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Full Length Research Paper

Responses of some selected Malaysian rice genotypes to callus induction under *in vitro* salt stress

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Tissue culture technique can be used as a source for genetic variability by means of genetic modifications through the process of *in vitro* cultures. This technique has been widely used for breeding purposes, especially for stress tolerance selection, which severely limits rice production. Also, the establishment of a suitable plant regeneration system is a prerequisite for successful genetic transformation. The aim of this study is to identify the most suitable medium and to assess the genotype performance for *in vitro* salt stress responses in some selected Malaysian rice genotypes. Differences in culture conditions, growth rate, plant hormone responses and accumulation of proline content were monitored. All the selected genotypes showed that the callus-growth capacities were significantly affected by the genotypes and the culture media. Evidently, callus was best induced on the MS medium added with 10 µM dichlorophenoxyacetic acid (2,4-D) and 0.4 gm/l casein hydrolysate. In addition, the shoot regeneration capacity from the callus was the most effective in ½ MS added with 10 µM 6 benzylaminopurine (BAP). The two genotypes, that is, MR219 (line 4) and MR219 (line 9), consistently performed the best in both callus culture (93.51 and 92.22%) and plant regeneration capacity (27.03 and 26.34%), respectively. When the callus were transferred to different concentrations of NaCl (0 to 250 mM) supplemented medium in order to examine their responses to salinity, the two genotypes, that is, MR219 (line 4) and MR219 (line 9), showed a significant decline in the callus growth (18.83 and 23.5%) and regeneration capacity (7.33 and 7.68%), respectively. A similar trend was also observed for the proline content. All the genotypes significantly resulted in proline accumulation. MR211 showed the highest accumulation, whereas MR219 (line 4) revealed the lowest proline accumulation. These proline content analyses further suggest potential salinity tolerance in the rice genotypes.

Key words: Plant regeneration, embryogenesis, salinity, callus.

INTRODUCTION

Rice (*Oryza sativa* L.) is one of the most important staple crops which provides the source of carbohydrates for more than half of the world's population (Tyagi et al., 2004). The world's projected demand of rice by 2020 is 880 million tons in proportion to the increased population (Anbazhagan, et al., 2009). Although with the increasing population and decreasing land availability, agriculture is suffering from severe damage of biotic and abiotic stresses. Conventional breeding is essential to improve

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Abbreviations: 2,4-D, Dichlorophenoxy acetic acid; **BAP,** 6 benzylaminopurine; **Kin,** kinetin.

rice but progress is slow due to several barriers (Wang et al., 2005). According to Shavindra et al. (2005), these challenges can be met by using advanced biotechnologies as a result of improved stress resistance with a high stable yield potential and good grain quality. Having mentioned this, rice is a salt sensitive plant, whereby the growth and productivity are unfavourably affected by salt stress, which prevents the crop from reaching its full genetic potential as well as limits its yield (Blumwald and Grover, 2006). Hence, the use of *in vitro* technique has been employed in rice tissue culture in order to understand the mechanism of salinity stress in the plant.

In tissue culture, production of callus and its subsequent plant regeneration are the prime steps to be manipulated by biotechnological means (Saharan et al., 2004) and to exploit somaclonal variation of the unorganized white, yellow or brown callus (Monirul Islam et al., 2005). *In vitro* selection of salt tolerant cell lines and regenerated plants has been reported in several species, for instance Hordeum (Sibi and Fakiri, 2000), sunflower (Alvarez et al., 2003) sugarcane (Gandonou et al., 2005) and rice (Lutts et al., 1990). However, there is a need to understand the response of rice tissue culture to salinity conditions before *in vitro* selection studies can be carried out.

Proline is an osmoprotectant use to conserve osmotic stability and prevent damage. Plants cultured under salt stressed show high proline accumulation (Praderm et al., 2003). It is an extensive phenomenon, whereby the proline accumulation in plants exposed to salt stress has been correlated in many species with their adaptation to osmotic stress (Delauney and Verma, 1993). The occurrence of high proline content coupled with reduced chlorophyll content under salt stress condition was reported by Harinasut et al. (2000). However, its role in imparting resistance to salt stress is controversial, and perceived as a symptom of stress injury rather than an indicator of stress tolerance (Yong et al., 2005). Hence, the proline accumulation in salt stress must be more precisely investigated. Keeping these points in view, the objective of this investigation is to elucidate the responses of five selected Malaysian rice genotypes for efficient callus induction, reproducible plant regeneration and proline content under salinity conditions.

MATERIALS AND METHODS

Callus induction and plant regeneration

Mature seeds of the five selected rice genotypes, that is, MR219 (control), MR219 (line 4), MR219 (line 9), MR220 and MR211, were dehusked and surface sterilized with 70% ethanol for 2 min followed by 40% sodium hypochlorite for another 20 min. The seeds were rinsed five times with sterile distilled water before inoculation on a callus induction medium containing the MS medium (Murashige and Skoog, 1962) supplemented with 3% sucrose, 2.75 gm/l gelrite and 2,4 dichlorophenoxy acetic acid (2,4-D) of different concentrations, that is, 0, 5, 10, 15, 20 and 25 µM. The effects of different concentrations of casein hydrolysates were determined. In addition, the pH of the medium was adjusted to 5.8 prior to autoclaving at 15 psi for 20 min. The callus induced from the seeds was aseptically removed using forceps, and then transferred to the same medium. Furthermore, the callus was classified as embryogenic and non-embryogenic as described by Van et al. (1990). After four weeks of culture, the embryogenic callus was recorded for each genotype. The fresh weight and dry weight were measured for the callus growth curve determination at weekly interval. The best callus growth medium was chosen according to its stable and continuous growth in all genotypes. Conversely, to find out the plant regeneration capacity, the calli were transferred to the regeneration medium. The regeneration capacity was investigated in full and ½ strengths of MS medium added with 6 benzylaminopurine (BAP) and kinetin (Kin). The regenerated green shoots per plantlet were then counted and the frequency of callus induction as well as regeneration was calculated as follows:

Callus induction frequency (%) = number of seeds producing callus/ number of seeds plated x 100

Regeneration frequency (%) = number of plants recovered/number of callus plated x 100.

Effects of salinity on callus growth and plant regeneration

The embryogenic callus of all genotypes was cultured onto the best callus reproduction medium obtained from the above experiment. Four-week-old callus was divided into pieces of 50 mg. These pieces were transferred onto the same medium supplemented with different NaCl concentrations, that is, 0, 50, 100, 150, 200 and 250 mM, for salt stress responses. At the end of the four-week period, the callus was taken for growth analysis. For the callus growth analysis, the fresh weights of callus were recorded at the beginning and the end of the culture period. The relative growth was calculated on the basis of the initial and final growths, as follows:

$$\text{Relative growth} = \frac{(\text{Final growth} - \text{Initial growth}) \times 100}{\text{Initial growth}}$$

For plant regeneration, the callus was transferred to the best regeneration medium obtained from the above results and supplemented with NaCl at different concentrations (that is, 0, 50, 100, 150, 200 and 250 mM). The plant regeneration capacity was measured on the basis of plant formation.

