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IN VITRO SELECTION OF RICE SOMACLONAL VARIANTS FOR SALT TOLERANCE

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Abstract

An experiment was conducted at the Advanced Plant Breeding and Tissue Culture Laboratory of Bangabandhu Sheikh Mujibur Rahman Agricultural University to evaluate the effects of NaCl on rice callus induction and plant regeneration, along with genetic variability assessment through molecular markers. Dehusked seeds of five distinct rice genotypes (Dakshahi, Gondhakasturi, Guamasuri, Duksail, and Khazar) were put on MS medium supplemented with five levels (0, 0.2, 0.4, 0.6 and 0.8%) of NaCl. Developed calli were placed in MS medium for regeneration around 3-6 weeks later. Callus induction and plant regeneration declined with the increase of salinity levels. Dakshahi outperformed the other cultivars in both parameters at different salt concentrations, and it was the only cultivar with callus and regenerated plants at 0.08% NaCl concentrations. To amplify the genomic DNA of parents and somaclones of different varieties, five random amplified polymorphic DNA (RAPD) primers were used. Polymorphic bands were visible in OPD-07 primer. Dakshahi and Khazar were found to have the maximum genetic diversity (0.941). The genetic similarity coefficients between lines demonstrate that the degree of genetic diversity within rice genotypes was quite distinct. The cluster analysis grouped five genotypes into two clusters. The presence of genetic variation in the somaclones of Guamasuri implies the potential of RAPD markers in assessing genetic diversity and validating somaclonal variation. Also, Dakshahi demonstrating the highest tolerance for NaCl stress in both callus induction and plant regeneration, making it a promising candidate for salt-tolerant rice breeding in future.

Keywords: Callus, Cluster, Regeneration, RAPD, Rice, Salt, Somaclone.

Introduction

Rice (*Oryza sativa*) is a staple food consumed by more than half of the world's population. Many people rely on rice as a key source of carbohydrate and protein, particularly in Asia. The land type, ideal atmosphere, and people's preferences for rice as their primary dish create a good market, leading to extensive rice monoculture in many Asian countries including Bangladesh (Shelley *et al*., 2016; Rahman *et al*., 2020). Currently, Bangladesh ranks as the third-largest global rice producer after China and India, covering an area of 11.69 million hectares in 2022, with an average yield of 4.89

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tons/ha (FAOSTAT, 2022). However, in order to fulfill the growing demands due to the rapid population growth, it is estimated that production of rice would need to increase from 6.31 to 7.17 tons/ha by 2030 and 8.06 tons/ha by 2040 (Rabbi *et al.,* 2021). Since Bangladesh is one of the world's most populous countries, there is very little chance that the amount of arable land will be increased, hence high-yielding varieties of rice must be developed (Rabbi *et al.,* 2021).

Rice is grown in almost every part of Bangladesh (Mamun *et al.,* 2021) However, farmers in the southern zone, particularly those in coastal areas, are cautious to grow rice for one key reason is salinity (Shelley *et al*., 2016). Salinity is to blame for the unproductiveness of vast amounts of land in the southern part of Bangladesh (Baten *et al*., 2015; Kabir *et al*., 2021). Salinity tolerance of rice is defined as moderate (Shaheen *et al*., 2005). Rice production in saline soils could not be increased without the development of saline tolerant varieties. It is one of the best options for bringing these lands under cultivation through growing the saline tolerant rice varieties in this area (Kabir *et al*., 2021). So far Bangladesh Rice Research Institute (BRRI) and Bangladesh Institute of Nuclear Agriculture (BINA) have developed six and three saline tolerant rice varieties (BRRI, 2023; BINA 2023). Nevertheless, research to develop salt-tolerant rice varieties must be continued, respectively.

