RESEARCH PAPER

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Effect of air desiccation and salt stress factors on in vitro regeneration of rice (*Oryza sativa* L.)

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Abbreviations: MS, Murashinge and Skoog; 2,4-D, 2,4-Dichlorophenoxy acetic acid; NAA, α-napthalene acetic acid; BAP, 6-Benzyl amino purine; Kin, kinetin; CH, casein hydrolysate; w, week; d, day; h, hour; VCn = number of viable callus; ICn = number of inoculated callus.

Enhancement of callus induction and its regeneration efficiency through *in vitro* techniques has been optimized for 2 abiotic stresses (salt and air desiccation) using 3 rice genotypes *viz.* BR10, BRRI dhan32 and BRRI dhan47. The highest frequency of callus induction was obtained for BRRI dhan32 (64.44%) in MS medium supplemented with 2, 4-D (2.5 mgL^{-1}) and Kin (1.0 mgL^{-1}). Different concentrations of NaCl (2.9, 5.9, 8.8 and 11.7 gL^{-1}) were used and its effect was recorded on the basis of viability of calli (VC), relative growth rate (RGR), tolerance index (TI) and relative water content (RWC). It was observed that in all cases BRRI dhan47 showed highest performance on tolerance to VC (45.33%), RGR (1.03%), TI (0.20%) and RWC (10.23%) with 11.7 gL^{-1} NaCl. Plant regeneration capability was recorded after partial air desiccation pretreatment to calli for 15, 30, 45 and 60 h. In this case BRRI dhan32 gave maximum number of regeneration (76.19%) when 4 weeks old calli were desiccated for 45 h. It was observed that air desiccation was 2-3 folds more effective for enhancing green plantlet regeneration compared to controls. Furthermore, desiccated calli also showed the better capability to survive in NaCl induced abiotic stress; and gave 1.9 fold (88.80%) increased regeneration in 11.7 gL^{-1} salt level for BRRI dhan47. Analysis of variance (ANOVA) showed that the genotypes, air desiccation and NaCl had significant effect on plant regeneration at $P < 0.01$.

Introduction

Rice (*Oryza sativa* L.) belongs to the family Gramineae is a cereal crop and staple food over the world and also main food crop in Bangladesh. The population of rice eaters are increasing day by day and the number of rice consumers will probably 2 fold by 2020.[1] In spite of many abiotic stresses are played negative role on rice and other crop production.[2-5] Drought and salinity are the major abiotic stresses that adversely affect the overall metabolic activities and cause plant demise.[6,7] Certain crops reduced their production capabilities in high saline conditions.[8] Over 2 million acres of agricultural land has been estimated which lost from production per every year due to occurrence of high Na^+ and Cl^- ions in soil.[9] The coastal area of Bangladesh is 20% of the country and over 30% of net cultivable area and it expends inside up to 150 km from the coast.[10] Agricultural genetics is one of the most important parts to solve the recent global issue. In biotechnology, genotype strongly influences the potentiality of tissue culture, and identification of superior performance is a key step to transform gene in rice.[11] *In vitro* production of rice plants provides efficient and convenient system to produce rice lines rapidly.[12] The application of biotechnology in combination with conventional breeding methods such as doubled haploid breeding may help to increase food production properly.[13-15] For rice improvement, *in vitro* culture method was successfully initiated by culturing of excised immature embryos in nutrients media.[16,17] Successful plant regeneration by embryo-derived calli has been reported by Raina,[18] Vasil[19], Crougham and Chu.[20] For the development of highly reproducible reproduction system through somatic embryogenesis using mature embryos in rice were studied by Verma et al.[21] Joyia et al.[22] optimized the responsive age of the cells to regeneration which was a prime character for efficient plant regeneration. However, many factors affect plant regeneration frequency in rice e.g. genotype, development stage of callus in the explants and hormonal composition of medium.[23-25] A comprehensive study on rice genotypes for both callus induction and plant regeneration has been done for 500 rice varieties by Kamia et al.[26] The identification and screening of useful cultivars for embryogenic callus formation and subsequent plant regeneration through *in vitro* system is a key step in rice genetic improvement.[27,28] An efficient plant regeneration in Bangladeshi *indica* rice is still poses a major

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problem for genetic manipulation through innovative approaches.[14,29] The optimal desiccation periods were 72 h and 48 h for Malaysian rice cultivars of MR232 and MR220 respectively, where plant regeneration enhanced up to 2-5 folds.[30] They suggested that partial desiccation can be useful in stimulating regeneration response. Desiccation treatment reduced hyperhydricity of the calli.[31] Tsukahara and Hirosawa[32] reported that dehydration for 24 h of cell suspension derived calli of *japonica* rice increased shoot regeneration from 3 to 47%. Three-fold increased in shoot regeneration frequency following partial desiccation for 24 h of suspension cells in *indica* rice.[33] Chand and Sahrawat[34] carried out partial desiccation of embryogenic calli prior to transfer to regeneration medium and found increasing regeneration frequency of desiccation treatment to callus cultures of cv. Safari17 and Kasturi. In sugarcane desiccation improves somatic embryogenesis and the calli exhibited a greater regeneration frequency that is very important for genetic transformation work.[35] Three and 4 hours desiccated calli reduced fresh weight due to reduction of water content and stimulated callus growth, globularization and embryo formation in 2 date palm cultivars.[31] Desiccation also has been reported to promote somatic embryo differentiation and development to other crops like as soybean,[36,37] wheat,[38] spruce[39] and cassava.[40] In recent years tissue culture techniques are being used as a useful tool to elucidate the mechanism involved in salt tolerance.[41,42] Several metals are essential nutrients for plants, yet they become toxic at high levels and deleteriously affect crop yield and quality.[43] Using Bangladeshi *indica* rice cultivars till there is not enough report on successful regeneration on high salt stress and other stress pretreatment factors for tolerance level. Therefore, the present study has been undertaken to determine the effect of partial air desiccation and by NaCl on somatic embryogenesis derived from mature seeds and their subsequent regeneration using 3 rice cultivars.