Proline analysis

The proline content in the tissue was estimated by utilising the colorimetric method as described by Bates at el. (1973). Acid ninhydrin was prepared by warming 1.25 g ninhydrin in 30 ml glacial acetic acid and 20 ml 6 M phosphoric acid. Approximately, 0.5 g of the leaf samples was homogenized in 10 ml of 3% aqueous sulfosalicylic acid and the homogenate was then filtered through the Whatman filter paper. 2 ml of filtrate was reacted with 2 ml of acid ninhydrin and 2 ml of glacial acetic acid in the test tubes for 1 h under the water bath condition at 100°C. Then, the reaction was terminated in the ice bath for 10 min. The reaction mixtures were extracted with 4 ml toluene and mixed vigorously for 30 sec. The chromophore containing toluene was aspirated from the aqueous phase and the absorbance was read at 520 nm using toluene as a blank. The standard curve for proline was prepared by dissolving it in distilled water covering the concentration range of 1 to 10 µg/ml. The proline concentration was determined by the standard curve and calculated on the fresh weight basis as follows:

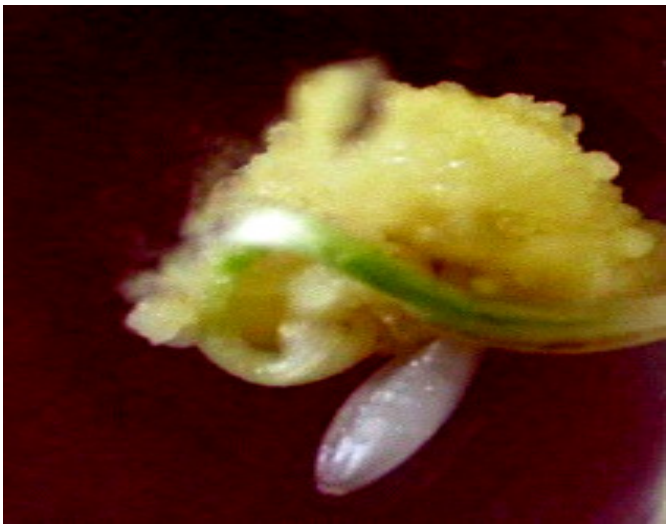

Figure 1. Callus initiation stage of MR219 (line 4). The bar scale corresponds to 0.5 cm.

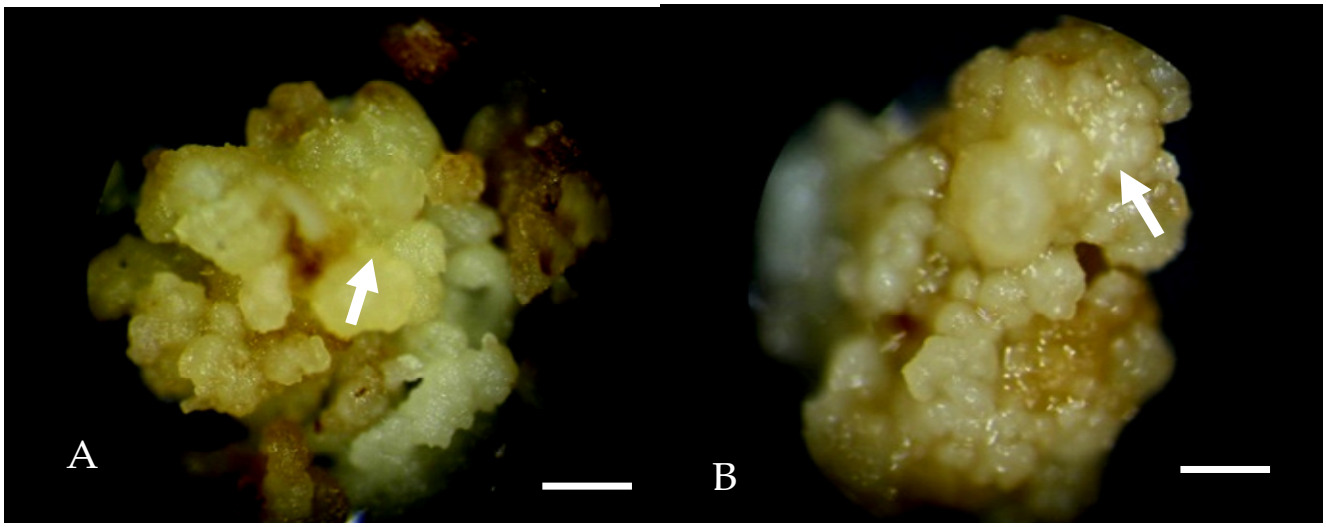

Figure 2. A, Embryogenic callus; **B,** non-embryogenic callus of MR219 (line 4). The bar scale in A and B corresponds to 0.5 cm.

[(µg proline / ml × ml toluene) / 115.5 µg / µg mole] / [(g sample) / 5] = µmoles proline / g of the fresh weight material

All the experiments were conducted in triplicates. The data were analyzed in a completely randomized design using the Statistical Analysis System (SAS) and MSTATC computer programmes. In addition, the comparison of means was tested for significance using the Least Significant Difference (LSD) test at 0.05 level of probability.

RESULTS AND DISCUSSION

Callus induction

Callus initiation started on the scutellum region of the seed embryo within one week of culture and grew in size over the next four weeks on the callus induction medium. The study indicated that the five selected rice genotypes were ready to form calli. Figure 1 shows the initiation of callus from genotype MR219 (line 4), as well as the embryogenic and non-embryogenic calli (Figures 2A and 2B). The embryogenic callus was isolated and selected based on the description by Van et al. (1990). Its feature is usually light yellow to white in colour, as well as dry, compact and nodular. On the other hand, the non-embryogenic callus appeared to be watery, light yellow and non-nodular. The total callus induction frequency in the MS medium ranged from 28.03 to 93.51%. The results indicated that the treatment of MS basal medium supplemented with 10 µM 2,4-D exhibited the highest callus induction for all the tested rice genotypes. These

Table 1. Callus induction percentage of five rice genotypes in the MS media supplemented with different concentrations of 2-4,D.

Media	MR219	MR219	MR219	MR220	MR211
	Control (%)	Line 4 (%)	Line 9 (%)		
MS (Control)	0	0	0	0	0
MS + 5 µM 2-4,D	41.91 ± 1.07 t	80.57 ± 1.41 f	74.04 ± 0.78 i	61.94 ± 1.41 op	68.87 ± 1.67 l
MS + 10 µM 2-4,D	85.23 ± 1.4 cd	93.51 ± 1.99 b	92.22 ± 0.68 b	72.46 ± 1.77 j	84.61 ± 2.14 d
MS + 15 µM 2-4,D	61.57 ± 1.64 p	74.99 ± 1.35 h	85.58 ± 0.61 c	65.88 ± 1.48 n	81.85 ± 1.08 e
MS + 20 µM 2-4,D	56.67 ± 1.29 q	76.21 ± 1.16 j	68.9 ± 0.56 l	31.82 ± 1.37 u	66.68 ± 1.21 m
MS + 25 µM 2-4,D	45.77 ± 0.75 s	70.45 ± 1.44 k	55.24 ± 0.78 r	28.03 ± 1.55 v	62.63 ± 0.52 o

Means ± SD was calculated from the means of the three replicates of the culture for callus induction percentage. The different letters indicate significant difference at $P < 0.05$.

Table 2. The variance analysis of the effects of culture media and genotypes on the callus growth rate.

Source	Df	Mean square	F value
Media	5	13380.44	6439.23**
Genotype	4	1714.52	825.1**
Media*Genotype	20	236.06	113.13**
Error	60	2.077	

*: Significant at P = 0.001; a: coefficient of variation = 2.56.

highest callus percentage values in the five genotypes were 93.51% for MR219 (line 4), 92.22% for MR 219 (line 9), 85.23% for MR219 (control), 84.61% for MR211 and 72.46% for MR220. In all the media combinations, the maximum percentage of callus induction was observed for genotype MR219 (line 4) in the MS medium added with 10 µM 2,4-D (93.51%). The treatment containing 5, 15, 20 and 25 µM 2,4-D concentrations resulted in the callus induction percentages, as follows 80.57, 74.99, 76.21 and 70.45%, respectively (Table 1). Mitchiba et al. (2001) reported that high concentration of 2,4-D would reduce the cell viability, which possibly could be due to its herbicide nature to cells. In this study, 10 µM 2,4-D was found to be the most effective concentration for callus induction. Also, according to the analysis of variance, as shown in Table 2, the effects of 2,4-D concentrations and all the genotypes, and the interaction between media and genotypes were significantly different at $P < 001$.

Generally, auxins, such as 2,4-D, have been used for embryogenic callus induction in many monocotyledonous plants (Kunitake and Mii, 1998). MS medium is commonly used for rice tissue culture studies, and the basal medium with 10 µM 2,4-D is effective for the establishment of embryogenic callus culture (Yin et al., 1993). In this study, overall, the callus from the MS medium had a good texture. Furthermore, some genotypes also produced better callus in other concentrations of 2,4-D treatment, however, the quality of callus was not as good as those in 10 µM 2,4-D. Notably, the success of *in vitro* cultures largely depends on the use of harmonious combinations of nutritional constituents and plant growth regulators. Evidently, MR219 (line 4) revealed the best callus growth percentage of 93.6% in the combination of casein hydrolysate (that is, 0, 0.1, 0.2, 0.3, 0.4 and 0.5 g/l) and 10 µM 2,4-D with 0.4 gm/l (Figure 3). On the other hand, Table 3 shows that the effects of casein hydrolysate concentrations and all genotypes, as well as the interaction of media and genotypes were significantly different ($P < 001$). The use of MS medium without hormone and with only casein hydrolysates did not respond to callus induction, but only resulted in seed germination.

