The International Journal of Biotechnology
2013 Vol.2, No.12, pp.193-205
ISSN(e): 2306-6148
ISSN(p): 2306-9864

SCREENING OF SALT TOLERANT POTENTIALITY AND DEVELOPMENT OF *IN VITRO* TISSUE CULTURE SYSTEM FOR SOME LOCAL RICE (ORYZA SATIVA.L) VARIETIES

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ABSTRACT

The present investigation was aimed to establishment of an efficient in vitro tissue culture system with high frequency callus and regeneration induction of some local rice (Oryza sativa L.) varieties. Mature Seed Scutellum (MSS) as explants for three rice varieties which were Rajashail, Katicota, and BRRI-22 were taken as an experimental material and cultured on MS based different PGRs and additives supplemented media. In this study, in vitro experiments were conducted for assessing the effects of salt stress on germination rate, length of shoot and root of germinated plant, callus induction, fresh weight of callus and finally regeneration. For germination and callus induction frequency, MSS was cultured on MS-based medium supplemented with different concentrations of NaCl from 0.2% to 1.5%. With increasing the NaCl concentration, both the rate of germination and callus proliferation were decreased. In case of callus induction, callus was formed only for the medium contained 0.2% and 0.5% and 1% NaCl. Gradual reductions in regeneration frequency as well as number of plantlet were observed with increasing the salt concentration in MS-based medium supplemented with 2.5 mgl^{-1} BAP. All cultivars responded well on medium supplemented with 0.2% and 0.5% NaCl. The best responded regarding the callus induction frequency, the regeneration frequency and the number of shoot per callus were obtained from cv. Rajashail and Katicota among three varieties. A number of regeneration media were also evaluated for regeneration. The optimized medium for plant regeneration was MS + 2.0 mgl^{-1}BAP + 3 mgl^{-1}NAA. These studies revealed that callus induction and plant regeneration frequency were determined by medium combinations as well as genotype of plant. Thus, for developing a new and more potent salt tolerant cell lines, these 2 varieties can be used as suitable sources.

Keywords: Rice, Salt tolerant, Embryogenic, *In-vitro* culture, Proline

1. INTRODUCTION

The term salinity includes all the problems due to salt present in the soil. South and Southeast Asia are technically suited for rice production but are left uncultivated or are grown with very low yields due to salinity. About 6.5% (831 million ha) is affected by salt in soils (FAO). Bangladesh is a country of 147, 570 km2and about 20% covers the coastal areas and 30% covers

net cultivable areas (Haque, 2006). According to salinity survey findings and salinity monitoring information, about 1.02 million ha (about 70%) of the cultivated lands are affected by varying degrees of soil salinity. About 0.282, 0.297, 0.191, 0.450 and 0.087 million hectares of lands are affected by very slight, slight, moderate, strong and very strong salinity, respectively (Haque, 2006). Cropping intensity may be increased in very slight and slightly alkaline areas by adopting proper soil and water management practices with introduction of salt tolerant varieties of different crops. According to SRDI, there are lots of areas that contain different levels of salinity. 27%, 73%, 93%, and 100% areas of total cultivated areas are affected at non-saline with very slightly saline, very slightly saline with slightly saline, slightly saline with moderately saline, moderately saline with strongly saline category, respectively. The need of the improvement of salt tolerance in crop plants, rice in particular is well documented. The aims in present investigation are: characterization of the best salt tolerance capability within species by using different concentrations of NaCl and development of a potent and reproducible *in vitro* tissue culture system by various hormonal combinations which is prerequisite for *Agrobacterim*-mediated transformation.

2. MATERIALS AND METHODS

Seeds of three indica rice cultivars as Rajashail, Katicota and BRRI-22 were collected from the coastal area of Sandwip, Bangladesh. In this study, Matured Seed Scutellum (MSS) was used as experimental material.