Plant breeders frequently employ local varieties and landraces as parents to generate new varieties since they are prime sources of necessary alleles and traits (Breseghello *et al*., 2013; Hasan *et al*., 2015). Aside from conventional breeding, tissue culture methods are also commonly used in breeding program, particularly in stress tolerance breeding program. Tissue culture is a source of genetic variability that arises from genetic modifications occurred during the *in vitro* culture process, a phenomenon known as somaclonal variation. The technique has been utilized by many researchers to create somaclonal variants and screen salt tolerance (Anwar *et al*., 2010; Pérez-Clemente *et al*., 2012). This screening can be done by selecting variants derived from *in vitro* somaclonal materials such as protoplasts, cell colonies, or callus. Moreover, generating somaclonal variants and combining them with conventional breeding produce fast and effective results (Krishna *et al.,* 2016), making it a powerful strategy for developing salt-tolerant varieties (Pérez-Clemente *et al.,* 2012. Production of new salinity resistant varieties using diverse biotechnological approaches would also require the establishment of regeneration protocols. The goal of this work was to create an effective *in vitro* culture system under various salt concentrations that would serve as a platform for future tissue culture research on improving salt tolerance in rice varieties.

Molecular markers provide genetic information that may be used to determine a species' distinctiveness and rank based on the number of adjacent relatives and phylogenetic relationship (Rahman *et al*., 2007). For determining the level of genetic diversity in rice, different types of molecular markers have been utilized, viz., RFLP (Botstein *et al*., 1980), RAPD (Williams *et al*., 1990), AFLP (Vos *et al*., 1995), SSR (Tautz 1989) and SNP. The RAPD analysis provided a simplistic and rapid way for determining genetic diversity among the genotypes. It nevertheless indicated a significant level of polymorphism even without any prior knowledge of the DNA sequence (Karp *et al*., 1997). Considering the above circumstances, the study was conducted to investigate

the consequences of NaCl at various concentrations on callus growth and plant regeneration. Also, the response of five rice genotypes to *in vitro* culture medium, along with their molecular diversity and validate their somaclones by RAPD markers.

Materials and Methods

Experimental location and materials

The experiment was carried out at Bangabandhu Sheikh Mujibur Rahman Agricultural University (BSMRAU), Salna, Gazipur, Bangladesh, at the Advanced Plant Breeding Laboratory of the Department of Genetics and Plant Breeding. In this study, the following rice genotypes were used as experimental materials.

Table 1. List of rice genotypes

SI. No.	Name of genotypes	Place of Collection
1.	Dakshahi	Rice Gene Bank of BRRI
2.	Gondhakasturi	
3.	Gua masuri	
4.	Duksail	
5.	Khazar	

Media preparation and salt concentrations

Healthy, disease-free explants for the study were chosen and then the seeds were dehusked. For callus induction, MS (Murashige and Skoog, 1962) media used supplemented with sucrose (30 g/L), 2, 4-D (2 mg/L), agar (10g/L), and five different salt (NaCl) doses. Callus pieces of suitable size were moved to MS regeneration medium treated with NAA (l mg/L), BAP (1 mg/L), sucrose (30 g/L), agar (l0 g/L), and five different salt concentrations for plant regeneration. The effect of salt was studied using five different amounts of NaCl salt solution (0, 0.2, 0.4, 0.6, and 0.8 %). (0, 34.2mM, 68.4 mM, 102.7 mM and 136.9 mM) or (0. 2000, 4000, 6000 and 8000 ppm)

Callus induction and regeneration

In a laminar air flow cabinet, the dehusked seeds were air dried and sterilized by wiping with 70% ethanol for 10 minutes before being placed on callus induction media. Those seeds were put in the culture to accomplish callus induction while keeping the temperature at 25±2°C in the dark. The seeds of the responsive cultivars began to generate callus after 3-6 weeks of inoculation. The frequency of callus induction was determined using the formula provided. For this calculation, multiple callus generated from a single seed were treated as one. Calli with a minimum diameter of 2 mm were placed in regeneration medium and incubated in a temperature-controlled growing environment to accomplish plant regeneration at 25±2°C under a 16-hour light photoperiod with a light strength of around 2000-3000 lux. Observations were made on a daily basis to track the response. The number of callus-producing plantlets was used to calculate the number of regenerated plants.