In addition to the maintenance and multiplication of callus tissues in the same morphological appearance, the growth patterns of the callus were investigated. This experiment was to examine the developmental stage of the transformational study and the optimum stage for sub-culturing. Variations in response for callus growth on the basis of fresh weight and dry weight were clearly observed when the average values of weight gain were compared (Table 4). The observation showed significant differences of fresh and dry weights among the genotypes. Apparently, the highest fresh weight (0.043 g) and dry weight (0.166 g) were found in MR219 (line 4). Meanwhile, the fresh weight and dry weight of other genotypes were 0.231 g and 0.067 gm for MR219 (control), 0.420 and 0.163 g for MR219 (line 9), 0.394 and 0.157 g for MR220 and 0.407 and 0.158 g for MR211 in the MS medium added with 10 µM 2,4-D. All the results agreed with the above study whereby, the concentration of 10 µM 2,4-D gave better response for fresh and dry weights than other combinations.

Furthermore, the callus growth duration for fresh weight

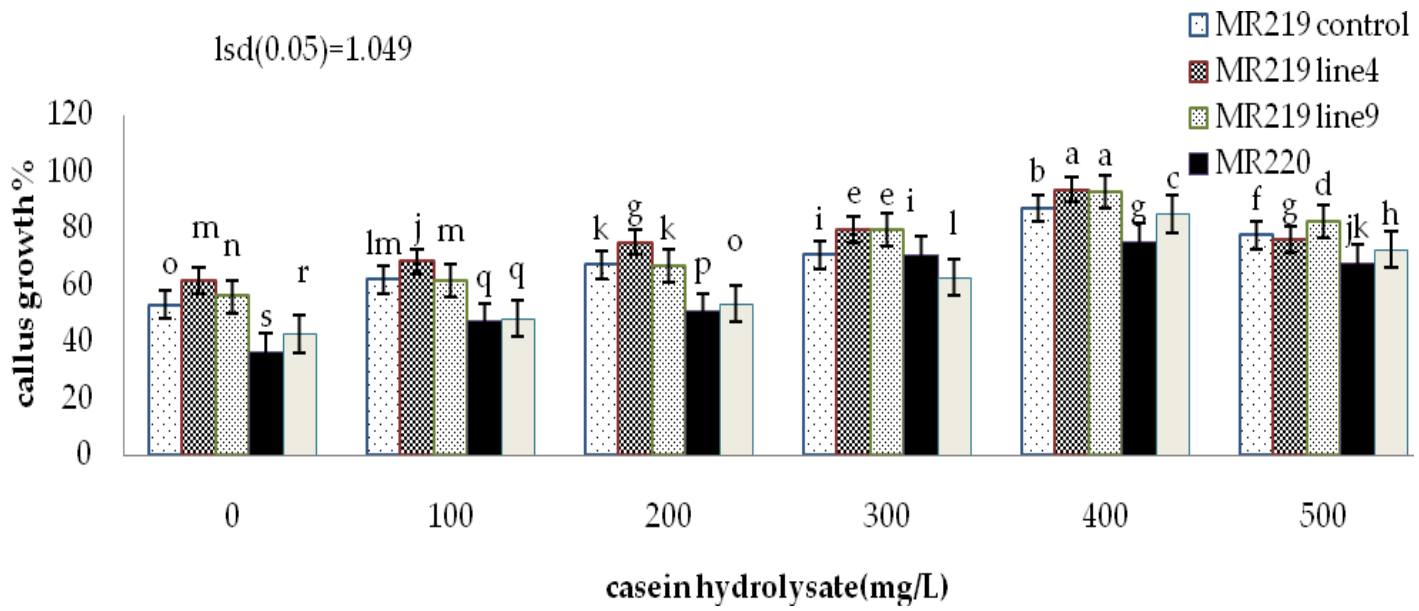

Figure 3. Effects of different concentrations of casein hydrolysate in the callus of the five selected rice genotypes on the MS medium added with 2,4-D. The data of mean was taken from the total of the three replicates. The different letters indicate that the values were significantly different at P < 0.05.

Table 3. The variance analysis of the effects of the casein hydrolysate media and genotype on the callus growth rate.

Source	Df	Mean square	Fvalue
Media	5	2659.53608	644.84**
Genotype	4	1057.83673	256.49**
Media x Genotype	20	43.03516	10.43**
Error	60	4.12431	

*: Significant at P = 0.001; a: coefficient of variation = 3.01.

and dry weight was tested in all genotypes at one week interval. This duration was important for plant regeneration. From this observation, all the callus tissues could be suitably maintained and multiplied using the induction medium and transferred to a fresh medium at one week interval. Figures 4 and 5 show that the best growth of callus was observed in the fourth week of the culture period, which revealed the highest fresh and dry weights in all the genotypes. In addition, the highest growth rate was found in MR219 (line 4), that is, 0.430 and 0.166 g for fresh and dry weights, respectively.

All these results indicated that the callus growth depended on the genotypes, which was the same with the finding for durum wheat, bread wheat and sugarcane (Gandonou et. al., 2005a). Furthermore, both the genotypes and media compositions, and their interaction largely affected callus induction and subsequent plant regeneration. Pandey et al. (1994) reported that the success of *in vitro* culture largely depends on the nutritional media, growth regulators, genotypes and the interaction of genotype x medium. Likewise, this revelation is in agreement with the finding of Monirual Islam et al. (2005) and this present study. The results of this present experiment also showed that 10 µM 2,4-D containing the media responded well for callusing and plant regeneration although 2,4-D was used at different doses (that is, 5, 15, 20 and 25 µM, respectively). Moreover, Pandey et al. (1994) also found that the mature dehulled rice seeds were the best source for callusing, while the use of 10 µM 2,4-D was the best medium for callus. Michiba et al. (2001) observed that 2,4-D in high concentration caused damage to the cells in their study of *Doritaenopsis* cell suspension. Notably, the induction of embryogenic callus in rice is considered the most critical step. At the same time, casein hydrolysate was found beneficial for the generation of embryogenic callus in *Japonica* (Toki, 1997) and *Indica* rice (Zhang et al., 1996). In this present investigation, the MS basal medium containing 10 µM 2,4-D and 0.4 g/l casein hydrolysate was successfully used for the induction of embryogenic callus.

Plant regeneration

Plant regeneration efficiency system needs to be determined in the attempt to use the embryogenic callus as the target tissue in a reliable genetic modification system. In this research, the plant regeneration study was carried out by establishing the callus culture in full and half strengths of MS medium under light condition. After the transfer of embryogenic callus to the regeneration medium, green spots began to develop on the callus surfaces

Table 4. Fresh weight and dry weight of the callus of the five rice genotypes in the MS medium combination with 2,4-D. The data of mean was taken from the total of the three replicates.