2.1. Growth Condition

At first Mature Seeds were sterilized by using 0.2% $HgCl_2$ solutions for 8-10 mins. Then Calli were initiated from mature seeds of all varieties on Murashige and Skoog (1962)(MS) medium supplemented with 2.5 mgl^{-1}2,4-D containing different level of salt at 25±2^{0}C in dark. Four weeks old calli were transferred to the fresh regenerating media supplemented with different level (0.2%, 0.5%, 1%, 1.5%) of NaCl and kept at 25±2^{0}C under intensity of light from two 40W fluorescent tubes maintained at a level of 30 cm above the culture bottles. After few weeks, small buds like shoots were appeared and developed multiple shoots were transferred to the rooting medium.

The media for callus induction and regeneration:

- MS + 2.5 mgl^{-1} 2, 4-D (as control)
- MS + 2.5 mgl^{-1}2, 4-D + 0.2% NaCl
- MS + 2.5 mgl^{-1}2, 4-D + 0.5% NaCl
- MS + 2.5 mgl^{-1}2, 4-D + 1% NaCl
- MS + 2.5 mgl^{-1}2, 4-D + 1.5% NaCl
- MS + 2.5 mgl^{-1}BAP + 0% NaCl (as control)
- MS + 2.5 mgl^{-1}BAP + 0.2% NaCl

• MS + 2.5 mgl^{-1}BAP + 0.5% NaCl
• MS + 2.5 mgl^{-1}BAP +1% NaCl
• MS + 2.0 mgl^{-1}NAA.
• MS + 2.0 mgl^{-1}BAP + 3 mgl^{-1}NAA

2.2. Determination of Different Growth Frequency

A. CIF (Callus Induction Frequency)

The frequency of callus induction was determined as the percentage of explants producing callus.

CIF (%) = (No. of explants producing callus/ No. of explants plated) × 100

B. EF (Embryogenic Frequency)

The embryogenic frequency was determined as the percentage of explants producing embryogenic callus.

EF (%) = (No. of explants producing embryogenic callus/ No. of explants plated) × 100

C. PRF (Plant Regeneration Frequency)

The frequency of regeneration was determined as the percentage of calli producing fully regenerated plants

PRF (%) = (No. of calli regenerated plantlets/ No. of calli plated for regeneration) × 100

2.3. Statistical Analysis

The data for the parameters were analysis using MSTAT-C statistical software. Differences among the means were compared following Duncan's Multiple Range Test (DMRT) at 5% level of significance.

3. RESULTS

Experiment -1

Effect of different concentrations of NaCl supplemented with 2, 4-D and MS medium on callus induction of 3 rice cultivars as Rajashail, Katicota and BR-22.

4. METHODS

4.1. Treatment

There were 2 factors in this experiment. Factors A consisted of 3 rice genotypes and factor B consisted of five concentrations of NaCl.

Factor A: Genotype Rajashail, Katicota, and BR-22.

Factors B: MS + 2.5 mgl^{-1}2, 4-D (as control), MS+2.5 mgl^{-1}2,4-D + 0.2% NaCl, MS + 2.5 mgl^{-1}2, 4-D +0.5% NaCl, MS + 2.5 mgl^{-1}2,4-D + 1% NaCl, and MS + 2.5 mgl^{-1}2, 4-D + 1.5% NaCl.

Explants: MSS (Mature Seed Scutellum) of rice.

Design: Factorial in Completely Randomized Design (CRD).

4.2. Data Recording

The following data are recorded:

(a) Number of MSS produced callus

(b) Percentage of callus induction

(c) Percentage of embryogenic callus

(d) Nature of callus

4.3. Statistical Analysis

The data for the parameters were analyzed using MSTAT-C statistical software. Differences among the means were compared following Duncan's Multiple Range Test (DMRT) at 5% level of significance.

5. RESULT AND DISCUSSION

The relative growth of callus of the three rice varieties was observed at the different treatment levels of NaCl as 0%, 0.2%, 0.5%, 1% and 1.5% with the supplement of MS and 2, 4-D. Callus initiation was begun within 10 days after inoculation. After 14 days subculture was done on the basis of embryogenic callus. After 28 days of total aged of callus, data were taken.

5.1. Influence of Varieties

The main effects of varieties have been presented in (Table 1 and Fig.1). In case of callus production BR-22 showed the best result (54.05%) whereas in case of embryogenic properties Katicota showed the best result (41.61%). In the percentage of embryogenic callus formation Rajashail and BRRI-22 showed significant differences statistically as (40.66%) and (40.97%), respectively. Both of these two varieties have significant difference from the highest value showed by Katicota. But in case of Katicota and Rajashail have no significant differences statistically in case of callus formation. Their statistical values are 52.99 % and 52.73%, differ from BRRI-22, 54.05% which is the maximum value.

