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Research Article

Evaluation of seedling growth in fragrant rice (*Oryza sativa*) germplasms under salinity stress

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Abstract

Fragrant rice is said to be associated with poor agronomic traits and low tolerability to salt stress. This study was conducted to examine the relationship between fragrant intensity of Sri Lankan rice germplasms and their seedling growth under salt stress. It is reported that functional *badh2* homologue is associated with more salt tolerance than the nonfunctional fragrant alleles. Fifteen accessions that exhibited different levels of fragrance were selected and they were categorized into four fragrant intensity groups. Rice seedlings were grown under three salinity levels, 0.73 dSm^{-1} (control), 4 dSm^{-1} and 8 dSm^{-1} and their survival percentage, root length and shoot length were measured. A novel formula of Survival Index (SI) was derived from survival percentages and analysis of variance revealed that survival index under salinity stress was significantly associated with the fragrant intensity groups of rice. As the fragrant intensity increases, SI, root length and shoot length decreases. A fragrant accession that was exceptionally salt tolerant was also disclosed. Most of the Sri Lankan fragrant rice germplasms do not possess the predominant *badh2.1* fragrant allele but appeared containing a rare allele of *badh2.7*. These findings would be useful in rice improvement breeding programs designed for fragrance and salt tolerance**.**

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Keywords: *badh*2 homologue, fragrant intensity groups, allele, breeding, Sri Lanka, survival index.

Introduction

The fragrant trait of *Oryza sativa* L. constantly adds value to the quality of rice attracting consumer preference. Demand for high quality aromatic rice is increasing day by day together with the living standard of people. Basmati and Jasmine rice are the most popular two aromatic rice varieties in the world. 2-Acetyl-1-Pyrroline (2-AP) is the major compound responsible for the fragrance in Basmati and Jasmine style rice [1, 2, 3, 4]. However there are many other traditional aromatic rice which exhibit different volatile compounds other than 2AP [3, 5, 6, 7] and hence, fragrant intensity of a variety may be associated with presence of multiple volatile compounds produced by different biochemical pathways.

Bradbury *et al.* [8] reported that an 8 bp deletion in the 7th exon of *badh2* gene, referred as fragrant gene, located on 8th chromosome is responsible for 2AP production. The wild type *badh2* gene encodes the betaine aldehyde dehydrogenase enzyme (BADH2) which is reported to be associated with salt tolerance n rice [9, 10]. Recently, Michael *et al.,* [11] revealed other mutations that cause BADH2 enzyme non-functionality and these mutations scattered from 1st exon to 14th exon of *badh2*, were named as *badh2.1, badh2.2, badh2.3, badh2.4 and badh2.5, badh2.6, badh2.7, badh2.8, badh9* and *badh10.* It is reported that *badh2.2,* is associated with fragrance of some germplasms in China [12]. However, most of the popular cultivars including Basmati and Jasmine rice contain the predominant *badh2.1*.

Rice is salt sensitive crop and sensitivity of rice to salinity stress varies with the growth stage. In general, rice plants are sensitive to salinity stress at young seedling stages and less sensitive to it at reproduction [13, 14]. Heenan *et al*., [15] have reported that most rice varieties are extremely sensitive to salinity during young seedling stage and early developmental stages. Hence, improvement of salt tolerance of rice should be targeted at these growth stages that are more sensitive to salinity stress since it can significantly affect grain yield.

Basmati is the usual donor of the fragrant gene in the rice breeding programs although it shows the poor combining ability with some high yielding varieties [16]. It has been reported that aromatic rice are more susceptible to saline soil than non-fragrant rice [9, 10]. Therefore, it is necessary to evaluate growth performances of Sri Lankan traditional aromatic rice as many of them are commercially unexploited and they have the potential to be used in quality improvement breeding programs instead of Basmati. However, there are only few reports on high-quality aromatic rice linked to salt stress. Thus, this study was conducted to examine the relationship between fragrant intensity and seedling growth of Sri Lankan rice germplasms under salt stress and to detect the fragrant allele at *badh2* locus.