Media and genotype	MR 219 (c)		MR 219 (4)		MR 219 (9)		MR 220		MR 211	
	FW	DW	FW	DW	FW	DW	FW	DW	FW	DW
MS+5µM 2-4,D	0.171 ± 0.003	0.085 ± 0.003	0.303 ± 0.004	0.126 ± 0.002	0.315 ± 0.003	0.151 ± 0.001	0.294 ± 0.001	0.141 ± 0.001	0.307 ± 0.002	0.139 ± 0.002
MS+10µM 2-4,D	0.231 ± 0.002	0.107 ± 0.003	0.43 ± 0.001	0.206 ± 0.002	0.42 ± 0.001	0.203 ± 0.001	0.394 ± 0.003	0.197 ± 0.001	0.407 ± 0.003	0.198 ± 0.004
MS+15µM 2-4,D	0.224 ± 0.004	0.096 ± 0.003	0.412 ± 0.002	0.184 ± 0.003	0.329 ± 0.002	0.158 ± 0.002	0.364 ± 0.002	0.175 ± 0.002	0.325 ± 0.003	0.163 ± 0.002
MS+20µM 2-4,D	0.214 ± 0.003	0.086 ± 0.001	0.371 ± 0.001	0.155 ± 0.003	0.325 ± 0.0012	0.145 ± 0.002	0.353 ± 0.004	0.151 ± 0.001	0.315 ± 0.002	0.144 ± 0.001
MS+25µM 2-4,D	0.204 ± 0.003	0.08 ± 0.001	0.291 ± 0.001	0.101 ± 0.002	0.285 ± 0.001	0.134 ± 0.001	0.341 ± 0.001	0.121 ± 0.001	0.276 ± 0.002	0.139 ± 0.001

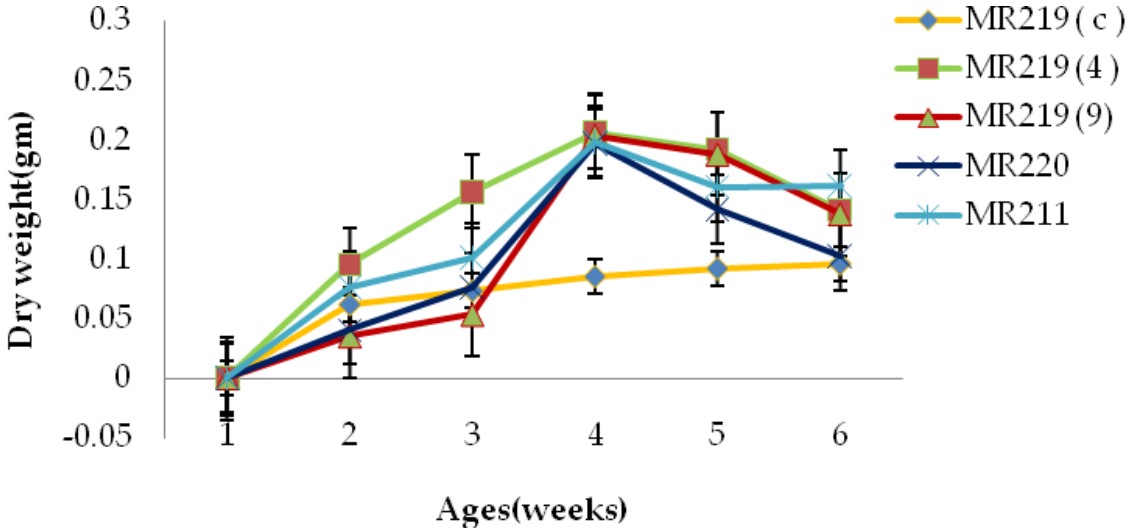

Figure 4. Fresh weight of five rice genotypes of the callus growth percentage in the MS medium added with 10 µM 2,4-D in one week interval.

within 7 to 8 weeks. After that, green plants developed from these green spots (Figures 6A and B). Simultaneously, some calli began to turn brown and eventually died. It appeared that the non-embryogenic callus did not regenerate when transferred to the regeneration medium. In addition, striking differences were also observed in the regenerated plants between the genotypes tested.

Effects of Kin and BAP on plant regeneration

Determining the suitable medium for plant regeneration for rice callus was conducted using the MS medium with different concentrations and combinations of BAP and Kin. In this study, full and ½ strengths of MS medium supplemented with BAP (5 µM) or Kin (5 µM) were used as the preliminary test. Of all the genotypes, MR219 (line4) showed the best regeneration capacity followed by MR219 (line 9), MR 219 (control), MR211 and MR220 in most of the media combinations. A half strength MS combined with BAP (13.35%) gave the best regeneration percentage compared with Kin (11.27%), as shown in Figure 7. However, due to minor regeneration

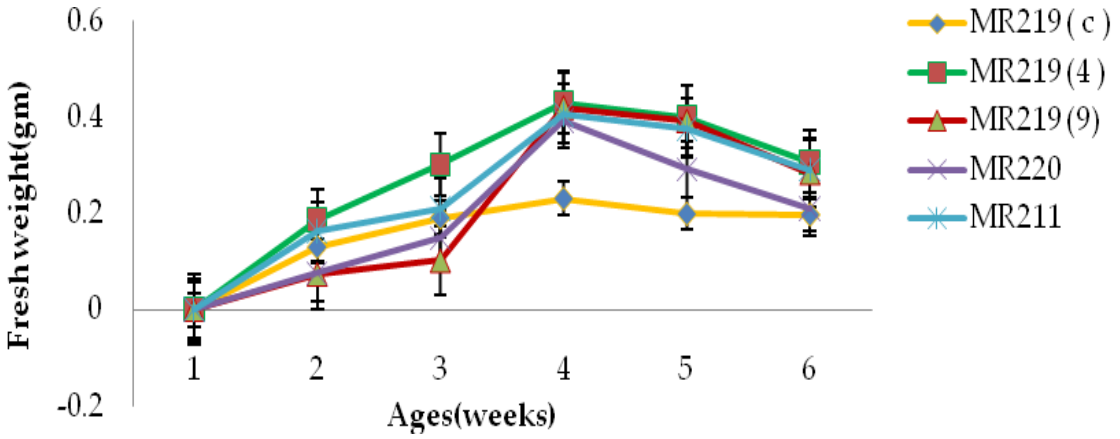

Figure 5. Dry weight of five rice genotypes of the callus growth percentage in the MS medium added with 10 µM 2,4-D in one week interval.

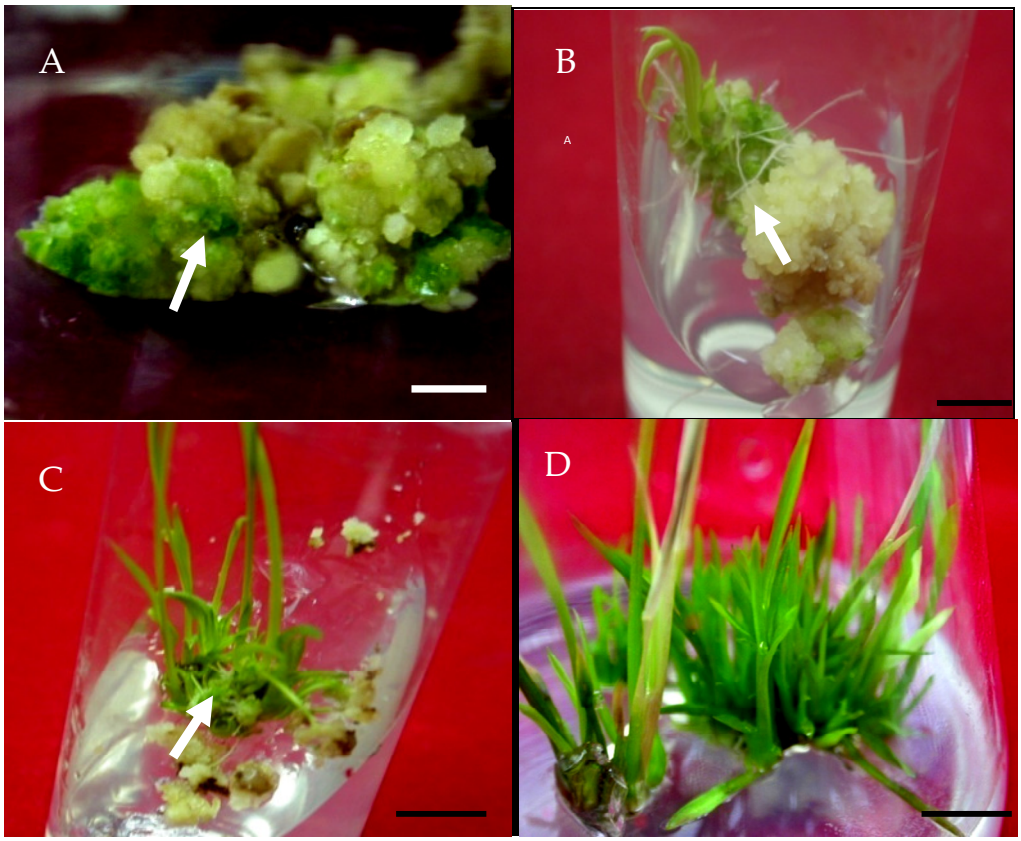

Figure 6. (A) The green plant regeneration from mature seeds; (B) green callus and plant regeneration of MR219 (line 4); (C) and (D) green plant formation in NaCl treated media. The bar scale in A and B corresponds to 0.5cm, while in C and D, it corresponds to 1 cm.