Table 1: Responses on callus induction of 3 rice cultivars as Rajashail, Katicota and BRRI -22

Treatment	% of Callus	% of embryogenic callus
Variety		
Rajashail	52.99ab	40.66b
Katicota	52.73b	41.61a
BRRI-22	54.05a	40.97b

Mean in a column followed by uncommon letter (s) varied significantly at 5% level of significance.

5.2. Influence of NaCl concentration

Influence of NaCl concentrations on callus production and embryogenicity were observed and described in the Table 2 and Fig.1. Callus growth for all 3 varieties was decreased with increasing salt concentration in the medium described in the Table 2 and Fig.1.

The maximum values were found 91.32% and 82.15% for callus induction percentage and embryogenic callus formation, respectively in control medium where NaCl concentration was 0 and 2, 4-D was 2.5mgl^{-1} supplemented with MS medium. No callus formation was occurred in 1.5% NaCl concentration where NaCl was inhibitory for seed germination and callus formation. The second highest was found in 0.2% NaCl, which was followed by 0.5% and 1% NaCl, respectively on both of callus production and percentage of embryogenicity. So, it is seen from this experiment that well proliferated big embryogeniccalli were found in 0.2% and 0.5% NaCl concentrations, although the percentage was less than the control medium. The least percentage was observed in 1% NaCl concentration as 31.87% and 14.70% (Table 2).

So, there are significant differences among their media concentrations which were analyzed by statistical software. Therefore, it can be concluded from the above results that with the increasing of salt concentration callus induction of different varieties and their embryogenic potentiality were decreased gradually. The non-embryogeniccalli were watery, whitish, friable and less compact and have no regeneration capability.

Table-2. Influence of different NaCl concentrations on percentage of total callus induction and embryogenic callus production of 3 rice cultivars as Rajashail, Katicota and BRRI-22.

Con. of 2, 4-D+ NaCl	% of Callus	% of embryogenic callus
MS+2.5 mgl^{-1} 2,4-D (as control)	91.32a	82.15a
MS+2.5 mgl^{-1} +0.2% NaCl	76.40b	62.38b
MS+2.5 mgl^{-1} +0.5% NaCl	66.69c	46.16c
MS+2.5 mgl^{-1} +1% NaCl	31.87d	14.70d
MS+2.5 mgl^{-1} +1.5% NaCl	0.00e	0.00e

Mean in a column followed by uncommon letter (s) varied significantly at 5% level of significance.