Results

Effect of media on callus induction (CI)

Two basal media (MS and N6) consisting of 4 hormonal combinations (H_1 - H_4) were used for their effectiveness on callus induction. All the steps in callus induction, somatic embryogenesis (direct) and plant regeneration in rice are shown in **Fig. 1A–H**. The results indicated that all of the responding genotypes showed well embryogenic response to induce callus (**Fig. 1A and B**). Out of 3 genotypes, BRRI dhan32 (64.44%) and BRRI dhan47 (52.78%) performed highest number of CI in MS + H_2. The maximum callus induction (53.26%) was recorded for BR10 in MS + H_1 (**Fig. 2**). On the other hand, BR10, BRRI dhan47 and BRRI dhan32 gave minimum callusing 47.13, 47.71 and 55.34% in N6 + H4, respectively. It was observed that all the responding genotypes showed better performance on callusing in MS medium than N6. Analysis of variance showed the significant differences within the studied genotypes and the media examined at $p < 0.01$ level (**Table 1**).

Response to *in vitro* Abiotic Stresses

Viability of callus to NaCl induced stress

To observe the viability of calli 4 concentrations of NaCl were used and data shown in **Table 2**. The varieties BRRI dhan47, BR10 and BRRI dhan32 gave 53.33, 14.67 and 2.67% viable calli after one week cultured in the highest concentration of NaCl (11.7 gL^{-1}) respectively. In the same salt level, the viability was decreased remarkably after 4 weeks of culture in BRRI dhan47 (45.33%), BR10 (10.67%) and BRRI dhan32 (0.00%). In stress condition, the survival rate of callus was significantly differed on NaCl concentrations, culture periods and rice genotypes (**Table 1**).

Relative growth rate, tolerance index and relative water content to NaCl stress

Three weeks old calli were used for this study and grown in 4 concentrations of NaCl (2.9, 5.9, 8.8, 11.7 gL^{-1}) up to 4 weeks. Relative growth rate (RGR), tolerance index (TI) and relative water content (RWC) were determined and shown in **Figure 3A–C**. It was observed that in all cases, significant differences were found among the genotypes. Furthermore, significant differences were also observed in absence of NaCl in the medium (control) on RGR, TI and RWC. However, 1.03, 0.23 and 0.11 RGR values were recorded at 11.7 gL^{-1} salt stress in BRRI dhan47, BR10 and BRRI dhan32 respectively. On comparison to the controls, RGR values were decreased at 79.88% in BRRI dhan47, 94.26% in BR10 and 97.59% in BRRI dhan32. Since, BRRI dhan47 grew with highest capability in the top most level of NaCl stress (11.7 gL^{-1}). The same genotype carried the highest TI (0.20) which expressed the high capability to grow in abiotic stress condition developed by NaCl. Comparatively lower TI numbers were recorded in other 2 genotypes BR10 (0.02) and BRRI dhan32 (0.06). The WRC values were also lower for BR10 (7.22%) and BRRI dhan32 (7.03%) than BRRI dhan47 (10.23%).

On spite of showing highest RGR and TI values, BRRI dhan47 was taken to conduct related another extended experiment. To observe the changing pattern of RGR, its values were determined in contrast of stress levels and calli exposure periods (every week up to 4). The results showed that RGR was increased till 2 weeks of calli exposure periods, at all NaCl levels tested. After 2 weeks, RGR was restricted in 5.9, 8.8 and 11.7 gL^{-1} stress levels, while in 2.9 gL^{-1} lower rate of increment in RGR value was found (**Fig. 3D**).

Effect of partial air desiccation to regeneration

Calli of different age groups (3, 4, 5 and 6 w) were partially desiccated (15, 30, 45 and 60 h) and cultured on RM. The significant differences were found within the genotypes, age groups and air desiccation period on regeneration (**Table 1** and **Fig. 1C and D**). The results showed that 4 w old calli of BRRI dhan32 performed highest regeneration (76.19%) among the genotypes when it was desiccated by 45 h (**Table 3**). The gained regeneration value was 2 fold higher than the control (38.10%). In the same desiccation pretreatment (45 h), other 2 genotypes BR10