efficiency, a second study was carried out using only BAP combination. At this time, full and ½ strengths of MS were used with only 5 to 25 µM of BAP addition. Significantly, the cultures treated in ½ MS medium added with BAP resulted in higher percentage of plant regeneration as compared to the hormone free medium added with Kin. Moreover, the addition of BAP also revealed the best percentage values in plant regeneration efficiency, for instance, 28.17% for ½ MS with 10 µM BAP and 12.01% for full MS with 10 µM BAP, as compared with other

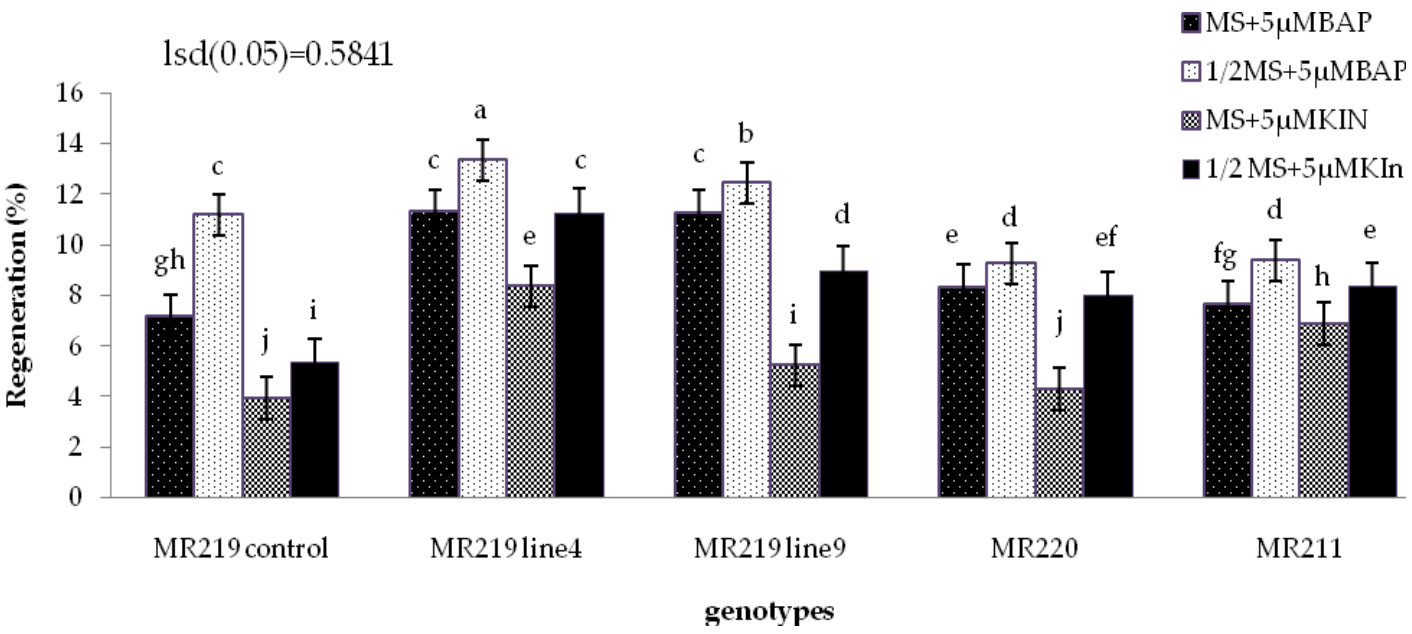

Figure 7. Percentage of better regeneration of five rice genotypes in 5 µM BAP and 5 µM Kin hormone combination in full and half strengths MS medium.

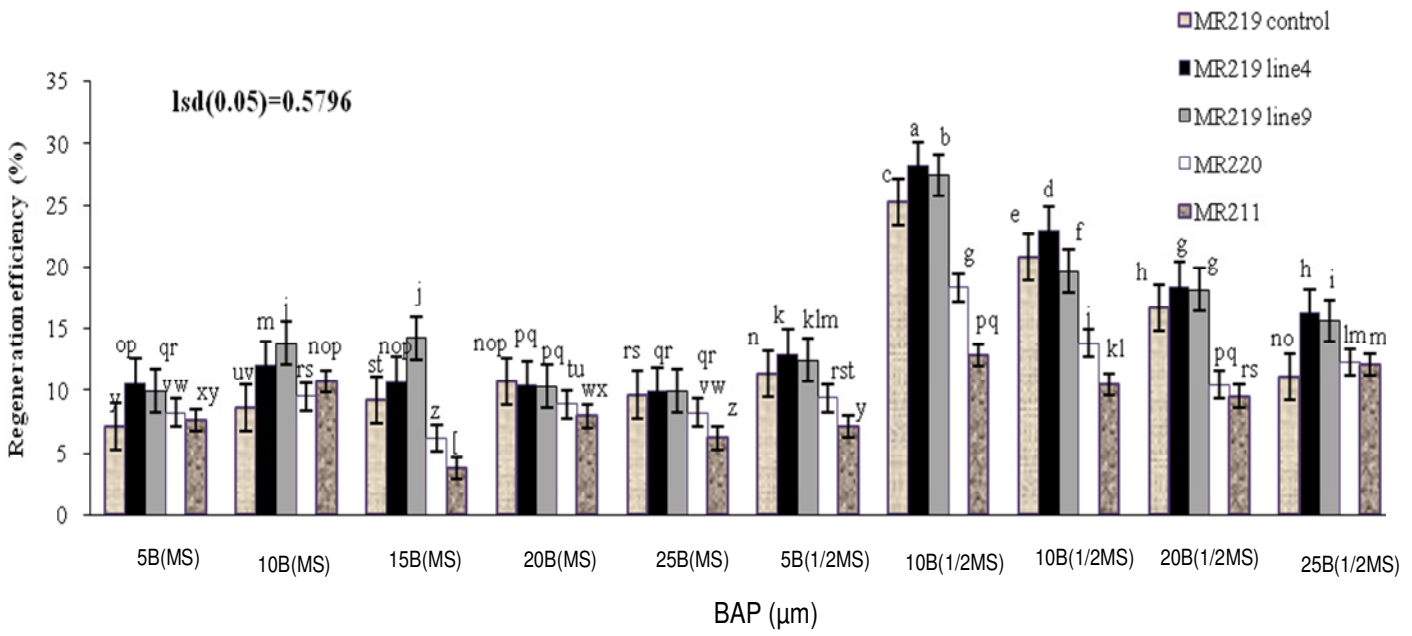

Figure 8. Regeneration percentage of five rice genotypes in BAP (5-25 µM) with full and ½ strengths MS media combination. The mean and standard deviation were calculated using the data taken from the three replicates. The different letters indicate the significant difference.

combinations (Figure 8). From the results, BAP was the adenine type in cytokinin that was more effective on plant regeneration. Evidently, BAP was the most effective cytokinin in enhancing plant regeneration efficiency.

Previous studies on *Saccharum* sp (Gandonou et al., 2005a,b), *Sorghum bicolor* (Mishra and Khurana, 2003) and *Triticum* (Zale et al., 2004) demonstrated the quality of callus, the embryogenic response and the regeneration potential, which were claimed to be influenced by the genotype. Similar findings were also shown in *Oryza sativa* (Hoque and Mansfield, 2004; Rachmawati and Anzai, 2006). Likewise, Hoque et al. (2007) recommended

that suitable genotypes and media combi-nation should be used to increase the plant regeneration frequency in rice tissue culture. These variations would be due to the differences in the components and concentrations of endogenous phytohormones, as well as the differences in their responsiveness to 2,4-D. Although relatively high frequency of callus was observed in this study, overall, the regeneration was quite low. The callus exhibited high regenerative potential and the capacity to proliferate in later subcultures (Huan et al., 2004). The early subculture of the newly initiated callus would have a minimal proliferation rate and may lead to the death of the callus culture. Therefore, the newly formed callus was only sub-cultured onto the fresh media for it to grown into a mass. Furthermore, it was observed that the green plant regeneration ability of the plated callus depended on the genotypes and the callus induction media. Among the studied rice genotypes, MR219 (line 4) regenerated the maximum regeneration percentage (28.17%) followed by MR219 (line 9) (27.41%) MR219 (control) (25.26%), MR220 (18.32%) and MR211 (12.79%) (Figure 8).