Statistical analysis

The data for the callus induction and plant regeneration was statistically examined whenever reasonable. The experiment was carried out with three replications following Factorial Completely Randomized Design (CRD) in a growth room. Duncan's Multiple Range Test (DMRT) was used to compare the means of the Analyses of Variance for dissimilar characteristics.

$$Percent\ callus\ induction = \frac{Number\ of\ seeds\ induced\ calli}{Number\ of\ seeds\ incubated}\ X\ 100$$

$$Percent\ plant\ regeneration = \frac{Number\ of\ calli\ with\ plantlets}{Number\ of\ incubated\ calli}\ X\ 100$$

Molecular characterization

RAPD analysis was done on five parents and three somaclonal genotypes in this study. The modified CTAB (cetyltrimethylammonium bromide) technique of Doyle and Doyle (Doyle and Doyle, 1987) was utilized to extract genomic DNA from leaf samples obtained from two-week-old plants. Five RAPD markers with notable amplifications were used to analyze genetic diversity. Five ten-mer RAPD markers (Chinna Gen, RAPD markers. 2016) were used in the PCR. For the process, a 25 µL mixture containing 25 ng template DNA, 2.5 µL of 2.5 mM dNTPs, 2.5 µL of 10x buffer, 2 µL of 25 mM MgCl2, 2 µL of each of the primers, and 0.25 µL of *Taq* polymerase (add the company of the Taq polemerase) were prepared. The PCR fragment size was estimated using a DNA molecular weight marker (100-bp ladder) (Roche, DNA ladders. 2016). The PCR reaction was carried out at 94°C for 5 minutes, followed by 42 cycles of 94°C for 1 minute, 36°C for 1 minute, and 72°C for 2 minutes, then 72°C for 10 minutes. The final products were electrophoretically analyzed in TAE buffer using a 1% agarose gel dyed with ethidium bromide (5 g/mL). Gels were photographed using a UV transilluminator after being stained with a 0.5 g/mL ethidium bromide solution. The presence or absence of polymorphic bands, as well as the strength of these polymorphic bands, were analyzed in the genomes by Alpha Ease FC 4.0 software. Using NTSYS-pc software an index of genetic variation was determined, and dendrograms were built through the UPGMA method. In the beginning, five decamer random primers (Table 02) were checked for the existence of bands. One primer (OPD 07) was chosen since it showed polymorphic and repeatable banding profiles.

Table 2. List of Primers

SL NO	Primer code	Sequence
01	OPD 02	GGACCCAACC
02	OPD 05	GGACCAACCG
03	OPD 07	TTGGCACGGG
04	OPD 08	GTGTGCCCCA
05	OPD 12	TATTGCCGTT

Results and Discussions

Effects of salt (NaCl) for callus induction and regeneration

The MS medium without salt had the highest frequency of callus induction (90%) and plant regeneration (72%). The lowest result was observed (3 % for callus induction and 1% plant regeneration) in a 0.8% salt concentration. Both callus induction and plant regeneration showed a diminishing trend as salt concentrations increased (Fig. 1.). Similar findings were also stated by Adil *et al.*, (2009); Zinnah *et al.*, 2013; Hasan *et at.*, (2013); Sankepally *et al.*, 2016; Arefin *et al.*, 2018. The effect of NaCl on callus induction and plant regeneration in rice is multifaceted and depends on various factors, including concentration, duration of exposure, and the specific characteristics of the rice cultivar. High concentrations of NaCl can inhibit callus induction in rice. Salinity stress may negatively affect cell division and differentiation, crucial processes in callus formation. The effect of NaCl on plant regeneration is often more complex. While high salt concentrations can hinder regeneration, low to moderate levels may induce stress tolerance mechanisms that lead to improved regeneration efficiency. Moreover, NaCl can induce both osmotic stress and ionic stress. Osmotic stress results from reduced water availability due to increased salt concentration, while ionic stress occurs due to the accumulation of sodium ions. Both types of stress can impact cell physiology and tissue culture responses.