Materials and Methods

Plant materials

Seeds of 80 rice accessions were obtained from the Plant Genetic Resources Centre (PGRC), Sri Lanka and Rice Research and Development Institute (RRDI), Batalagoda, Sri Lanka. Fifteen accessions were selected for this study out of 80 accessions of which the fragrance was evaluated by KOH treated phenotypic method. The 15 accessions were comprised of 2 high yielding varieties, At401 and At405 that were derived from same parental lines, Pokkali, the well known salt tolerant variety, two Basmati varieties and 10 traditional accessions that are popular among commercial growers in Sri Lanka.

Fragrance evaluation

Aroma in leaves was determined according to method described by Sood and Siddiq [17] with 10 ml of 0.5% potassium hydroxide (KOH) solution. Basmati217 was used as the reference for the sensory aroma evaluation and the smell of Basmati217 was categorized as highly fragrant. The KOH treated leaf samples were smelt and fragrant intensity score (FIS) was assigned ranging from 0 to 3 which is indicated by 0: Non-fragrant, 1: Fragrant, 2: Highly fragrant and 3: Superior fragrant according to the procedure described by Amarawathi *et al*., [18] by a sensory evaluation panel of five members.

Detection of badh2 alleles

DNA was extracted from each accession and subjected to PCR amplification with the mixture consisted of 1.5 µl of 10X buffer, 1.2 µl of (2.5 mM) dNTPs, 1 µl (20 pmol/µl) of primers (**ESP**: TTGTTGGAGCTTGCTGATG, **IFAP**:CATAGGAGCAGCTGAAATATATACC, **INSP**:CTGGTAAAAAGATTATGGCTTCA, **EAP:**AGTGCTTTACAAGTCCCGC; [8] and 0.2 µl of (5 units/µl) Taq DNA Polymerase in total volume of 15 µl. Amplification was conducted in a single tube with four specific primers using thermal profile consisted with an initial denaturing at 95°C for 5 minutes followed by 35 cycles of 1 minute at 95°C, 30 seconds at 58°C of annealing temperature, 1 minute at 72°C and final extension cycle of 5 minutes at 72°C. Amplified PCR products were electrophoressed by 1.3% agarose gel followed by ethidium bromide staining. Same PCR protocol was applied to detect the 14th exon region amplicon with the primer BadHapG9 (Forward: CATTGAATTGGCCAACGATACTC and Reveres: GGCGTACTCCGTCACTTGCT [11] under 55°C annealing temperature and amplified fragment was subjected for sequencing.

Preparation of saline soil and establishment of seedlings

Saline soil was prepared based on the procedure described by Sirisena and Hemachandra [19] with modifications. The un-amended soil consisted of 75.2% of clay, 18.6% of silt and 6.2% of sand and the pH was 8.1 while the Electric Conductivity of Saturated Extract (ECe) of the soil was 0.73 dSm^{-1}. The calibration graph was plotted in order to determine the correct amount of NaCl to be added and accordingly, 0.1 g, 0.2 g, 0.3 g, 0.4 g, 0.5 g and 0.6 g of NaCl were added separately to 250 g soil samples with distilled water up to saturated level and samples were stirred overnight. ECe of each sample was measured at room temperature (30°C) after extracting water by a vacuum pump and the graph was plotted on ECe vs added weight of NaCl per 250 g of soil. Required amount of NaCl to maintain 4 dSm^{-1} and 8 dSm-^{1} was calculated by the graph. Plastic trays (42 cm x 32 cm x 6 cm sized) were filled with 5 kg of soil and three levels of ECe with 0.73 dSm^{-1} (control), 4 dSm^{-1} and 8 dSm^{-1} were established by saturating the soil with artificially prepared saline water added with estimated NaCl. Electrical Conductivity (EC) of prepared soil treatments also measured by inserting the portable EC meter- probe into the saturated soil and another graph was plotted on ECe vs EC to maintain the correct ECe throughout the experimental period. EC of saturated soil was monitored and de-ionized water was added if necessary to the soil tray to maintain correct EC. Each salinity treatment was replicated two times.

Seeds were soaked and after emerging radicals, five day old seedlings were transferred into trays prepared with three salinity levels, 0.73 dSm^{-1}, 4 dSm^{-1} and 8 dSm^{-1}. Seeds of each genotype were planted in a line keeping about 2 cm gap between plants comprising of 20 seedlings per accession.