5.1. Interaction between Variety and NaCl

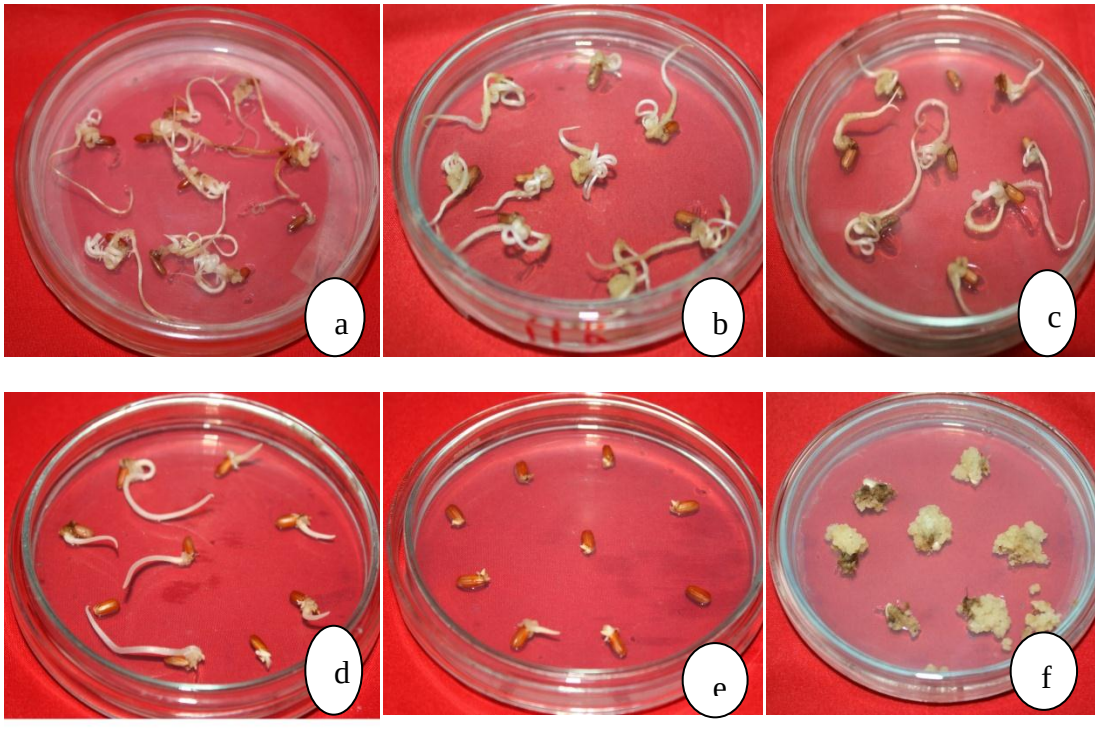
It was found that varieties were interacted significantly with NaCl in all parameters studied (Table 3 and Fig.1). All the varieties induced callus production at a maximum rate when they were with 2.5 mgl^{-1}2,4-D with no salt. On the other hand, all the varieties induced no callus production when salt concentration was increased at the highest peak as 1.5%. Among the salt concentrations, all the varieties interacted with 0.2 and 0.5% NaCl at a higher rate which were followed by 1% NaCl at a least value as 31.45%, 12.15% for callus induction frequency in percent and embryogenicity, respectively. All the levels of NaCl have significant differences at each level, which were analyzed by using statistical software MSTAT-C.

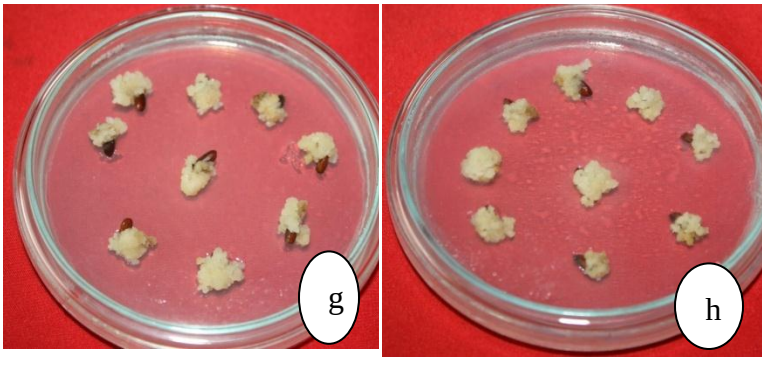
Table-3. Interactions between varieties and NaCl treatments on total callus and embryogenic callus production.

Variety	Different Concentrations of NaCl with 2.5mgl^{-1} 2, 4-D	% of callus	% of embryogenic callus
Rajashail	2.5mgl^{-1} 2,4-D + 0.00% NaCl	90.74a	81.21b
	2.5mgl^{-1} 2,4-D + 0.2% NaCl	74.17c	63.82d
	2.5mgl^{-1} 2,4-D + 0.5% NaCl	68.58d	46.11h
	2.5mgl^{-1} 2,4-D + 1% NaCl	31.45f	12.15k
	2.5mgl^{-1} 2,4-D + 1.5% NaCl	0.00g	0.00 l
Katicota	2.5mgl^{-1} 2,4-D + 0.0% NaCl	92.19a	85.14a
	2.5mgl^{-1} 2,4-D + 0.2% NaCl	75.77c	63.18e
	2.5mgl^{-1} 2,4-D + 0.5% NaCl	64.71e	47.28g
	2.5mgl^{-1} 2,4-D + 1% NaCl	30.98f	12.44k
	2.5mgl^{-1} 2,4-D + 1.5% NaCl	0.00g	0.00l
BRRI-22	2.5mgl^{-1} 2,4-D + 0.0% NaCl	91.03a	80.10c
	2.5mgl^{-1} 2,4-D + 0.2% NaCl	79.27b	60.15f
	2.5mgl^{-1} 2,4-D + 0.5% NaCl	66.77de	45.08i
	2.5mgl^{-1} 2,4-D + 1% NaCl	33.19f	19.52j
	2.5mgl^{-1} 2,4-D + 1.5% NaCl	0.00g	0.00l l

Mean in a column followed by uncommon letter (s) varied significantly at 5% level of significance.