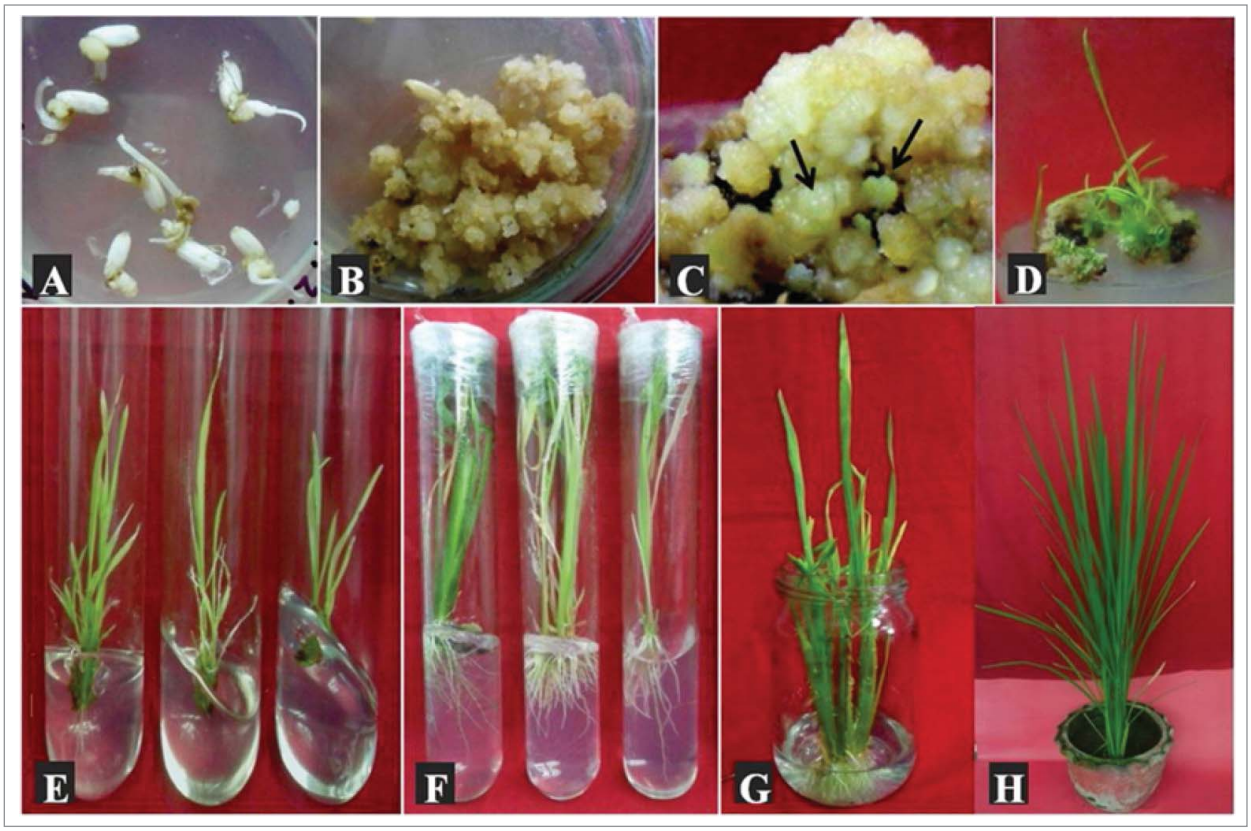

Figure 1. Callus induction, somatic embryogenesis (direct) and plant regeneration in rice. (**A**) Calli derived from mature seeds after one week of culture, (**B**) Proliferation of embryogenic calli (4–5 weeks old), (**C**) Development of somatic embryos on mature embryo induced calli (indicated by arrows), (**D**) Formation of shoots from germinated somatic embryos, (**E**) Elongated shoots, (**F**) Rooted shoots (regenerated plantlets), (**G**) Acclimatized plantlets, (**H**) Hardening of regenerated plantlets in pot culture.

and BRRI dhan47 gave 73.02% and 58.73% regeneration respectively; and the results were more than 2 fold higher (**Fig. 4**). In contrast to the controls, desiccated calli showed around 2–3 folds higher regeneration. Considering our recorded data, the calli of lower age, needed to higher desiccation pretreatment within a range than comparatively aged callus to perform maximum regeneration. However, analysis of variance (ANOVA) showed that the effect of partial air desiccation, age of calli and rice genotype on plant regeneration differed significantly at $P < 0.01$ (**Table 1**).

Regeneration response by partially desiccated calli in salt stress

Desiccated callus performed with increased regeneration in NaCl induced stress condition (**Table 4**). The callus age of 4 weeks were pretreated at 45 h air desiccation and transferred to regeneration medium (RM) supplemented with NaCl levels (2.9, 5.9, 8.8, 11.7 gL^{-1}). The variety BRRI dhan47 gave the highest regeneration (26.98%) at 11.7 gL^{-1} salt level after desiccation pretreatment. The result was 1.89 fold higher than the control (14.29%). In the same stress level, other 2 varieties BR10 and BRRI dhan32 could not be regenerated from undedicated calli; whereas, after desiccation pretreatment they were been capable for regeneration at 11.11% and 4.76%, respectively.

Discussion

In the present study high frequency of callus induction (CI) was found in 3 Bangladeshi *indica* rice varieties *viz.* BR10, BRRI dhan32 and BRRI dhan47 (**Fig. 1 and 2**). Zuraida et al.[44] reported that CI frequency depends on genotype, and most *indica* rice cultivars had poor callusing potentiality. In this case we examined the CI potentiality using 4 hormonal combinations (H_1, H_2, H_3 and H_4). Results showed that all the responding genotypes induced callus at high frequency in 2, 4-D than NAA and IAA. Makerly et al.[30] recorded highest percentage of CI (41% and 37%) for Malaysian *indica* rice cultivars MR232 and MR220, respectively, in MS supplemented with NAA; and also reported that the varieties responded lower in 2, 4-D. Present investigation differs with their reports, and mention that in 2, 4-D studied varieties responded high to induce calli. Tiwari et al.[45] reported that the optimum hormonal combination as 1.5 mgL^{-1} 2, 4-D + 0.1 mgL^{-1} NAA + 0.1 mgL-1 mgL^{-1} BAP with MS for maximum CI. They have recorded 85% and 90% CI for