Evaluation of seedlings by survival index (SI)

Seedling survival percentage of each accession was calculated once every three days after transplanting (DAT) till 21 days of the experiment. The results of seedling survival percentages were expressed as a weighted Survival Index [SI] to convert them into quantitative data. This index provides maximum weight to the seedlings that survive throughout the 21 DAT, and is calculated from the formula as follows; SI = (3xS1+6xS2+.....21xS7)/(3+6+..... 21) 100, where S1, S2...S7 are the survival percentages that had observed on 3rd, 6th,21st DAT. The generalized form of the formula was derived as,

$$SI = \sum_{k=1}^{n} D_k S_k \Big/ \left(\sum_{k=1}^{n} D_k\right) 100$$

where, D is the DAT, S is the survival percentage, n is the total period in DAT (in this experiment n is 21 DAT), D_k is the DAT at k^{th} data collection, S_k is the survival percentage of k^{th} data collection and k=1,2,3….n. The maximum index is 1.0 if all seedlings survived throughout the whole period, while the minimum index is 0 if all seedlings died on the first day of data taken.

Evaluation of seedlings by Standard Evaluation Score (SES) of visual salt injury

SES of visual salt injury at seedling stage was assigned to each accession from 1 to 9 which is indicated by 1: Highly Tolerant, 3: Tolerant, 5: Moderately Tolerant, 7: Susceptible and 9: Highly Susceptible as described by Gregorio *et al*., [20] for salt tolerance on the basis of the morphological appearance of seedling growth after 21 days of salinity treatment.

Evaluation of seedling growth by shoot length and root length

After 21 days of the salt stress, remaining seedlings were taken from soil media without damaging the roots. Shoot length and root length of seedlings of each accession were measured. Mean shoot length and mean root length were calculated and were analyzed to study the seedling growth performances under salinity stress over different FIS.

Statistical analysis

Three salinity treatments of 0.73 dSm^{-1}, 4 dSm^{-1} and 8 dSm^{-1} and four types of FIS, 0, 1, 2 and 3 were followed in a factorial experiment with completely randomized design. Mean SI, mean root length and mean shoot length were analyzed by analysis of variance (ANOVA) using Minitab version 15. The mean SI over FIS was analyzed by regression model after creating indicator variables for fragrant categories by Minitab version 15.

Results and Discussion

Fragrant evaluation and detection of badh2 alleles

The fragrant intensity of leaves was evaluated and assigned into four categories as shown in Table 1 and the results revealed that the strengths of the aroma were different among accessions. Out of 80 accessions tested (Data not shown) the traditional variety, Kuruluwee gave the highest fragrant intensity exceeding even the fragrance of Basmati217, the reference variety. There were several fragrant and highly fragrant accessions when compared to the Basmati217. Of all 80 accessions, only two local accessions, At 405 and Suwanda-samba exhibited the *badh2.1* allele. None of the Sri Lankan traditional fragrant accessions revealed the *badh2.1* allele at 7 th exon region and instead wild type allele was detected at the locus. Three traditional accessions, Suwanda-Al (Acc. No. 004366), Suwandal (Acc. No. 001064) and Kuruluwee (Acc. No. 004903) revealed a "G" insertion at the 14^{th} exon region and it was identified as the *badh2.7* allele reported by Michael *et al*., [11].

Evaluation of seedlings by survival index (SI)

In this study, in order to detect the relationship between fragrant intensities and seedling growth under salt stress, **s**urvival percentages of different accessions under salt stress were examined (Figure 1). SI was calculated using survival percentages in order to convert the data into a quantitative parameter. Analysis of variance revealed that SI is significantly (P <0.001) associated with different level of fragrant scores (Table 1). The regression analysis was conducted separately for4ECe and 8ECe salinity levels and the regression models were obtained as follows by using FIS as indicator variables

1. SI at 4ECe = $0.463 + 0.495\ (FIS_0) + 0.287\ (FIS_1) + 0.360\ (FIS_2)$
2. SI at 8ECe= $0.111 + 0.652\ (FIS_0) + 0.487\ (FIS_1) + 0.406\ (FIS_2)$

Table 1. Fragrant intensity score (FIS), alleles of *badh2*, standard evaluation score (SES) and survival index (SI) of tested accessions.