Fig-1. Effects of different concentrations of NaCl on callus production supplied with MS+2.5mgl^{-1} 2, 4-D of cultivar Rajashail. (a) and (f) Callus production on control medium without salt at 14 and 28 days respectively; (b) and (g) Callus production in 0.2% NaCl; (c) and (h) Callus production in 0.5% NaCl; (d) 1% and (e) 1.5% NaCl.





Experiment-2

Effect of different concentrations of BAP and NaCl on the percentage of shoot regeneration from callus.

6. METHODS

Treatments: There were 2 factors in this experiment. Factor A consisted of three rice genotypes and factor B consisted of four concentrations of Benzyl Amino Purine (BAP).

A.Genotype: Rajashail, Katicota, and BR-22.

B.BAP and NaCl concentrations: MS + 2.5 mgl^{-1}BAP as control, MS + 2.5 mgl^{-1}BAP + 0.2% NaCl, MS+ 2.5 mgl^{-1}BAP + 0.5% NaCl and MS + 2.5mgl^{-1}BAP + 1% NaCl.

Total number of treatments were 12 (4X3), and replicated 3 times.

Explants: Callus from different 2, 4-D treatments.

Design: Factorial in Completely Randomized Design (CRD).

6.1. Data Recording

The followed data are recorded

(a) Number of explants regenerated

(b) Number of callus responded

(c) Percentage of shoot regeneration

6.2. Statistical Analysis

The data for the parameters were analyzed using MSTAT-C statistical software. Differences among the means were compared following Duncan's Multiple Range Test (DMRT) at 5% level of significance.

6.3. Influence of Varieties

The main effects of varieties have been represented in the Table 4 and Fig.2. After 4 weeks, embryogeniccalli ware transferred to regeneration medium, where MS medium supplemented with 2.5 mgl^{-1}BAP and 0.2%, 0.5% and 1% NaCl. The calli were kept for 10 days at dark conditions with using 1% (w/w) agar. Then the calli were transferred to same regeneration

medium except changing the concentration of agar from 1% to 0.8% (w/w) at the light conditions. At the third week of culture, data were scored. It was observed from the Table 4, Rajashail showed the best response. It has the maximum value as 13.00, 10.00 and 39.18% for all three parameters which are number of callus response, number of shoot per callus, percentage of shoot regeneration. Rajashail is followed by Katicota and BRRI-22. In case of callus responded Rajashail and Katicota showed no significant differences although the values were different but in case of shoot produced and percentage of shoot regeneration, Rajashail showed significant difference. Also BRRI-22 and Katicota differ from each other significantly in all three parameters.

Table-4. Responses of varieties on different concentrations of BAP and NaCl treatment in the percentage of shoot regeneration

Variety	No. of callus response	No. of shoot per callus	% of shoot regeneration
Rajashail	13.00a	10.00a	39.18a
Katicota	11.25a	7.917b	34.98b
BRRI-22	8.417b	6.250c	26.11c

Mean in a column followed by uncommon letter (s) varied significantly at 5% level of significance

6.4. Influence of Cytokine and NaCl

Significant effects of NaCl were observed in shoot regeneration, which were depicted in Table 5.Addition of NaCl at regeneration medium decreased the relative growth of plantlet at a significant rate. Regeneration medium without NaCl showed a marked increase in both the number of shoot per callus and also percentage of shoot regeneration. The greatest values observed in control media MS+2.5 mgl^{-1}BAP were as 20.11%, 13.67% and 64.55% for number of callus responded, number of shoots per callus, percentage of shoot regeneration, respectively.