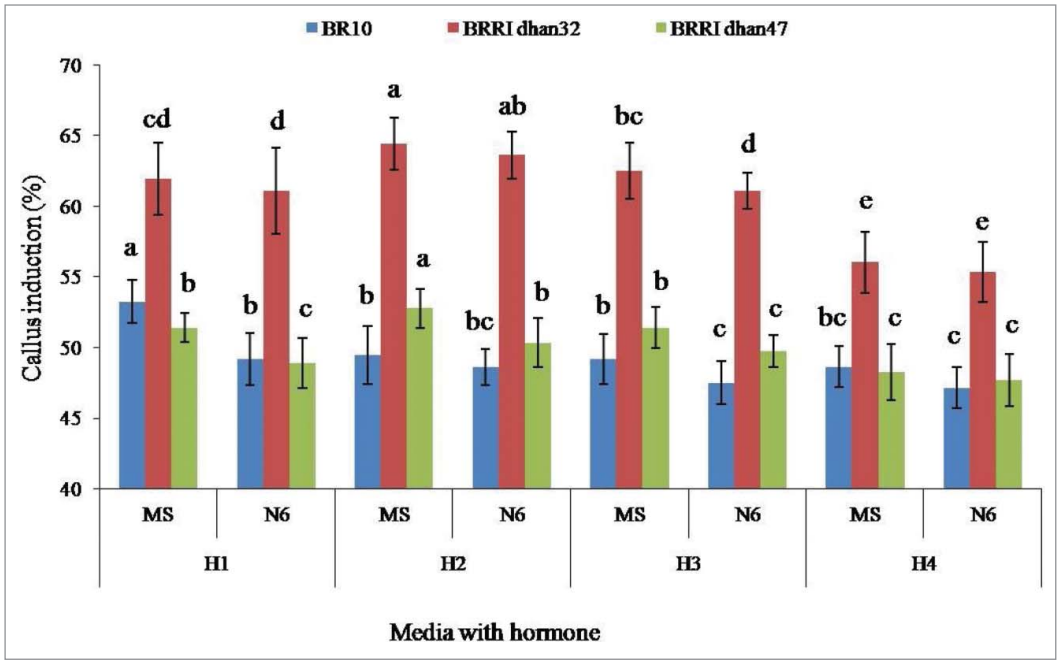

Figure 2. Effect of media and hormonal combinations on callus induction in 3 rice genotypes. $H_1 = 0.5$ mgL^{-1} 2, 4-D + 1.0 mgL^{-1} Kin, $H_2 = 2.5$ mgL^{-1} 2, 4-D + 1.0 mgL^{-1} Kin + 300 mgL^{-1} L-proline + 400 mgL^{-1} CH, $H_3 = 0.5$ mgL^{-1} NAA + 2.0 mgL^{-1} Kin + 400 mgL^{-1} L-proline + mgL^{-1} CH, $H_4 = 2.5$ mgL^{-1} IAA + 0.5 mgL^{-1} Kin + 300 mgL^{-1} L-proline + 400 mgL^{-1} CH. Within the bars of same color, different letter (s) shows significant difference at $p < 0.05$ according to DMRT.

indica rice varieties of Pusa Basmati1 and Kalanamak respectively. In this case, we found that BRRI dhan32 showed the highest CI capability at 64.44% and 63.61% in MS and N6 media respectively (**Fig. 2**). Out of responding genotypes BRRI dhan37, recorded CI was 52.78% in MS + H_2 and 50.33% in N6 + H_2. The results of present study defers to the previous reports with lesser frequency of CI. It might be occurred due to the effect of different media with hormonal combinations and the genetic variability of the rice genotypes.

In the experiment on viability test, the calli expressed the survival capability in salt stress condition. Soheilikhah et al.[46] recorded 47-64%

Table 1. Analysis of variance (ANOVA) subjected to callus induction, partial air desiccation and NaCl treatments in 3 rice genotypes

Stress	Subject of ANOVA (Data source)	Source of variation	DF	Mean sum of square
—	Callus Induction (CI) (**Fig. 1**)	Genotype (G)	2	442.476**
		Media (M)	7	2.858**
		G × M	14	—
Salt induced stress	Viability of callus (VC) (**Table 1**)	Genotype (G)	2	7422.85**
		Salt concentration (SC)	4	8543.69**
		Callus culture period (CCP)	3	193.27**
		G × SC	8	581.68**
		G × CCP	6	4.36^{NS}
		SC × CCP	12	7.27^{*NS}
		G × SC × CCP	24	8.48
	Relative growth rate (RGR) (**Fig. 3A**)	Genotype (G)	2	1.741**
		Salt concentration (SC)	4	9.414**
		G × S	8	0.132
	Tolerance index (TI) (**Fig. 3B**)	Genotype (G)	2	0.039**
		Salt concentration (SC)	4	0.453**
		G × S	8	0.005
	Relative water content (RWC) (**Fig. 3C**)	Genotype (G)	2	3.327**
		Salt concentration (SC)	4	38.644**
		G × SC	8	2.119
Partial air desiccation stress	Plant regeneration (PR) (**Table 2**)	Genotype (G)	2	604.34**
		Age of callus (AC)	3	803.94**
		Partial air desiccation (PAD)	4	686.17**
		G × AC	6	18.45^{NS}
		G × PAD	8	45.93^{NS}
		AC × PAD	12	124.71*
		G × AC × PAD	24	35.09

** = Significant at $P < 0.01$, * = Significant at $P < 0.05$, NS = Non-significant.

Table 2. Effect of salt on viability of seed derived callus exposed in different concentrations of NaCl and grown one to 4 weeks for 3 genotypes

Variety	NaCl (gL^{-1})	Viable calli (% ± SE)			
		1 w	2 w	3 w	4 w
BR10	Cont.	93.33 ± 1.33ab	88.00 ± 2.31a	86.67 ± 3.53a	85.33 ± 2.67a
	2.9	78.67 ± 3.53d	77.33 ± 2.67cd	74.67 ± 1.33bc	73.33 ± 1.33b
	5.9	41.33 ± 1.33h	36.00 ± 2.31g	33.33 ± 2.67f	33.33 ± 2.67e
	8.8	32.00 ± 2.31i	32.00 ± 1.33g	26.67 ± 1.33f	24.00 ± 2.31f
	11.7	14.67 ± 2.67j	12.00 ± 2.31i	12.00 ± 2.31gh	10.67 ± 2.67gh
BRRI dhan32	Cont.	86.67 ± 2.67bc	84.00 ± 2.31ab	81.33 ± 2.67ab	81.33 ± 2.67a
	2.9	62.67 ± 3.53f	52.00 ± 2.31f	45.33 ± 2.67e	33.33 ± 1.33e
	5.9	25.33 ± 1.33i	22.67 ± 1.33h	18.67 ± 1.33g	16.00 ± 2.31g
	8.8	13.33 ± 1.33j	8.00 ± 2.31i	8.00 ± 2.31h	5.33 ± 1.33hi
	11.7	2.67 ± 1.33k	0.00 ± 0.00j	0.00 ± 0.00i	0.00 ± 0.00i
BRRI dhan47	Cont.	94.67 ± 1.33a	89.33 ± 1.33a	86.67 ± 3.53a	85.33 ± 1.33a
	2.9	81.33 ± 3.53cd	80.00 ± 2.31bc	77.33 ± 1.33bc	74.67 ± 1.33b
	5.9	74.67 ± 1.33de	73.33 ± 1.33d	72.00 ± 2.31c	72.00 ± 2.31b
	8.8	70.67 ± 3.53e	64.00 ± 2.31e	64.00 ± 2.31d	58.67 ± 1.33c
	11.7	53.33 ± 1.33g	49.33 ± 1.33f	46.67 ± 2.67e	45.33 ± 3.53d