Accession No.	Accession name	FIS	Allele at 7th exon	Allele at 14th exon	SES of visual salt injury		SI	
					4Ec	8Ec	4Ec	8Ec
**	At 401	0	wild	*	1	1	1.000000	0.924286
**	Pokkali	0	wild	*	1	1	1.000000	0.857143
003521	Hondarawal	0	wild	*	1	5	0.896429	0.719643
006775	Basmati-fine	0	wild	*	1	5	0.892857	0.626786
011041	Kaluheenati	1	wild	*	3	3	1.000000	0.683929
003989	Kaluheenati	1	wild	*	3	3	1.000000	0.905357
003507	Suwanda-samba	1	*badh2.1*	*	3	5	0.928571	0.700000
003867	Hondarawala	1	wild	*	3	5	0.630357	0.532143
010729	Suwadal	1	wild	*	3	9	0.480357	0.480357
04366	Suwanda -Al	1	wild	*badh2.7*	5	9	0.708929	0.369643
010646	Suwadal	2	wild	*badh2.7*	5	9	0.692857	0.635714
008921	At 405	2	*badh2.1*	*	3	7	0.910714	0.626786
004759	Kuruluthuda	2	wild	wild	3	7	0.916071	0.510714
007018	Basmati217	2	*badh2.1*	*	5	9	0.771429	0.294643
004903	Kuruluwee	3	wild	*badh2.7*	7	9	0.462500	0.110714

** Obtained from RRDI. * Not sequenced

According to the coefficient obtained at 8ECe (Model: 1), non-fragrant rice (FIS_0) has an average of 0.652 higher SI than superior fragrant rice (FIS_3) while fragrant rice (FIS_1) has average of 0.488 higher SI than superior fragrant rice. The average difference between highly fragrant rice (FIS_2) and superior fragrant rice (FIS_3) was 0.406 at the ECe level of 8 dSm^{-1}. In general, accessions with higher fragrant scores showed the higher reduction in SI when the salinity stress was increased from 4 dSm^{-1} to 8 dSm^{-1}.

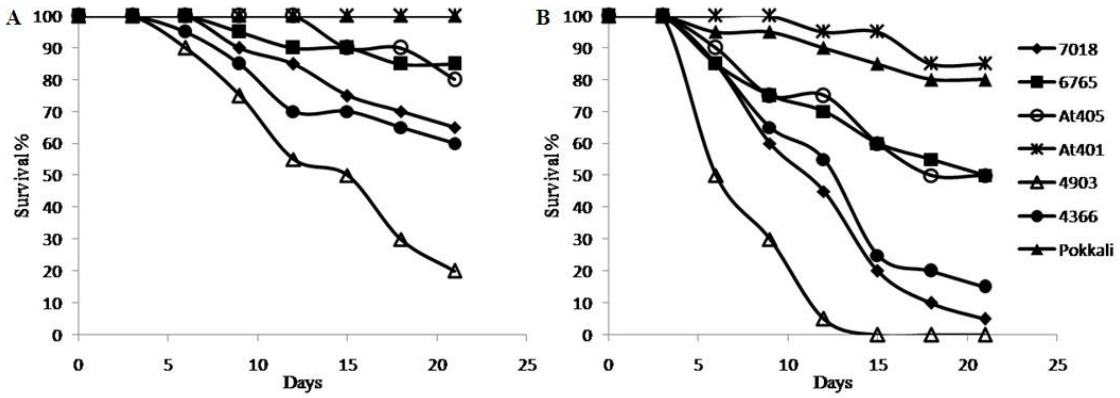

Figure 1. Survival percentages of different accessions under salt stress.

Non-fragrant accessions, At 401 and Pokkali, exhibited the highest SI in both 4$dSm^1$to 8 dSm^{-1} levels. Although Kaluheenati (Acc. No. 003989) was a fragrant accession it demonstrated highest SI as that of Pokkali in both 4 dSm^{-1} to 8 dSm^{-1} levels. Least SI was observed in Kuruluwee (Acc. No. 004903) which was the one and only superior fragrant accession detected out of 80 accessions. Basmati217 (Acc. No. 007018) also gave very low SI at 8ECe level. The results showed that the trend of the SI decreases as the fragrant intensity increases.