The second highest value was observed in 0.2% NaCl concentration for all parameters, which was followed by 0.5% NaCl concentration. In 1% NaCl concentration no callus was regenerated from the callus. The 1% showed the highest inhibitory effect. Thus it is clearly proved that with the increasing of salt concentration the regeneration frequency is gradually decreased and show significance difference in each level. The highest percentage is 0.5% for cultivar Rajashail, Katicota and BR-22.

Table-5. Effect of different concentrations of BAP and NaCl treatment on the percentage of shoot regeneration

Con. of BAP & control	No. of callus response	No. of shoot per callus	% of shoot regeneration
MS+2.5mgl^{-1} BAP (as control)	20.11a	13.67a	64.55a
MS+2.5mgl^{-1} BAP + 0.2% NaCl	14.00b	10.22b	43.26b
MS+2.5mgl^{-1} BAP + 0.5% NaCl	9.444c	8.333c	25.89c
MS+2.5mgl^{-1} BAP + 1% NaCl	0.000d	0.000d	0.000d

Mean in a column followed by uncommon letter (s) varied significantly at 5% level of significance

6.5. Interaction between Variety and BAP

It was examined and found that all varieties significantly interacted with BAP and NaCl concentrations in all parameters (Table 6 and Fig. 2). All the varieties regenerated at maximum level when they were cultured on MS medium supplemented with 2.5mgl^{-1}BAP but no salt. Variety Rajashail showed the best results (17%) in 0.2% NaCl for all the parameters. From the table it is found that there were a negative relation among varieties and NaCl concentration. Shoot production was declined at the highest NaCl concentration (1% NaCl). So, there is a significant difference in each treatment which was analyzed by analytical statistical software MSTAT.

Table-6. Data on Interaction between variety and BAP on different experimental parameters.

Variety	Hormone	No. of shoot per callus	% of shoot regeneration	% of shoot regeneration
Rajashail	MS+2.5mgl^{-1} BAP (as control)	22.67a	16.00a	70.07a
	MS+2.5mgl^{-1} BAP + 0.2% NaCl	17.00bc	13.006	51.67d
	MS+ 2.5 mgl^{-1} BAP + 0.5% NaCl	12.33cd	11.00c	35.00f
	MS+ 2.5 mgl^{-1} BAP+ 1% NaCl	0.00F	0.00f	0.00i
Katicota	MS+ 2.5 mgl^{-1} BAP+ as control	20.67ab	14.00b	67.68b
	MS+ 2.5 mgl^{-1} BAP+0.2% NaCl	14.00cd	8.667d	43.77e
	MS+ 2.5 mgl^{-1} BAP+ 0.5% NaCl	10.33de	9.00d	28.48g
	MS+ 2.5 mgl^{-1} BAP+ 1% NaCl	0.00f	0.0f	0.00i
BRRI-22	MS+ 2.5 mgl^{-1} BAP (as control)	17.00bc	11.00c	55.91c
	MS+ 2.5 mgl^{-1} BAP 0.2% NaCl	11.00d	9.00d	34.34f
	MS+ 2.5 mgl^{-1} BAP 0.5% NaCl	5.667e	5.00e	14.21h

Mean in a column followed by uncommon letter (s) varied significantly at 5% level of significance

Fig-2. Effect of different concentrations of BAP and NaCl on the percentage of shoot regeneration from callus (a) and (b) Green spot and small bud appeared in the callus (c) and (d) Shooting in the control media(no salt) (e) at 0.2%; (f) at 0.5% salt and (f) Rooting of plantlets.

7. DISCUSSION

In vitro tissue culture is a prerequisite for plant transformation. In recent years considerable efforts are being directed toward the improvement of important agronomic traits of rice through biotechnological techniques. Callus cultures were exposed to plant regeneration medium containing various concentrations of NaCl, as it is a good parameter for analysis of the effect of salt at regeneration level. During this study 0.2%, 0.5% and 1% NaCl level with 2.5 mgl^{-1}BAP were used. All three varieties respond at 0.2% and 0.5%. But at 1.0% level, it showed no regeneration capability. Among these 3 varieties Rajashail and Katicota responded well in regeneration than BRRI-22. Rajashail showed (13.00%) and Katicota (11.25%). Although the values were different but they showed no significant differences. BRRI-22 showed 8.41 values in