The treatment of auxin to cytokinin ratio in the medium can lead to the development of shoots, roots or somatic embryos from which the whole plants can subsequently be produced. Benzyl aminopurine (BAP) has been reported as being favoured for adventitious shoot formation in numerous plants. Until now, most of the plant regeneration studies have been done on *japonica* rice. However, the *Indica* rice varieties are considered persistent to tissue culture manipulation. The high rate of callus induction and the low rate of regeneration were observed in this study, and the results could be attributed to the related auxin source used in the callus induction medium. Therefore, the exact level of hormone in the callus induction medium requires some degree of concession between callus induction and plant regeneration frequency. The differences in response of genotypes for plant regeneration capability is important, especially when carried out after a short time of callus maintenance as reported in maize (Muhammad et al., 2005). Availability of an efficient regeneration system is a prerequisite for undertaking any transformation study.

In Vitro responses for salinity condition

Callus growth

For salt treatment studies, attempts to produce embryogenic callus were conducted in the saline medium containing 0, 50, 100, 150, 200 and 250 mM NaCl concentrations for each genotype. The induced callus were excised after inoculation of explants, transferred to a fresh medium for proliferation, and then transferred again to the best regeneration medium containing different concentrations of NaCl according to the above results.

The addition of NaCl into the culture medium caused an increase in callus necrosis for all genotypes (Figures 6C and D) and a significant difference was observed among the genotypes. The calli of all genotypes were severely reduced at 150 to 250 mM. The highest percentage of callus growth was found in MR211 (54.16%) followed by MR220 (29.33%), MR219 (line 9) (23.5%), MR219 (control) (25.16%) and MR219 (line 4) (18.83%) at 250 mM NaCl concentration (Figure 9). Also, in proportion to the analysis of variance, Table 5 shows that the effects of NaCl concentration and all genotypes, as well as the interaction between media and genotypes were significantly different ($P < 001$). Furthermore, the control callus treated with NaCl showed normal growth compared with the NaCl treated media. This condition could be due to the growth inhibition and non-availability of nutrients or accumulation of other metabolites due to the NaCl treatment. In addition, Bhattacharya (1991) observed that the possibility of reduction in the callus growth could be due to stress, ionic imbalance and reduction of protein or increase and decrease in the concentration of other metabolites. Also, Niluffer and Zapata (1994) reported that the reduction of callus growth was caused by the increase in the concentration of NaCl. The callus growth percentage decreased as the concentration of NaCl increased in the culture medium. This decrease was more important for MR219 (line 4) and MR219 (line 9) in comparison with the other three genotypes. According to the results, for 50 mM NaCl (i.e. the lowest NaCl concentration used), the callus growths were about 72.88 and 46.23% at the highest NaCl concentration using 250 mM for MR219 (line 4). After measuring the rice callus, 150, 200 and 250 mM NaCl concentrations already inhibited the growth of the rice callus and the regeneration. In the MS medium with 50 and 100 mM NaCl concentrations, the embryogenic callus of all cultivars seemed to develop normally. In addition, in sunflower (Alvarez et al., 2003) and sugarcane (Gandonou et al., 2005), it was found that NaCl reduced the callus growth and responded differently to salt stress. On the other hand, in this study, NaCl effect resulted in callus necrosis and a reduction of its growth.

Plant regeneration

When the NaCl treated calli of the five selected rice genotypes were transferred to the selected regeneration media, they resulted in complete plant regeneration. The various rice genotypes responded to different plant regeneration capacities. But the frequency of plant regeneration was reduced with increased NaCl concentration. According to the results of plant regeneration among the five rice genotypes, sensitivity to salt tolerance at 250 mM NaCl was in the order of; MR 211, MR219 (control), MR219 (line 9), MR219 (line 4) and MR220; that is, 9.91, 8.77, 7.68, 7.33 and 7.29%, respectively (Figure 10). Furthermore, base on the

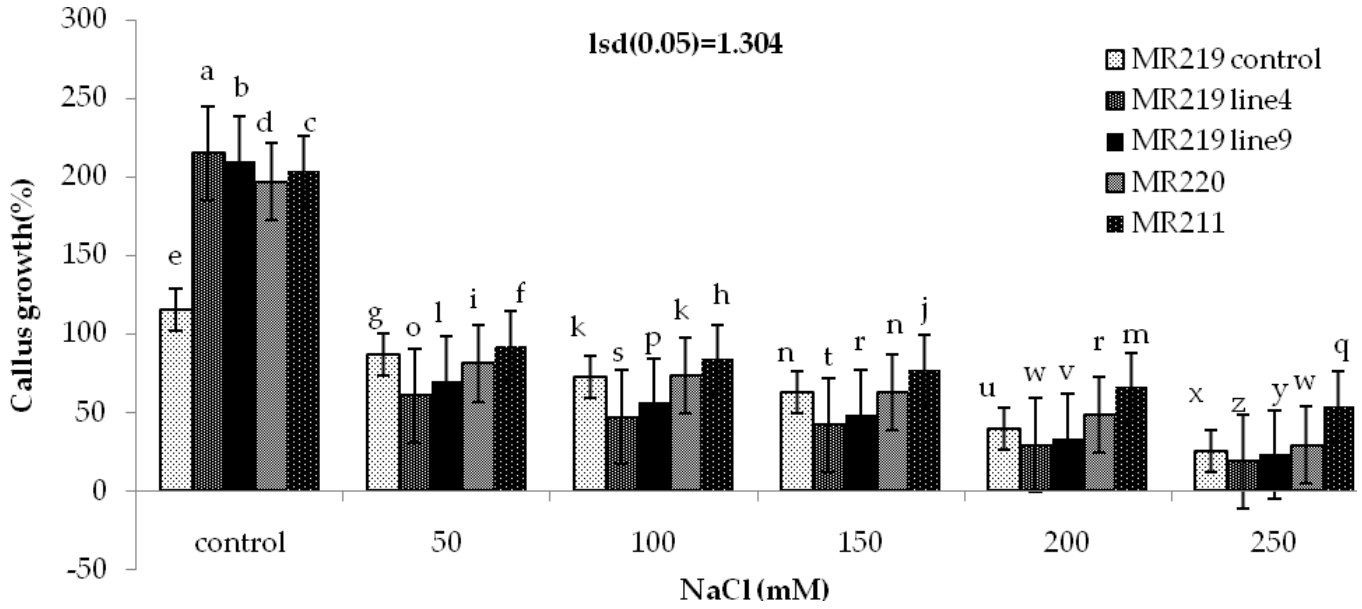

Figure 9. The callus growth (%) of five rice genotypes in different concentrations of NaCl treated media. The mean ± SD was calculated based on the three replicates. The different letters show the significant difference.

Table 5. The analysis of variance on the effects of NaCl treated media and five rice genotypes on the callus growth rate.

Source of variation	Df	Mean square	F value
NaCl callus media	5	48531.83	7612.7**
Genotype	4	2525.79	396.2**
Media x Genotype	20	1091.63	171.23**
Error	60	6.375	

*: Significant at P = 0.001; a: coefficient of variation = 3.25.