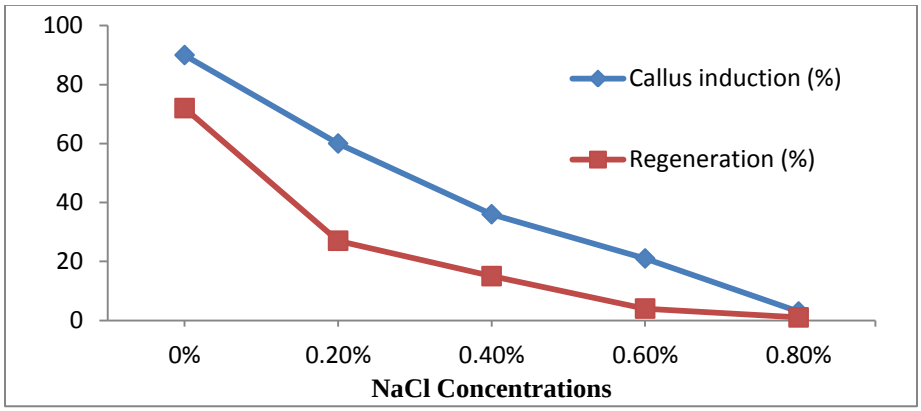


Fig. 1. Mean effects of NaCl salt concentration on callus induction (%) and plant regeneration (%)

Effects of salt on rice cultivars (In vitro)

The selection of an appropriate genotype is a critical factor in the success of in vitro culture techniques in rice. Despite the salt concentrations, Dakshahi had the highest callus induction (53%) and regeneration (31%), while Khazar was found to have the lowest (callus induction 36% and regeneration 17%) (Fig. 2). Guamusuri (42%), showed a moderate result for callus induction, which was statistically similar to Duksail (41%),

and Gondhakasturi (38%). Niroula *et al*. (2005), Puhan *et al*. (2013) & Muhammad *et al.* (2013) reported that the callus induction rate varies to ricegenotypes. Similar observations were found in the case of plant regeneration for Guamusuri (26%), Gondhakasturi (24%) and Duksail (21%). Adil *et al.* (2009); Hasan *et al.* (2013); Taratima *et al.* (2022) discovered genotypic alterations and influences in *in vitro* plant regeneration, which matched the present findings.

The response of different genotypes to callus induction and plant regeneration in rice can be varied. Genetic factors play a crucial role in determining the success of tissue culture processes. Some genotypes are more amenable to tissue culture techniques because of their inherent regeneration capacity and natural high propensity for callus induction and plant regeneration, while others may show limited or poor responses. Moreover, some other crucial factors, like response to stress, growth regulator sensitivity, embryonic potential (ability to form somatic embryos), and the molecular and genetic makeup of a cultivar can also influence callus induction and plant regeneration.

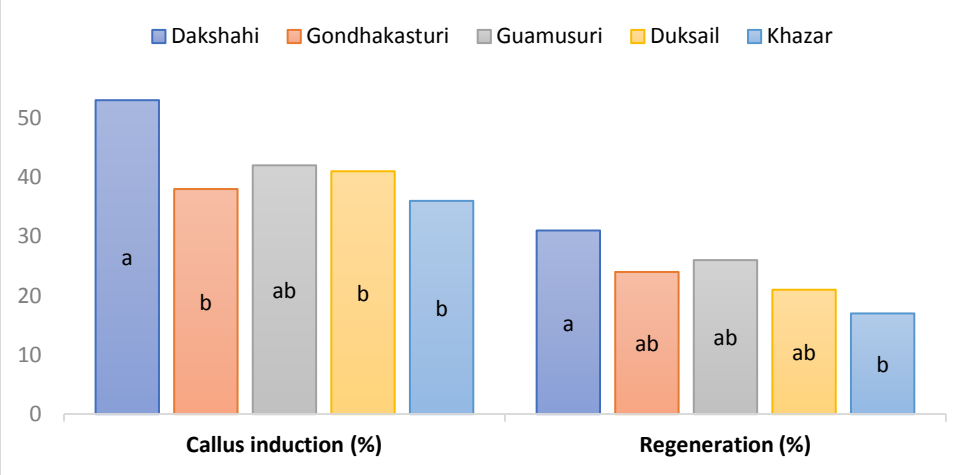


Fig. 2. Mean Effects of different genotypes on callus induction and plant regeneration