Culture medium was MS +2.5 mgL^{-1} 2, 4-D + 1.0 mgL^{-1} kin + 0.3 gL^{-1} L-proline + 0.4 gL^{-1} CH + NaCl. For each NaCl concentration, number of used callus was 75 in 3 replications and in a column the mean values followed by same letter (s) are not significantly different at $p < 0.05$ according to DMRT.

decreased cell viability in NaCl induced stress for Safflower (*Carthamus tinctorius* L.) varieties, and mentioned that the accumulation of Na^+ ions and osmolytes could play an important role in osmotic adjustment in cells under saline stress. The present investigation significant differences were found among the rice genotypes on cell viability as well as the viability of the calli in NaCl induced stress. The calli of BRRI dhan47 exhibited with highest viability (45.33%) after 4 weeks cultured in 11.7 gL^{-1} of NaCl (**Table 2**). BR10 survived with 10.67% viable calli and BRRI dhan32 could not be exhibited when the calli were exposed in 11.7 gL^{-1} up to 4 weeks. We have observed that the phenomenon was happened due to presence of necrotic cells in the calli. A huge number of necrotic cells turned the calli deep brown or blackish in color, together with survival disability was appeared. It was observed that lower frequency of viable calli was appeared in higher salt concentration than the lower one. On spite of lesser osmotic potentiality and genotypic effect, the varieties might disable to adapt in salt stress condition. The calli of BR10 and BRRI dhan32 were begun to necrosis within a week, and after 4 weeks cultured in 11.7 gL^{-1} of NaCl

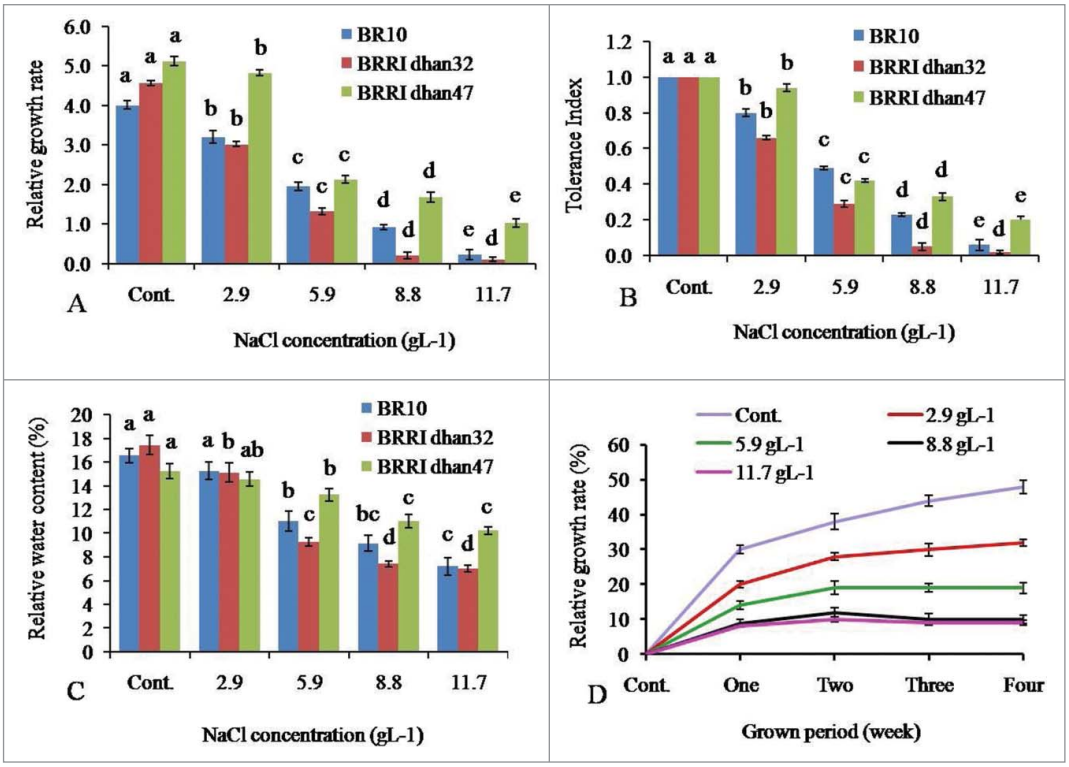

Figure 3. Effect of different concentrations (2.9, 5.9, 8.8, 11.7 gL^{-1}) of NaCl subjected to (**A**) relative growth rate, (**B**) tolerance index, (**C**) relative water content of 3 rice genotypes and (**D**): Relative growth rate (%) of BRRI dhan47 in different NaCl concentrations in contrast to calli exposure periods. Within the bars of same color, different letter (s) shows significant difference at $p < 0.05$ according to DMRT.

