	3.50 hi	Deep
15.	IET 21255	3.00 hi	Deep	3.00 fg	Deep	3.13 ef	Deep
16.	Heera-2	3.88 m	Deep	4.00 k	Deep	4.00 k	Deep
17.	BRI-dhan-29	4.00 m	Deep	4.00 k	Deep	4.00 k	Deep
18.	IET 22838	1.13 ab	Partial	1.25 ab	Partial	1.38 a	Partial
19.	Gobindabhog	1.00 a	Partial	1.13 ab	Partial	1.25 a	Partial
20.	Kalabhog	1.00 a	Partial	1.13 ab	Partial	1.13 a	Partial
21.	Khasha	1.13 ab	Partial	1.13 ab	Partial	1.25 a	Partial
22.	Badshahbhog	1.00 a	Partial	1.13 ab	Partial	1.13 a	Partial
23.	Radhunipagal	1.25 b	Partial	1.38 b	Partial	1.38 a	Partial
24.	Kalajeera	2.88 h	Deep	3.00 fg	Deep	3.13 ef	Deep
25.	Mohanbhog	1.63 c	Partial	1.75 c	Partial	1.88 b	Moderate
26.	Chinikamani	2.63 g	Moderate	2.75 ef	Moderate	2.88 cde	Deep
27.	Pusa Basmati-1	1.00 a	Partial	1.00 a	Partial	1.13 a	Partial
28.	UBKVR-11	2.13 e	Moderate	2.13 d	Deep	2.13 b	Moderate
29.	UBKVR-15	3.00 hi	Deep	3.00 fg	Deep	3.00 def	Deep
30.	UBKVR-18	1.13 ab	Partial	1.13 ab	Partial	1.13 a	Partial
31.	UBKVR-19	2.38 f	Deep	2.50 e	Moderate	2.63 c	Moderate
32.	UBKVR-16	1.00 a	Partial	1.13 ab	Partial	1.13 a	Partial
33.	UBKVR-4	4.00 m	Deep	4.00 k	Deep	4.00 k	Deep
34.	UBKVR-8	4.00 m	Deep	3.88 jk	Deep	3.88 jk	Deep
35.	UBKVR-3	3.63 l	Deep	3.63 ij	Deep	3.63 ij	Deep
36.	UBKVR-9	3.00 hi	Deep	3.00 fg	Deep	3.00 def	Deep
37.	UBKVR-1	3.88 m	Deep	3.88 jk	Deep	3.88 jk	Deep
38.	UBKVR-6	1.50 bc	Partial	2.50 e	Moderate	2.75 cd	Moderate
39.	KMR-3	3.25 jk	Deep	3.25 gh	Deep	3.38 ghi	Deep
40.	IVT4007-B	3.00 hi	Deep	3.13 gh	Deep	3.25 fgh	Deep
	Range	1.00-4.00		1.00-4.00		1.13-4.00	
Mean		2.66		2.74		2.83	

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

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.

In this present endeavour, 30 ppm of Al in nutrient solution has been considered for classification of the genotypes into three classes, namely tolerant, moderately tolerant and susceptible. A single visual inspection of stained root apices from contrasting phenotypes revealed that hematoxylin was sufficient to discriminate between Al tolerant and Al susceptible genotypes. Based on staining the genotypes were classified into three groups: (i) tolerant (no staining or partial staining), (ii) moderately tolerant to Al toxicity (moderate staining) and (iii) susceptible (dark staining) (Table 4). Tolerant genotypes are IET 22838, Gobindabhog, Kalabhog, Khasha, Badshahbhog, Radhunipagal, Mohanbhog, Pusa Basmati-1, UBKVR-18, UBKVR-16 and UBKVR-6. Moderately tolerant genotypes are Parijat, Krishna Hamsa, Kalajeera, Chinakamani, UBKVR-11 and UBKVR-19. Remaining 23 genotypes were susceptible to higher concentrations of Al.

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 [10-13], including maize [14]. Wenzl *et al*. [15] also used hematoxylin staining to screen *Brachiaria* grass genotypes for Al toxicity tolerance ability.

Hematoxylin staining has been widely used for direct visualization and localization of Al in root tissues [12]. It is a useful approach for macroscopically detecting Al accumulation in root tips by the formation of an intense blue coloration in the root tips of sensitive genotypes. This reaction occurs by the oxidation (in the presence of $NaIO_3$) of hematoxylin to hematyn, which in the presence of Al produces nucleic acid coloration.

Hematoxylin staining is an early indicator of Al toxicity effects on the apices of young, developing roots grown in nutrient solution. Al-toxicity tolerance is largely expressed by the exclusion of Al from roots or its binding, thereby avoiding absorption and toxification. This dye has the property of turning blue when it forms a complex with Al so that the penetration and retention of this ion in the roots can be assessed [13]. Therefore, the color intensity of stained root apices grown in nutrient solution can be a direct and quantitative measure of Al sensitivity [16] because susceptible genotypes tend to accumulate higher amounts of Al in their root tissues [11]. 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.