Evaluation of seedlings by SES of visual salt injury

The genotypes classified into five groups from highly tolerant (SES: 1) to highly susceptible (SES: 9) according to visual salt injury observations are presented in Table 1. None of the non-fragrant genotypes were ranked as susceptible or highly susceptible. All highly fragrant or superior fragrant genotypes exhibited either susceptible or highly susceptible phenotypes. Kaluheenati showed the exceptional tolerance even at 8 dSm^{-1} as that of salt tolerant check variety, Pokkali.

Evaluation of seedlings by shoot length and root length

Analysis of variance revealed that both shoot length and root length were significantly associated (P <0.001) with the fragrant intensity groups of rice. Means of the shoot length and root length are presented in Table 2. At 8 dSm^{-1} level, both mean shoot length and mean root length showed significant difference between non-fragrant and fragrant groups. In general, decreasing trend of shoot length and root lengths were observed as the fragrant intensity increases.

Table 2. Means of survival index, shoot length and root length of fragrant rice under salt stress.

Fragrance score / Salt level (ECe)	Non-fragrant	Fragrant	Highly fragrant	Superior fragrant
		Mean survival index		
4	0.9580[a]	0.7870[b]	0.8134[b]	0.6150[c]
8	0.76[a]	0.5975[b]	0.517[b]	0.1105[c]
		Mean shoot length (cm)		
4	18.3[a]	18.96[a]	16.27[b]	10.85[c]
8	13.78[a]	12.52[a]	11.81[a]	6.44[b]
		Mean root length (cm)		
4	4.09 [a]	3.06 [a]	3.58 [a]	4.03 [a]
8	3.42[a]	2.23[b]	2.56[b]	1.63[c]

Means followed by different letters (a, b and c) within each row show significant difference at P <0.05

This study was able to identify different intensity groups of rice and this probably may be due to the factor that fragrance is generated by other volatile compounds in addition to 2AP. Widjaja *et al*. [3] have reported that there are various volatile compounds associated with rice fragrance and hence, overall fragrance is likely to be rendered by complex interactions of volatile compounds leading to different fragrant intensities.

The *badh2.1* allele was detected only in two local rice varieties out of 80 accessions and one of them, At405, has been bred from Basmati parental line. This confirms that *badh2.1* allele is not a predominant allele in the Sri Lankan rice gene pool. Michael *et al*. [11] reported that there are other alleles of *badh2* causing the fragrance in rice. Proving that, Suwanda-Al (Acc. No. 004366), Suwandal (Acc. No. 001064) and Kuruluwee (Acc. No. 004903) contained a novel allele which was identified as *badh2.7* and it can probably be the causal factor for the fragrance of those lines.

The association between salt tolerance and fragrant phenotype was previously reported by Niu *et al*. [9] and Fitzgerald *et al*. [10]. Accordingly, the reason for the low tolerance to salinity has been explained by the loss of the function of BADH2 enzyme in fragrant rice. The role of BADH2 is the metabolism of gamma aminobutyraldehyde which is supposed to play a role in assisting abiotic stress tolerance in plants. Therefore, it was proposed that the absence of functional BADH2 leads to increased levels of 2AP and decreased level of GABA which ultimately lead to loss of salt tolerance

[10]. In this study most of the aromatic accessions appeared possessing non-functional *badh2* either at 7^{th} or 14^{th} exon and hence, it could be hypothesized there is a relationship between BADH2 and salt stress tolerance. The results of this study showed that SI decreases as the fragrant intensity increases. The similar trend of increasing salt susceptibility was observed with increasing fragrant intensity, when other parameters, SES, shoot length and root length were examined. Therefore, it is evident that Sri Lankan fragrant rice varieties have an association with the salt susceptibility at seedling stage.

Previously, germination index was used by the Khan *et al*. [21] to measure the growth performance of rice exposed to salinity stress. In this study, we derived the SI to measure the salinity stress for the first time based on the survival % of each accession. SI was created by giving the maximum weight to the plants that survived until the last day of the data taken and minimum weight was given to the plants that survived until only the first day of data taken. As the index distributed quantitatively, the variation of survivals among accessions were easily observed and analyzed.

Conclusion

In this study Kuruluwee was identified as a superior fragrant variety and thus this particular variety is worthy of being used in fragrance improving breeding programs. Kaluheenati showed highest SI as that of Pokkali, the salt tolerant check variety and therefore, further experiments are necessary to investigate Kaluheenati as a novel source of salt tolerance in gene mapping studies.

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