Interaction effect of NaCl and genotypes

Guamusuri had the maximum callus induction (100%) in the no salt medium, followed by Dakshahi (95%). The other three cultivars, i.e. Gondhakasturi (85%), Duksail (85%), and Khazar (85%), showed a moderate but statistically similar result in no salt medium. The lowest result was observed in Gondhakasturi (0%), Guamusuri (0%), and Khazar (0%) in 0.8% salt concentrated medium (Table 3). In our study, the callus induction of Dakshahi was better compared to the rest of the cultivars in four salt concentrations, and even in 0.8% NaCL. Duksail performed after Dakshahi and also sowed some results in the top salt concentration.

Table 3. The effect of five genotypes and salt concentrations on callus induction

Genotype	Salt concentrations (%)				
	0	0.2	0.4	0.6	0.8
Dakshahi	95 ab	70 bcd	55 def	35 efg	10 ghi
Gondhakasturi	85 abc	60 cde	30 fgh	15 ghi	0 i
Guamusuri	100a	55 def	35 efg	20 ghi	0 i
Duksail	85 abc	55 def	35 efg	25 ghi	5 hi
Khazar	85 abc	60 def	25 ghi	10 ghi	0 i

In the case of plant regeneration, the highest result was observed in Guamusuri (85%) at 0% salt medium, which was statistically similar to Gondhakasturi (95%) (Table 4). Though Dakshahi (65%) was lower at no salt medium, it was the best performer in all four salt-concentrations. Dukshahi was the only cultivar that regenerated at 0.8% salt concentration. The lowest result was found in Gondhakasturi (0%) and Khazar (0%) in a 0.6% salt concentrated medium. Salinity stress, induced by NaCl, is known to have both positive and negative effects on plant tissue culture processes. Summart *et al.,* (2010) noted that the complex inhibitory action of salt stress on rice is mostly responsible for the decrease in callus induction and plant regeneration. Considering the parameters, it might be said that there is a good chance of having salt stress-responsive genes in Dakshahi, which might trigger cell proliferation in an attempt to survive the stress conditions. This cultivar could also be used in breeding programs to develop salt tolerant rice varieties.

Table. 4. The effect of different genotypes and salt concentrations on plant regeneration

Genotype	NaCl Salt concentrations (%)				
	0	0.2	0.4	0.6	0.8
Dakshahi	65 a-c	45 cd	30 de	10 ef	5 ef
Gondhakasturi	80 ab	25 def	15 ef	0 f	0 f
Guamusuri	85 a	25 def	15 ef	5 ef	0 f
Duksail	70 ab	20 ef	10 ef	5 ef	0 f
Khazar	60 bc	20 ef	5 ef	0 f	0 f

Molecular characterization through RAPD markers

In this study, five decamer operon primers (Table 2) were utilized. One primer (Table 5) generated amplicons and was chosen for genetic diversity analysis after initial screening and taking into account the sharpness, intensity, and repeatability of bands. All the bands produced by the primer were polymorphic. A wide range of polymorphisms in rice genotypes was also found by Zakiyah *et al.* (2019) and Mazumder *et al.* (2020). The

somaclone of Guamasuri (0.2%) showed a distinct polymorphic band compared to its parent (Fig. 3). The difference in the callus induction of Guamashuri at 0% and 0.2% level of salt concentrations (Fig. 5), plant regeneration (Fig. 6) and established plant (Fig. 7) reinforce the findings of somaclonal variation. The possible causes of somaclonal variation include chromosome aberrations, DNA amplification, and the occurrence of transposable elements.