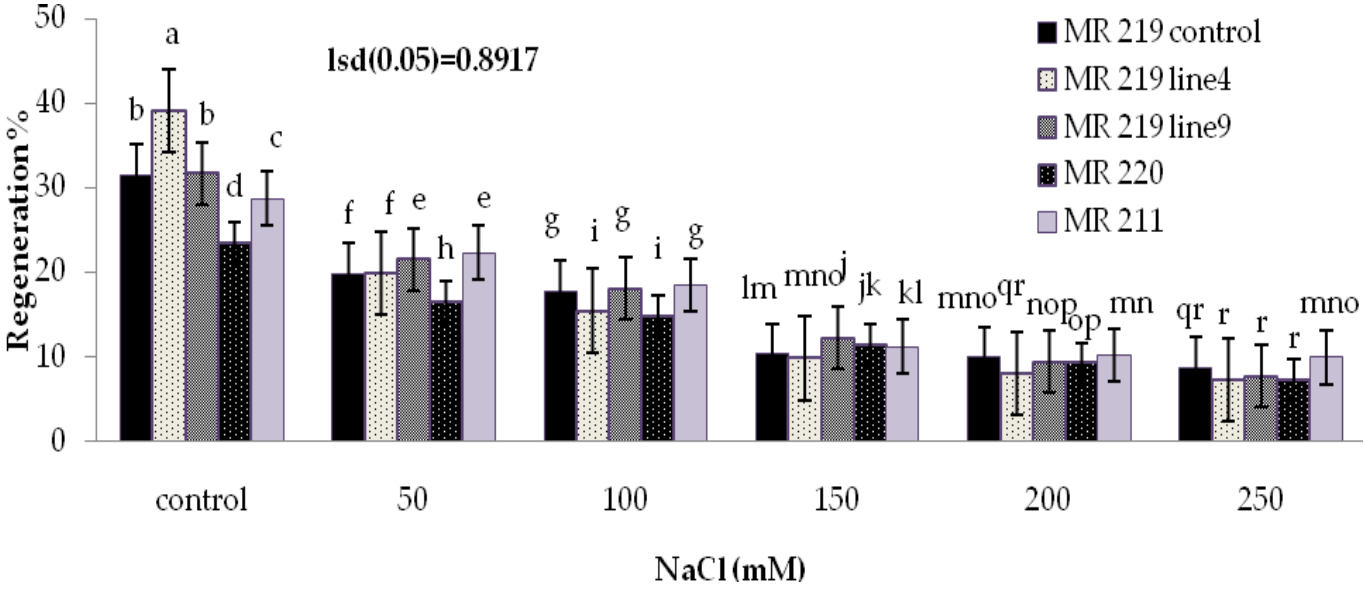

Figure 10. Regeneration (%) of five rice genotypes in NaCl treated media. The different letters show the significant difference.

Table 6. The analysis of variance on the effects of NaCl treated regeneration media and genotypes on plant regeneration.

Source of variation	Df	Mean square	F value
NaCl Reg media	5	1107.22878	371.47**
Genotype	4	29.243379	9.81**
Media x Genotype	20	19.578275	6.57**
error	60	2.980652	

*: Significant at P = 0.001; a: coefficient of variation = 10.74.

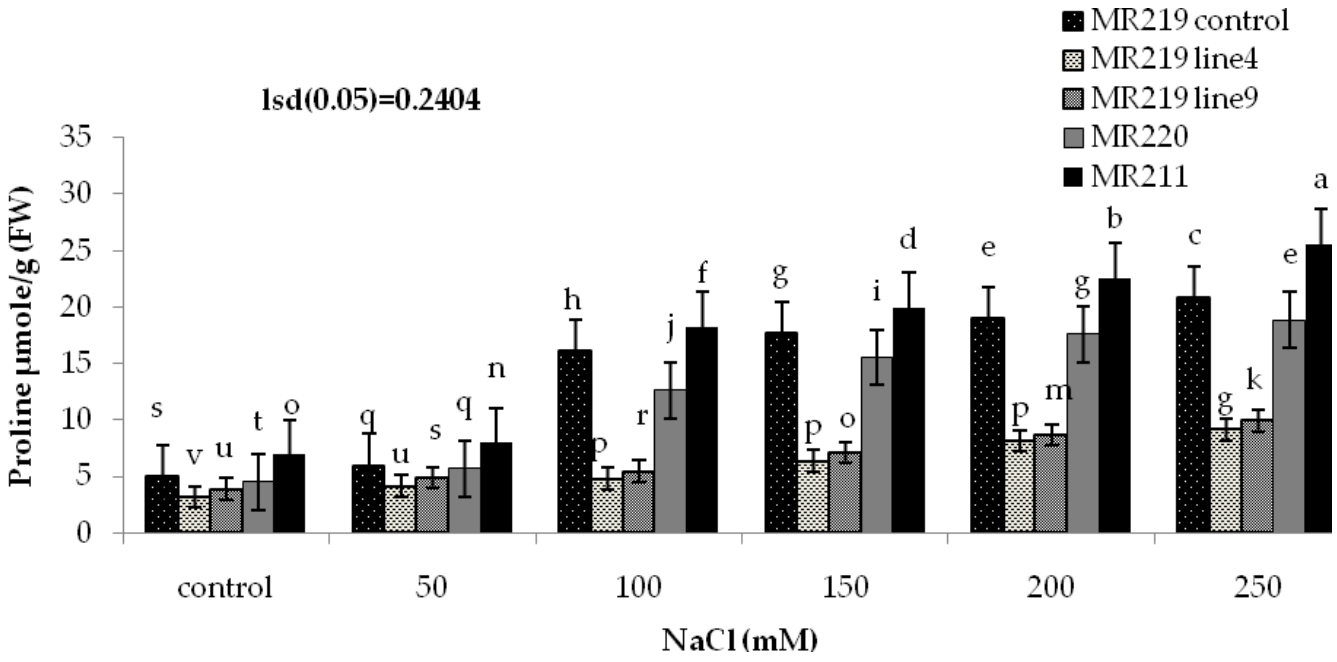

Figure 11. Effects of salinity on proline accumulation of five rice genotypes. The data represent the means + S.E. of the three replicates. The different letters show the significant difference.

analysis of variance, Table 6 shows that the effects of NaCl concentration with plant regeneration media and all genotypes, as well as the interaction between media and genotypes were significantly different (P < 001). This result was more important for MR219 (line 4) and MR219 (line 9) in comparison with others. The differences in plant regeneration capacity with the increased NaCl concentration were obtained among the genotypes and resulted in the decrease of plant regeneration with increased NaCl concentration. Likewise, Niluffer and Zapata (1994) also found similar results in their study. The NaCl concentrations of 150, 200 and 250 mM appreciably inhibited the growth of rice callus and regeneration. In the MS medium with 50 and 100 mM NaCl, the embryogenic callus of all the genotypes seemed to develop normally. Based on the results, it was clear that 150 mM NaCl started to appreciably inhibit the growth of cells in the callus compared to the growth in the absence of NaCl. Evidently, the findings revealed that the superior genotype was MR211, whereas the inferior one was MR219 (line 4) for salt tolerance together with their high potential for embryogenic callus induction. This could suggest that, these varieties are good models for investigative study of physiological mechanisms associated with *in vitro* selection for salt tolerance in rice.

Proline accumulation

The accumulation of proline was affected by rice genotypes, as depicted in Figure 11. Apparently, the accumulation of proline was higher under salt stress treatment than the untreated plant for all the rice genotypes. The level of increase in the proline concentration in response to salt stress varied between the rice varieties. In particular, MR211 (25.55) was observed to have the highest proline accumulation under salinity stress treatment among the genotypes, while MR219 (line 4) (9.17)

Table 7. The analysis of variance on the effects of NaCl treated regeneration media and genotypes on proline concentration.

Source of variation	Df	Mean square	F value
NaCl Reg media	5	375.07	1731.17**
Genotype	4	403.31	1861.49**
Media x Genotype	20	22.38	103.3**
Error	60	0.216	

*: Significant at P = 0.001; a: coefficient of variation = 4.14

had the lowest proline accumulation among the other rice varieties. Following the analysis of variance, the effects of NaCl concentration with plant regeneration media and all genotypes, the interaction between media and genotypes, as well as proline concentration were significantly different at P < 001 (Table 7). Thus, this result conformed to the above study for callus induction and plant regeneration under salinity stress. Notably, proline is known to play an important role as osmoprotectant in plants subjected to hyperosmotic stresses such as soil salinity. The proline levels in the sensitive plants were already increased after the NaCl treatment compared to the control (sensitive) untreated plants. An increase in the proline content in the treated tolerant plants was observed, which could be due to the enhanced breakdown of proteins. The accumulation of proline in response to various stresses has been reported in a number of plant species under water and salt stress conditions (Kapchina et al., 2004). Thus, the main factors, i.e. rice genotype and NaCl concentration, strongly affected the proline content.