In this present endeavour it may be concluded that the hematoxylin staining is a rapid and non-destructive method for screening of large number of rice genotypes against Al-toxicity. Our results, taken together with other previous data [6, 7], clearly indicated that this dye infact able to distinguish, early in root development, Al-tolerant rice genotypes from Al-sensitive ones. The tolerant genotypes as classified based on hematoxylin staining may be directly introduced in Al-toxic soil for crop cultivation or those tolerant genotypes may be used as donor in breeding programmes for development of Al-toxicity tolerant rice.

Table 4. Classification of 40 rice genotypes based on staining score

Scoring value	Classes	Genotypes
< 1.75	Tolerant	IET 22838, Gobindabhog, Kalabhog, Khasha, Badshhabhog, Radhunipagal, Mohanbhog, Pusa Basmati-1, UBKVR-18, UBKVR-16, UBKVR-6
1.75-2.75	Moderately tolerant	Parijat, Krishna Hamsa, Kalajeera, Chinakamani, UBKVR-11, UBKVR-19
> 2.75	Susceptible	Annadan, Satabdi, IR64, MUT 1010, MTU 1075, MTU 7029, Gotra-Bidhan-1,IET 5656, Pratikha, Aiswarya, Masuri, IR58025B, IVT4007-B, IET 21255, Heera-2, BRI-dhan-29, UBKVR-1, UBKVR-3, UBKVR-4, UBKVR-8, UBKVR-9, UBKVR-15 and KMR-3

References

1. **Carver B. F. and Ownby J. D.** 1995. Acid soil tolerance in wheat. Adv. Agron., **54**: 117-173.
2. **Jayasundara H. P. S, Thomson B. D. and Tang C.** 1998. Responses of cool season grain legumes to soil abiotic stresses. Adv. Agron., **63**: 77-151.
3. **Macedo de C. C., Kinet J. M. and van Sint J. V.** 1997. Effects of duration and intensity of aluminum stress on growth parameters in four rice genotypes differing in aluminum sensitivity. J. Plant Nutr., **20**: 181-193.
4. **Yamamoto Y., Masamoto M., Rikiishi S., Hachiya A., Yamaguchi Y. and Matsumoto H.** 1996. Aluminum tolerance acquired during phosphate starvation in cultured tobacco cells. Plant Physiol., **112**: 217-227.
5. **Ishikawa S. and Wagatsuma T.** 1998. Plasma membrane permeability of root-tip cells following temporary exposure to Al ions is a rapid measure of Al tolerance among plant species. Plant Cell Physiol., **39**: 516-525.
6. **Cançado G. M. A., Loguercio L. L., Martins P. R., Parentoni S. N., Paiva E., Borém A. and Lopes M. A.** 1999. Hematoxylin staining as a phenotypic index for aluminum tolerance selection in tropical maize (*Zea mays* L.). Theor. Appl. Genet., **99**: 747-754.
7. **Singh D., Rai A. K. and Panyang O.** 2009. Hematoxylin staining as a potential Screening technique for aluminium tolerance in pea (*Pisum sativum* L.). Curr. Sci., **96**: 1029-1030.
8. **Roy B. and Mandal A. B.** 2005. Towards development of Al-toxicity tolerant lines in indica rice by exploiting somaclonal variation. Euphytica, **145**: 221-227.
9. **van Sint J. V., Costa de Macedo C., Kinet J-M. and Bouharmont J.** 1997. Selection of Al-resistant plants from a sensitive rice cultivar, using somaclonal variation *in vitro* and hydroponic culture. Euphytica, **97**: 303-310.
10. **Wagatsuma T., Ishikawa S., Obata H., Tawaraya K. and Katohda S.** 1995. Plasma membrane of younger and outer cells is the primary specific site for aluminum toxicity in roots. *In*: Plant-soil interactions at low pH: principles and management, Date RA, Grundon NJ, Rayment GE, Probert ME (ed.). Kluwer Academic, the Netherlands: 271-278.
11. **Carver B. F., Inskeep W. P., Wilson N. P. and Westerman R. L.** 1988. Seedling tolerance to aluminum toxicity in hard red winter wheat germplasm. Crop Sci., **28**: 463-467.
12. **Rincón M. and Gonzales R. A.** 1992. Aluminum partitioning in intact roots of aluminum-tolerant and aluminum-sensitive wheat (*Triticum aestibum* L.) cultivars. Plant Physiol., **99**: 1021-1028.
13. **Delhaize E., Craig S., Beaton C. D., Bennet R. J., Jagadish V. C. and Randall P. J.** 1993. Aluminum tolerance in wheat (*Triticum aestivum* L.): uptake and distribution of aluminum in root apices. Plant Physiol., **103**: 685-693.
14. **Jorge R..A. and Arruda P.** 1997. Aluminum-induced organic acids exudation by roots of an aluminum-tolerant tropical maize. Phytochemistry, **45**: 675-681.
15. **Wenzl P., Arango A., Chaves A. L., Buitrago M. E., Patino G. M., Miles J. and Rao I. M.** 2006. A Greenhouse Method to Screen Brachiariagrass Genotypes for Aluminum Resistance and Root Vigor. Crop Sci., **46**: 968-973.
16. **Ruiz-Torres N. A. and Carver B. F.** 1992. Genetic expression of aluminum tolerance in hard red winter wheat. Cereal Res. Com., **20**: 233-240.