Table 5. Selected primer and genetic variations among rice genotypes by RAPD analysis

Primer code	Sequence	Total no. of bands	Polymorphic bands No.	Monomorphic bands No
OPD 07	TTGGCACGGG	3	3	0

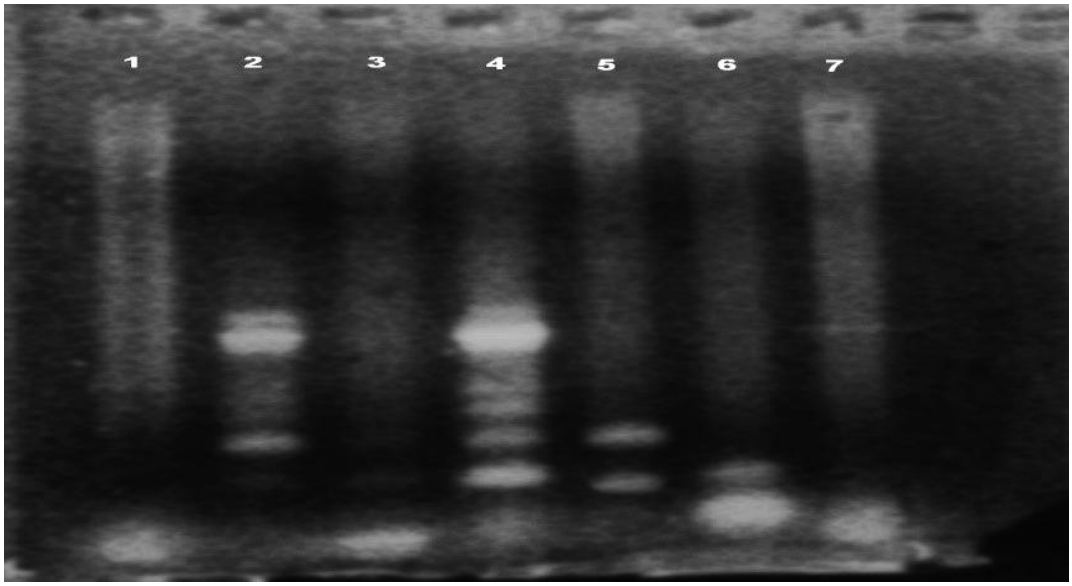


Fig. 3. RAPD profile of five rice varieties and two somaclones generated by OPD 07. Here,1: Guamasuri, 2: Dakshahi, 3: Duksail, 4: Gondhakasturi, 5: Khazar, 6: Somaclone of Guamasuri(0.2%), 7: Somaclone of Guamasuri (0.2%)

Similarity co-efficient analysis

The degree of relatedness between rice genotypes was determined using a similarity matrix based on the fraction of shared RAPD fragments. The similarity estimates for pairs varied from 0 to 0.941 (Table 6). Dakshahi and Khazar were found to have the most genetic diversity (0.941) in RAPD analysis. The genetic similarity coefficients between lines illustrate a lot of genetic variability in rice germplasm. Islam *et al.* (2017) assessed the genetic diversity of ten aromatic rices through RAPD markers and found 80% polymorphism. Again, Mazumder *et al.* (2020) looked over 16 rice genotypes

and discovered pair-wise estimations of genetic similarity among the 16 genotypes varied from 0.66 to 0.88. The range of 0.308 to 0.718 was found by Islam *et al*. (2013) during the evaluation of six rice genotypes.

Table 6. Analysis of Similarity matrix via Nei's original procedures of genetic Identity

No.	Variety	Guamasuri	Dakshahi	Duksail	Khazar	Gondhakasturi
01	Guamasuri	1	0.000	0.500	0.200	0.000
02	Dakshahi		1	0.364	0.941	0.500
03	Duksail			1	0.500	0.571
04	Khazar				1	0.462
05	Gondhakasturi					1

Cluster analysis

A cluster diagram was created using genetic similarities acquired from RAPD data. Using UPGMA, a cluster analysis based on Nei's similarity coefficients clustered 5 genotypes into 2 groups (Fig. 4). Cluster I contain only Guamashuri, but Cluster II has two genotypes in each of its two subclusters. Subcluster, I included Gondhakasturi and Duksail, while Dakshahi and Khazar represented Subcluster II (Table 7). Using the UPGMA approach, Islam *et al.* (2013) and Islam *et al.* (2017) analyzed six and ten rice genotypes using RAPD markers and found that the genotypes constituted three and two clusters, respectively. When assessing 16 rice genotypes, Mazumder *et al*. (2020) found four clusters.