Table 3. Effect of partial air desiccation period and 4 age groups of calli on plant regeneration for 3 rice genotypes (% ± SE)

		Genotypes		
Age of calli (w)	**Desiccation (h)**	**BR10**	**BRRI dhan32**	**BRRI dhan47**
3	Cont.	38.10 ± 2.75de	31.75 ± 3.17jk	33.33 ± 2.75def
	15	36.51 ± 1.59e	39.68 ± 1.59ghij	36.51 ± 3.17cde
	30	47.62 ± 2.75c	55.56 ± 1.59de	38.10 ± 2.75cde
	45	50.79 ± 3.17c	68.25 ± 3.17b	42.86 ± 2.75bc
	60	34.92 ± 1.59e	41.27 ± 1.59fghi	26.98 ± 1.59fg
4	Cont.	31.75 ± 1.59e	38.10 ± 2.75hijk	30.16 ± 1.59efg
	15	53.97 ± 3.17c	47.62 ± 2.75efg	34.92 ± 3.17cdef
	30	68.25 ± 3.17ab	65.08 ± 4.20bc	42.86 ± 2.75bc
	45	73.02 ± 4.20a	76.19 ± 2.75a	58.73 ± 3.17a
	60	46.03 ± 3.17cd	58.73 ± 1.59cd	47.62 ± 2.75bc
5	Cont.	30.16 ± 1.59e	34.92 ± 3.17ijk	30.16 ± 1.59efg
	15	47.62 ± 2.75c	47.62 ± 2.75efg	47.62 ± 2.75bc
	30	63.49 ± 4.20b	55.56 ± 1.59de	39.68 ± 1.59bcd
	45	49.21 ± 4.20c	49.21 ± 3.17ef	34.92 ± ± 3.17cdef
	60	34.92 ± 1.59e	39.68 ± 1.59ghij	31.75 ± 1.59defg
6	Cont.	31.75 ± 3.17e	30.16 ± 1.59k	26.98 ± 1.59fg
	15	38.10 ± 2.75de	44.44 ± 1.59fgh	34.92 ± 3.17cdef
	30	36.51 ± 1.59e	39.68 ± 3.17ghij	33.33 ± 2.75def
	45	34.92 ± 1.59e	38.10 ± 2.75hijk	26.98 ± 1.59fg
	60	33.33 ± 2.75e	31.75 ± 3.17jk	23.81 ± 2.75g

Used regeneration medium, MS + 2.0 mgL^{-1} BAP + 0.5 mgL^{-1} NAA + 1.0 mgL^{-1} Kin was constant for all the age of calli and partial air desiccation pretreatments. For each desiccation pretreatment number of callus was 63 in 3 replications and in a column the mean values followed by same letter (s) are not significantly different at $p < 0.05$ according to DMRT.

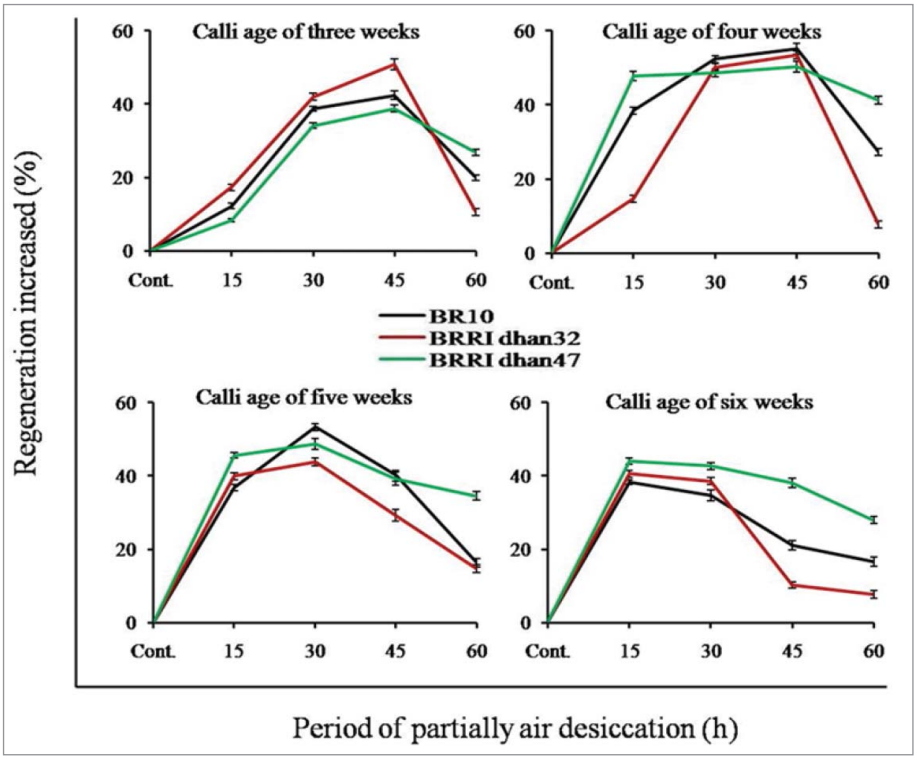

Figure 4. Effect of different period of partial air desiccation in relation to age of calli to enhance plant regeneration. Each curve indicates the percentage value of increased regeneration compare to the control (vertical bar expressed the SE of 3 replicates).

level, a few number of calli were existed. On the other hand BRRI dhan47 could be adapted to the NaCl stress and showed highest viability in all cases of salt stresses. Based on survival feature, BRRI dhan32 and BR10 showed sensitivity to NaCl induced *in vitro* stress; whereas, BRRI dhan47 was appeared as tolerant in nature.