respect to regeneration capability. Medium containing 0.2% and 0.5% NaCl had (14.00%) and (9.44%) value where in control had (20.11%). 1% showed no regeneration frequency. Therefore, plant regeneration decreases with the increasing of NaCl concentration, which was also reported by Shankhdhar *et al.* (2000)andAl-Forkan *et al.* (2005). Thus salinity affects the metabolism process in plant cells. High osmotic pressure from high salinity restricted plant cells to uptake water and some minerals and nutrients dissolved in the culture medium (Cicek and Cakirlar, 2002).

The resultant inhibition on relative growth of callus and plant may be due to high level of Na+ accumulation. Accumulation of Na+ in salt-stressed rice is generally dependent on salt concentrations (Djanaguiraman *et al.*, 2006 and Ahmad *et al.*, 2007), development stages (Castillo *et al.*, 2007), Salt exposure times (Golldack *et al.*, 2003)and salt tolerant abilities (Dionisio-Sese and Tobita, 1998; Hoai *et al.*, 2003; Khan and Panda, 2008). So the lower percentage of regeneration in the present study may be due to low Na: K ratio in the medium as addition of NaCl increased Na+ and reduced K+.

BAP is a potent growth regulator in regeneration medium as well as the cultivars showed a high frequency of regeneration when callus supplemented with 2.5 mgl^{-1}BAP on regeneration medium. Lee *et al.* (2002) reported that supplementation of high level of BAP was more effective on regeneration of rice. According to Xie *et al.* (1995)plant regeneration was dependent on the embryogenic callus which was affected by hormone combinations used in callus induction medium. Biswas and Mandal (2007) observed that addition of CH on regeneration medium inhibits the plant regeneration. However CH when added with ABA in regeneration medium green plant regeneration was significantly enhanced. In the current study 2.5 mgl^{-1}BAP was used and showed a significant amount of plant regeneration rate which was decreased gradually by the addition of 0.2% and 0.5% NaCl. At 1% level the regeneration frequency was zero.

Different concentrations of auxin and Cytokine were also used in regeneration medium for the three cultivars, as Rajashail, KaticotA and BRRI-22. These hormones were MS + 2.0 mgl-1 BAP + 3 mgl-1 NAA. There were several reports which indicated that nutrients compositions of differences are the most important factors for effective plant regeneration (Jain *et al.*, 1996; Khanna and Raina, 1998). In this study all the cultivars responded well.

According to Peyachoknagul *et al.* (1994), of the two cytokinins (BAP and Kn), BAP in combination with auxin (0.5 mgl^{-1}NAA) produced more green plantlets than Kn among ten rice cultivars.Agarwal *et al.* (2002) also reported the best response for regeneration when medium was supplemented with 1 mgl^{-1}NAA and 2.0 mgl^{-1}BAP. Those data support my present investigation for shoot regeneration at 2.5 mgl^{-1}BAP and multiple shooting at 2.0 mgl^{-1}BAP + 3 mgl^{-1}NAA medium concentration. NAA and IBA have been reported to induce roots from regenerated shoots of rice (Sahrawat and Chand, 1998; Burikam *et al.*, 2002). Well regenerated shoots when cultured on MS medium containing NAA and BAP, began to initiate roots.

According to SRDI, general salt content in coastal area of Bangladesh is 4 or 5 dS/m which is almost equal to 2400 or 3000 ppm. But Rajashail and Katicota already upland coastal cultivable

crop and in this experiment they showed the tolerance of 0.2% and 0.5% NaCl level which is equal to approximately 2000 to 5000 ppm, respectively. So, if a suitable salt tolerant gene can be transferred to Rajashail and Katicota and grown in *in vitro* by using this protocol more HYV can be developed which will be cultivable in more salt flooded areas.

8. ACKNOWLEDGEMENT

A deep gratitude goes to Dr. Md. Faisal, the senior scientific officer of Bangladesh Agricultural Research Institutes (BARI), Hathazari, Chittagong for the statistical analysis of the experimental data by using statistical software MSTAT-C.

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