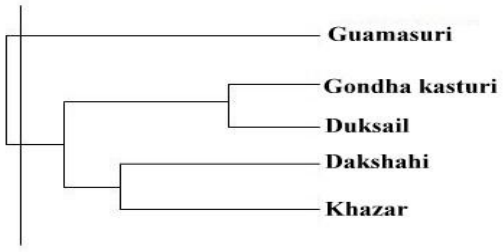
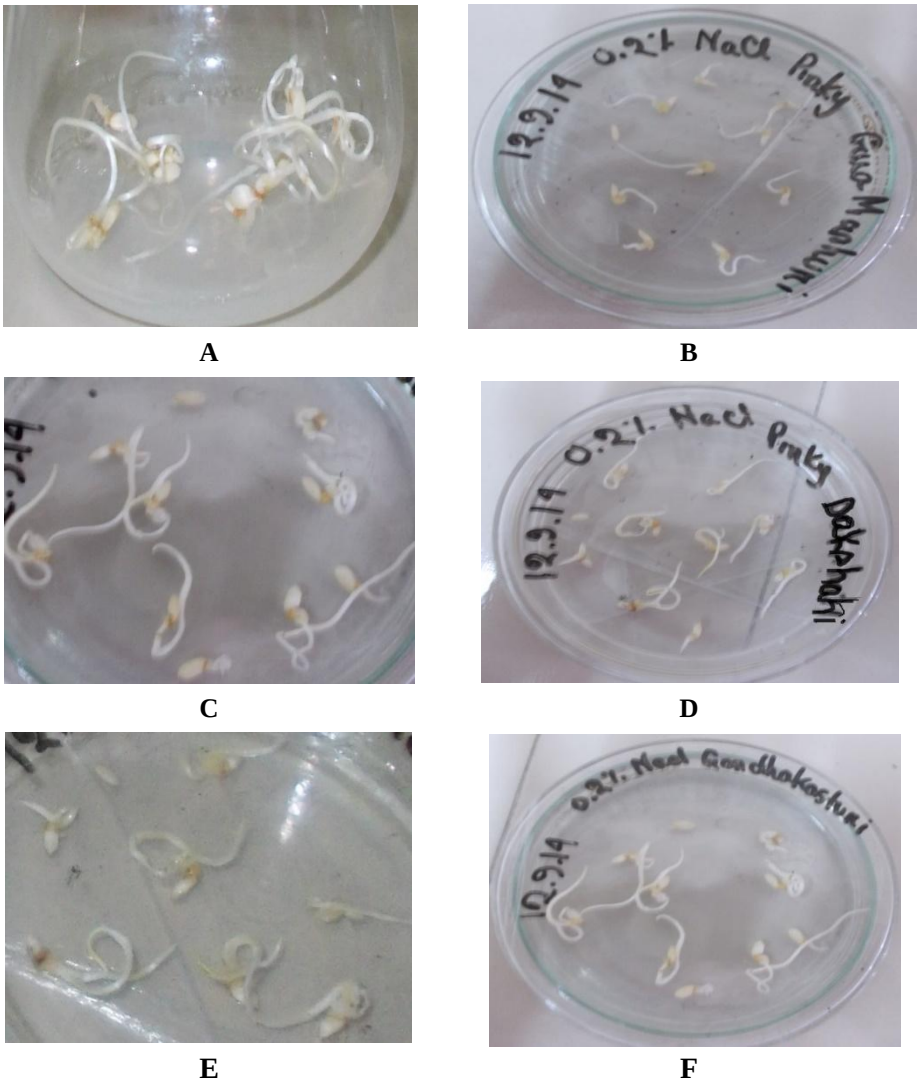


Fig. 4. RAPD analysis dendrogram (UPGMA) pattern in different rice genotypes.