Remarkable differences were found among the genotypes examined on relative growth rate (RGR), tolerance index (TI) and relative water content (RWC). In the top level of NaCl stress (11.7 gL^{-1}) recorded RGR values were 1.03, 0.23 and 0.11; TI were 0.20, 0.06 and 0.02; and WRC were 10.23, 7.22 and 7.03% for the genotypes BRRI dhan47, BR10 and BRRI dhan32, respectively (**Fig. 3A-C**). Among 3 genotypes BRRI dhan47 exhibited the highest potentiality to survive in NaCl induced abiotic stress with maximum RGR (1.03), TI (0.20), and WRC (10.23%). The recorded values of the parameters RGR, TI and WRC expressed the higher survival capability against the abiotic stress conducting the physiological activities of BRRI dhan47. On the other hand stress sensitivity was found in BRRI dhan32 and

Table 4. Effect of desiccation to NaCl stress on regeneration of 3 rice genotypes (% ± SE)

		NaCl (gL^{-1})				
Variety	**Desiccation (h)**	**Cont.**	**2.9**	**5.9**	**8.8**	**11.7**
BR10	Cont.	36.51 ± 1.59	31.75 ± 1.59	9.52 ± 2.75	3.17 ± 1.59	0.00 ± 0.00
	45	71.43 ± 4.76	68.25 ± 4.20	33.33 ± 2.75	14.29 ± 2.75	11.11 ± 3.17
BRRI dhan32	Cont.	36.51 ± 1.59	25.40 ± 1.59	9.52 ± 2.75	3.17 ± 1.59	0.00 ± 0.00
	45	74.60 ± 4.20	63.49 ± 3.17	20.63 ± 1.59	7.94 ± 3.17	4.76 ± 2.75
BRRI dhan47	Cont.	30.16 ± 1.59	28.57 ± 2.75	25.40 ± 3.17	23.81 ± 2.75	14.29 ± 2.75
	45	60.32 ± 4.20	58.73 ± 3.17	49.21 ± 4.20	38.10 ± 2.75	26.98 ± 3.17

Regeneration medium was MS + 2.0 mgL^{-1} BAP + 0.5 mgL^{-1} NAA + 1.0 mgL^{-1} Kin + different concentration of NaCl. In each case number of used callus was 63 in 3 replications.

BR10 considering lower value of the parameters. RGR, TI and RWC values were decreased in higher stress level than the lower one. The phenomena might be occurred due to reduction of water availability and lose of turgor pressure (TP) in the cells of the calli. Such physiological causes were reported in previous investigation for *Oryza sativa*,[47] *Carthamus tinctorius*,[46,48] *Saccharum sp*.,[9] *Tagetes minuta*[49] and *Triticum durum*[50,51]. Errabi et al.[9] mentioned that due to interference of Na^+ and Cl^- ions on uptake and translocation processes, nutritional imbalance might be created and the growth of callus is declined. However, NaCl treated calli of BRRI dhan47 was least affected by the highest dose of salt stress and exhibited high ability in terms of both cellular viability and growth of callus. BRRI[52] mentioned that BRRI dhan47 can tolerate at 12-14 dS m^{-1} (approximately 7.0-8.9 gL^{-1}) of NaCl stress and it has an ability to survive and grown in saline soil. So that it is considered as salt tolerant rice genotype. Therefore, our investigations are in agreement with the previous report for the genotype BRRI dhan47. Calli of BRRI dhan47 might accumulate less Na^+ ion than salt susceptible BR10 and BRRI dhan32. In several species K^+ is a major cation and contributor to adjust osmotic potential (OP) under stress condition.[50,53] In salt stress Na^+ concentration is increased which lead to decrease concentration of K^+ among rice genotypes. As a result an imbalance of essential ions be created and cell of salt sensitive varieties could not be survived. Such reports previously mentioned in rice,[47,54] sugarcane,[9,55] *Carthamus tinctorius*[46] and *Cynara cardunculus*.[56]

Before transfer the callus to regeneration medium, partial air desiccation pretreatment enhanced the frequency of plant regeneration. Rance et al.[57] reported that 2-4 folds higher regeneration from 3 h desiccated calli than the control in rice genotypes *viz.* PN1, IR72 and IR64. Compare to undesiccated calli, 2 and 5 folds higher regeneration was recorded from 48 and 72 h desiccation in MR220 and MR232 respectively.[30] Approximately similar increment of regeneration was recorded in maize,[58,59] sugarcane[35] and rice.[29,60,61] Under this study we have recorded around 2–3 folds higher regeneration from desiccated calli in BR10, BRRI dhan32 and BRRI dhan47 (**Fig. 4, Table 3**). Therefore, the obtained results are in agreement with previous findings. In our study, effect of partial air desiccation to regeneration was determined in contrast of callus age. However, an effective relationship was found, and investigated that callus of lower age (within a range) need to comparatively higher period of desiccation to perform maximum regeneration. On the other hand callus of relatively higher age gave maximum regeneration when it was pretreated at lower level of desiccation. The phenomena could be depended on water content (WC) in the cells of the calli. Callus of lower age might contain a big amount of water while they need to higher desiccation in which the calli dehydrated at optimum level. Makerly et al.[30] reported that the degrees of water loss differ against same desiccation period in different rice genotype, and an optimal level of water loss (partial air desiccation) could be beneficial to plant regeneration. They also noticed that regeneration varied depending on cultivars and duration of partial desiccation. In date plum cultivar 3 and 4 h partial desiccation reduced fresh weight of calli and stimulated calli growth globularization as well as embryo formation .[31] Under this study recorded results showed that partial desiccation strongly influenced the regeneration and played an effective role to enhance the somatic embryogenesis. To regenerate *in vitro* plant for *indica* rice genotypes genetic effect along with the age of explants has been reported earlier by Hoque and Mansfield.[27] Such effect was noticed in sugarcane,[62] coffee,[63] rice,[64,65] *Primula* ssp[66] However, genetic variability, optimal air desiccation and suitable age of calli might play a vital role to enhance regeneration in rice genotypes. Although at optimum level of desiccation promote the regeneration, yet over desiccation suppressed to embryo formation as well as plant regeneration. Over desiccation created the drought abiotic stress in which regeneration frequency was decreased up to 23.81% at 60 h desiccation to 6 w age of calli for BRRI dhan47 (**Table 3**). Kranner et al.[67] reported that loss of more than 20-50% water content of the cells is been lethal to most of the higher plants. However, the optimum period of partial air desiccation pretreatment was varied on the age of calli as well as the rice genotype significantly.