In summary, most of the rice genotypes resulted in significant variance for their *in vitro* response to callus induction, quality and subsequent plant regeneration. This depends on the initial callus induction and medium composition. It could be concluded that the genotypic differences sturdily influenced callus formation and regeneration potential. The results show that the embryogenic callus from the mature seed embryos is a very good source of material for efficient *in vitro* plant regeneration in rice. In this study, the recognized salt-tolerant plants selected from the callus were unstable with regard to salinity tolerance. However, it improved the ability of plants recovered from the stress medium to grow under salt stressful conditions, as revealed in the subsequent plant regeneration screen. The accumulation of proline in plants exposed to identical salt stresses appears to be cultivar specific in rice. Proline levels in stressed plants are inversely correlated with the capacity to withstand salinity stress. Therefore, the selection of better responsive rice genotypes like MR219 (line 4) will offer a great promise for the induction of higher level of callus and plant regeneration but lower accumulation of proline content. Hence, the identification of these genotypes with superior tissue culture performance is the key step in gene transfer for salinity gene in rice biotechnology.

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REFERENCES

Alvarez I, Tomaro, LM, Benavides PM (2003). Changes in polyamines, proline and ethylene in sunflower calluses treated with NaCl. Plant Cell, Tissue Organ Cult. pp. 1-9.

Anbazhagan M, Rajendran R, Kalpana M, Natarajan V, Panneerselvam R (2009). *Agrobacterium* mediated Transformation of rice, var. Pusa Basmati-1. J. Ecol. Biotechnol. 1(1): 7-11.

Bates LS, Waldren RP, Teare ID (1973). Rapid determination of free proline for water stress studies. Plant Soil. 39: 205-207.

Bhattacharya S (1991). Differential responses of rice(*Oryza sativa* L) callus derived from three cultivar in saline environment. Phytomorphol. 41(1-2): 133-137.

Blumwald EA, Grover (2006). Salt tolerance in Plant Biotechnology: Current and Future uses of Genetically Modified Crops, John Wiley and Sons Ltd., UK, pp. 206-224.

Delauney AJ, Verma DP (1993). Proline biosynthesis and osmoregulation in plants. Plant J. 4(4): 215-223.

Gandonou CH, Abrina J, Idaomar M, Skali Senhaji N (2005a). Response of sugarcane (*Saccharum* sp) varieties to embryogenic callus induction and *in vitro* salt stress. Afr. J. Biotechnol. 4(4): 350-354.

Gandonou CH, Errabi T, Abrini J, Idaomar M, Chibi F, Skali Senhaji N (2005b). Effect of genotype on callus induction and plant regeneration from leaf explants of sugarcane (*Saccharum sp*). Afr. J. Biotechnol. 4 (11):1250-1255.

Harinasut P, Srisunak S, Pitukchaisopol S, Charoensataporn R (2000). Mechanisms of adaptation to increasing salinity of mulberry: proline content and ascorbate peroxidase activity in leaves of multiple shoots. Sci. Asia, 26: 207-211.

Hoque ME, Mansfield JW (2004). Effect of genotype and explant age on callus induction and subsequent plant regeneration from root-derived callus of *Indica* rice genotypes. Plant Cell Tissue Org. Cult. 78: 217-223.

Hoque ME, Ali MS, Karim NH (2007). Embryogenic callus induction and regeneration of elite Bangladeshi *Indica* rice cultivars. Plant Tissue Cult Biotechnol. 17(1): 65-70.

Huan LVT, Takamura T, Tanaka M (2004). Callus formation and plant regenera6tion from callus through somatic embryo structure in Cymbidium orchid. Plant Sci. 166: 1443-1449.

Kapchina-Toteva V, Slavov S, Batchvarova R, Krantev A, Stefanov D (2004). Stress markers in chlorsulphuron tolerant transgenic tobacco plants. Belg. J. Plant Physiol. 30(1-2): 103-111.

Kunitake H, Mii M (1998). Somatic embryogenesis and its application for breeding and micropropagation in asparagus (Asparagus officinalis) Plant Biotechnol. 15(2): 51-61.

Lutts S, Bouharmont J, Kinet JM (1999). Physiological characteristics of salt-resistant rice (*Oryza sativa* L.) somaclone. Aust. J. Bot. 47: 835-849.

Michiba K, Okamoto T, Mii M (2001). Increasing ploidy level in cell suspension cultures of Doritaenopsis by exogenous application of 2,4-dichlorophenoxyacetic acid. Physiol. Planta. 112: 142-148.

Mishra A, Khurana P (2003). Genotype dependent somatic embryogenesis and regeneration from leaf base cultures of *Sorghum bicolor*. J. Plant Biochem. Biotechnol. 12: 53-56.

Monirul Islam M, Mahatalat A, Debabrata M (2005). *In Vitro* callus induction and plant regeneration in seed explants of rice (*Oryza sativa* L.). Res. J. Agric. Biol Sci. 1(1): 72-75.

Muhammad R, Tahira F, Tayyab H, Khurram B, Riazuddin S (2005).

Effect of different media on callus formation and regeneration of different genotypes of Maize (*Zea mays* L.) Plant Tissue Cult. 15(1): 57-65.

Murashige T, Skoog F (1962). A revised medium for rapid growth and bioassay with tobacco tissue culture. Plant Physiol. 15: 473- 49.

Zapata FJ, Nilufer HK (1994) Stress for regeneration: Effect of NaCl on rice (*Oryza sativa* L.) anther culture. Ind. J. Plant Physiol. 37(4): 242-245.

Pandey SK, Ramesh B, Gupta PK (1994). Study on effect on genotype and culture medium on callus formation and plant regeneration in rice (*Oryza sativa* L.). Ind. J. Genet. 54: 293-299.

Praderm W, Chalermpol K, Chawewan V (2003). Effect of Salinity on Biochemical and Physiological Characteristics in Correlation to Selection of Salt tolerance in Aromatic Rice (*Oryza sativa* L.). Sci. Asia. 29: 333-339.

Rachmawati D, Anzai H (2006). Studies on callus induction, plant regeneration and transformation of *Javanica* rice cultivars. Plant Biotechnol. 23: 521-524.

Saharan V, Yadav RC, Yadav NR, Chapagain BP (2004). High frequency plant regeneration from dessicated calli of *indica* rice (*Oryza sativa*). Afr. J. Biotechnol. 3(5): 256-259.

Shavindra J, Amitabh M (2005). Advanced in rice biotechnology in the new millennium, Plant Biotechnol. J. 3: 275-307.

Toki S (1997). Rapid and efficient *Agrobacterium*-mediated transformation in rice. Plant. Mol. Biol. Rep. 15:16-20.

Tyagi AK, Khurana JP, Khurana P, Raghuvanshi S, Gaur A, Kapur A, Gupta V, Kumar D, Ravi V, Khurana S, Vij P, Sharma S (2004). Structural and functional analysis of rice genome. J. Genet. 83: 79-99.

Van Sint Jan V, Skali-Senhaji N, Bouharmont J (1990). Comparaison de différentes variétés de riz (Oryza sativa L.) pour leur aptitude à laculture *in vitro*. Belg. J. Bot. 123(1/2): 36-44.

Wang Y, Xue Y, Jiayang LJ (2005). Toward molecular breeding and improvement of rice in China. Trends in Plant Sci. 10(12): 610-614.

Yin YH, Li SH, Chen YM, Guo HQ, Tian WZ, Chen Y, Li LC (1993). Fertile plants regeneration from suspension culture-derived protoplasts of an *indica* type rice (*Oryza sativa* L.). Plant Cell Tissue Org Cult. 32: 61-68.

Zhang Y, Chautler SE, Gupta S, Zhao Y, Hannah LC, Meyer C, Weston J, Wu MX, Preiss J, Okita TW (1996). Molecular approaches to enhance rice production through manipulation of starch metabolism during seed development. Rice genetics III, Proc. Third International Rice Genetics Symposium (ed. Khush GS.), IRRI, Manila, Philippines, pp. 809-813.