A **B**

C **D**

E **F**

Fig. 5. Callus induction of rice genotypes with and without Salt concentrations

A. Callus of Guamasuri without salt; **B.** Callus of Guamasuri with 0.2 % salt (NaCl) concentration; **C.** Callus of Dakshahi without salt; **D.** Callus of Dakshahi with 0.2% salt (NaCl) concentration; **E.** Callus of Gondha Kasturi without salt; **F.** Callus of Gondha Kasturi with 0.2% salt (NaCl) concentration

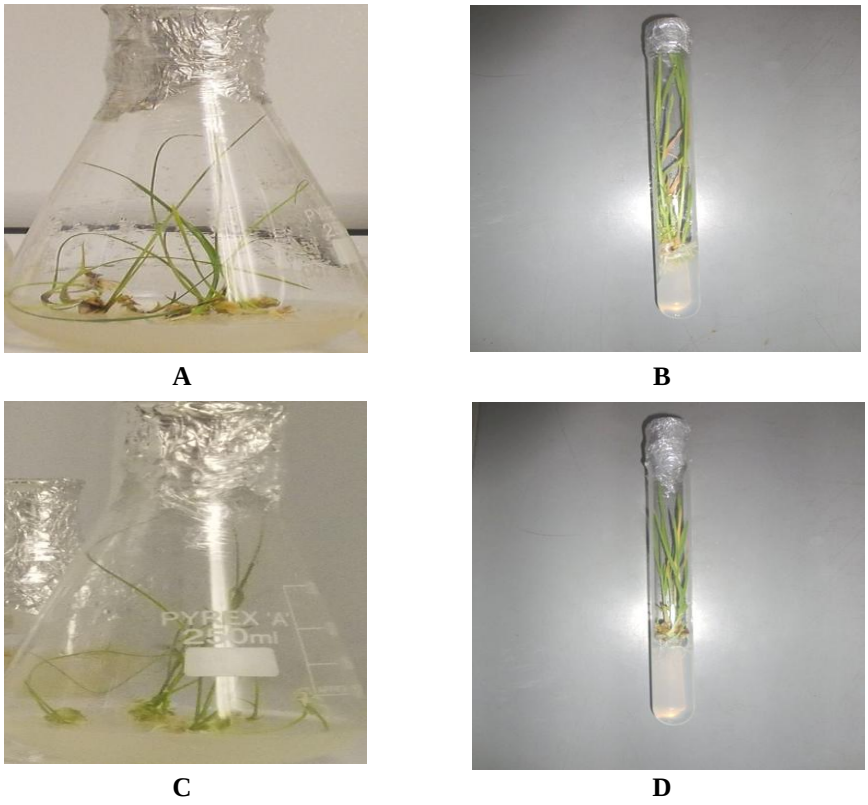


Fig. 6. Plant regenaration of rice genotypes with and without salt concentration

A. Guamasuri without salt; B. Guamasuri with 0.2% salt concentration; C. Dakshahi without salt; D. Dakshahi with 0.2% salt concentration

Fig. 7. Establishment of Guamashuri regenerated plants (0.2% and 0%), concentration of salt

Table 7. Distribution of five aromatic rice genotypes in different clusters

Cluster		Genotypes included in different clusters	No. of genotypes in cluster
I		Guamasuri	1
II	Subcluster I	Gondhakasturi, Duksail	2
	Subcluster II	Dakshahi, Khazar	2

Conclusions

The study underscores the impact of NaCl stress on rice callus induction and plant regeneration, revealing genotypic variability in salt tolerance among different rice genotypes. Dakshahi demonstrated superior performance under salt stress, indicating potential for breeding salt-tolerant rice varieties. Molecular characterization via RAPD markers provided insights into genetic diversity and validated somaclonal variation, with Dakshahi and Khazar showing significant genetic distance. This research highlights the efficacy of integrating tissue culture with molecular tools for developing resilient rice cultivars suitable for saline environments, which can be instrumental in sustaining rice production in salt-affected regions.

Author's contribution

M. M. Hasan and R. Sharmin conceptualized the study and designed the experiments. R. Sharmin and M. M. Hasan collected and analyzed the data and also interpreted the results. R. Sharmin and M. A. Hossain drafted the text. M. M. Hasan, M. G. Rasul, M. M. Rahman, and M. A. Hossain reviewed the results and approved the final version of the manuscript.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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