At optimum period, partially desiccated calli responded of enhanced regeneration in NaCl stress condition than the controls (**Table 4**). The variety BRRI dhan47 performed 88.80 per cent higher regeneration than the control at the top level of NaCl (11.7 gL^{-1}), after pretreatment of 45 h desiccation. The calli of other 2 genotypes BR10 and BRRI dhan32 were been able to regenerate at 11.7 gL^{-1} NaCl level; while both were not shown any regeneration without desiccation (control). Makerly et al.[30] reported that regeneration capability was varied depending on

the duration of desiccation. At optimal period of partial air desiccation characterized the cells of the callus to survive and adapt at adverse physiological stress. Because of reduction of water the cells of desiccated calli might acquired higher osmotic potential (OP). So that ability was developed to uptake water in salt stress condition and could be able to survive. However, in case of BRRI dhan47 partial air desiccation pretreatment was more effective to enhance the capability of regeneration in NaCl induced abiotic stress.

Conclusion

Out of studied 3 Bangladeshi indica rice genotypes, BRRI dhan32 showed better callusing and plant regeneration that might be considered for advance research in biotechnology especially in genetic transformation for varietal improvement. BRRI dhan47 expresses the tolerance features in salt stress, whereas BR10 and BRRI dhan32 showed susceptibility. At optimal level of partial air desiccation pretreatment played a positive role to enhance the plant regeneration along with increased the capability of calli adapt in NaCl stress condition for a suitable rice genotype. Desiccated calli are been enriched in osmotic potential resulting able to exist in salt stress condition.

Materials and Methods

Plant materials

For this study seeds of 3 rice varieties *viz.* BR10 (Progati), BRRI dhan32 and BRRI dhan47 were collected from Bangladesh Rice Research Institute (BRRI), Regional Station, Rajshahi, Bangladesh.

Methods

Sterilization of seeds and inoculation

Mature seeds were dehusked and surface sterilized with 70% (v/v) ethanol for 1 min and sodium hypochlorite (NaOCl) for 5 minutes. Seeds were then surface sterilized with 0.1% (v/v) mercuric chloride ($HgCl_2$) for 5 min and washed 2-3 times with sterile distilled water. The seeds were then inoculated on callus induction medium (CIM) in Petri dishes. Two basal media, MS and N6, and 4 types of hormonal combinations (H_1, H_2, H_3 and H_4) were used for experimental purpose as shown in **Fig. 2**. Petri dishes were sealed with parafilm and incubated at 25 ± 2°C in dark for callus induction. Ten (10) days old calli were sub-cultured using the same medium and after 3 weeks callus induction (CI) frequencies were recorded. The pH of all media adjusted 5.8.

Application of NaCl to medium and data collection

Four to 5 weeks old embryogenic calli were pretreated and subjected to abiotic stress as NaCl. Four different concentrations of NaCl (2.9, 5.9, 8.8, 11.7 gL^{-1}) were used to medium (MS + H_2). Data were recorded by one week interval and up to 4 weeks of culture initiation in salt stress condition. Through visual observation, the viable calli were counted and percentage value of viability was determined as (VCn / ICn) × 100. Calli ages of 4 weeks and approximately uniform size (100 mg) were weighed individually and placed to MS medium that supplemented with different hormones and NaCl. For each treatment calli were weighed individually which was known as initial fresh weight (FWi) and cultured into vessel singly. After 4 weeks, calli were rinsed with sterile distilled water 4-5 times. Excess water of calli was sacked by blotting paper and fresh weight (FWf) was recorded. Relative growth rate (RGR) of callus was determined on fresh weight (FW) using the standard formula RGR = (FWf − FWi) / FWi followed by Smith and McComb.[68] To compare variety-related responses to stress conditions, tolerance index (TI), based on RGR was computed according to formula TI = $RGR_{treatment}$ / $RGR_{control}$ as follows by Soheilikhah et al.[46]

Application of partial air desiccation to calli

To observe the effect of partial desiccation on plant regeneration, 4 (04) age groups of calli derived from mature seeds (3, 4, 5 and 6 w) and 5 (05) desiccation periods (15, 30, 45, 60 and 75 h) were applied to the suitable calli. For air desiccation calli were transferred to empty Petri dishes containing sterile whatman-1 filter papers followed by the standard protocol of Saharan et al.[61] The petri dishes were sealed with parafilm and kept at 27 ± 1°C in dark for different desiccation period. After the pretreatment duration the calli were transferred to regeneration medium MS + 2.0 mgL^{-1} BAP + 0.5 mgL^{-1} NAA + 1.0 mgL^{-1} Kin.

Determination of relative water content in calli

Targeted calli were incubated at 60°C for 48 h and after drying, dry mass of calli was weighted. The relative water content (RWC) of callus was calculated using the formula (FW – DW) / DW; where FW = fresh weight, DW = dry weight and percentage value was determined following the method of Al-Khayri and Al-Bahrany.[69]

Data recording and statistical analysis

The average or mean values were computed from 3 replications with standard error (SE). The experiments were laid out as completely randomized design (CRD). Analysis of variance (ANOVA) and Duncan's Multiple Range Test (DMRT) were done by SPSS16.0 software. To test the homogeneity of means accordance to DMRT, percentage values of replications were used. The seed derived calli without any pretreatment were considered as control.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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