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In vitro screening method for drought tolerance evaluation in two rice varieties (BRRI 28 and BRRI 29)

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Abstract: Abiotic stress is one of the major rate limiting factor for rice production worldwide especially in Asia and Africa. While drought tolerance is become of increasing importance in rice (*Oryza sativa L.*), selection under actual field conditions is tiresome due to low heritability and time required. Selection in tissue culture is thought to be one way to improve selection efficiency, but this requires standardized protocols. Rice cultivars BRRI 28 and BRRI 29 showed expressive callus induction, but the ability for callus induction and regeneration decreased under mannitol stress in both cultivars. Calli were induced on semisolid Murahige and Skoog (MS) medium supplemented with 2 m g/l 2,4-dichlorophenoxyacetic acid (2 ,4 -D). Embryogenic calli showed shoot regeneration on MS medium supplemented with 2 mg/l benzyl aminopurine (BAP) + 0. 5 mg/l kinetin + 0.5 mg/l naphthalene acetic acid (NAA). Increased levels of mannitol (0 gm/l, 10 gm/l, 20 gm/l, 30 gm/l, 40 gm/l) were used to create drought stress. There was reduction in callus induction ability and plant regeneration efficiency with increasing levels of mannitol stress. These results indicated that mannitol can be used as drought stress creating agent under in vitro conditions and rice variety BRRI 29 was relatively tolerant to drought stress as compared to BRRI 28. This study will serve as a base line for *in vitro* screening of drought tolerant transgenic rice.

Key words: *Oryza sativa, PEG, drought stress, callus induction, plant regeneration, BRRI, mannitol.*

1. Introduction

Water deficit is a main problem for crop production worldwide, limiting the growth and productivity of many crop species particularly in rain-fed agricultural areas [1]. The drought is a major environmental factor that determines the growth, the productivity and the distribution of plants. It is estimated to be one of the most serious yield reducing stresses in the agriculture. The drought affects more than 10 % of arable soil and desertification and salinization are rapidly increasing on a global scale [2]. The drought condition is continually distressed by the explosive increase in the world population, the continuing deterioration of arable land and scarcity of fresh water. This increase of the environmental stress poses serious threats to global agricultural production and food security. It has been estimated that two-thirds of the yield potential of major crops are routinely lost due to unfavorable growing environments. Rice (*Oryza sativa L.*) is subjected to a range of abiotic stresses that affect their growth and development. In particular, it is predicted that water deficit will continue to be a major abiotic factor affecting global crop yields [3]. One third of the world's population resides in water stressed regions, and with elevated CO_2 levels in the atmosphere and climatic changes predicted in the future, drought could become more frequent and severe.

In vitro selection for tolerance to abiotic stress depends on the development of efficient and reliable callus induction and plant regeneration systems. Hence this experiment was carried out to screen the indica varieties BRRI 28 and BRRI 29 for their inherent tolerance against drought.

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The importance of rice as staple food emphasizes its improvement undoubtedly. Rice is the synonym for food in Bangladesh and has been traditional source of carbohydrates and proteins since the prehistoric days. In Bangladesh, rice covers an area of 11,059 thousand hectares, yielded 3,448.2 kg ha^{-1} and the production is 38,134 thousand metric tons [4]. This sector generates about 35% of the total foreign exchange earnings and provides nearly 40% of total national employment (48% of rural employment), about 2/3 of total calorie supply, about 1/2 of the total protein intake of an average person and contributes 18% to the total GDP of the country [5].

Rice (*Oryza sativa L.*) is subjected to a range of abiotic stresses that affect their growth and development. In particular, it is predicted that water deficit will continue to be a major abiotic factor affecting global crop yields. One third of the world's population resides in water stressed regions, and with elevated CO_2 levels in the atmosphere and climatic changes predicted in the future, drought could become more frequent and severe. In tolerant plants, there are many defense mechanisms such as osmoregulation, ion homeostasis, antioxidant and hormonal systems, helping plants to stay alive and development prior to their reproductive stages [6].

Biochemical [proline, glycine betaine, soluble sugars, photosynthetic pigments and defensive proteins] and physiological [water use efficiency, osmotic adjustment, chlorophyll a fluorescence, and net-photosynthetic rate (Pn)] changes in plants growing under salt or water-deficit conditions have been broadly investigated in many crop species such as rice[7-9]. Those parameters in crop species cultivated in water deficit or salt stresses have been developed as effective indices for tolerant selection in breeding programs [10]. Culture media or nutrient solutions in order to induce water deficit conditions for protein expression or proteomic studies. Mannitol, a member of sugar alcohols, is an osmotic adjustment chemical to control osmotic potential in the culture media or nutrient solutions in order to induce water deficit conditions for protein expression or proteomics studies [11].

Plant tissue culture plays an important role in the production of agricultural and ornamental plants and in the manipulation of plants for improved agronomic performance. In vitro culture of plant cells and tissue has attracted considerable interest over recent years because it provides the means to study plant physiological and genetic processes in addition to offering the potential to assist in the breeding of improved cultivars by increasing genetic variability. Regenerated plants are expected to have the same genotype as the donor plant; however, in some cases somaclonal variants have been found among regenerated plants [12-13].

Media composition – mainly the hormonal balance – is an important factor influencing in vitro culture initiation and plant regeneration from embryos. The auxin 2, 4-dichlorophenoxy acetic acid (2, 4-D) alone or in combination with cytokinins, is widely used to enhance callus induction and maintenance. Genetic factors are considered to be a major contributor to the in vitro response of cultured tissues. Differences in the production of embryogenic calli and the regenerated plantlets have been observed, depending on the genotype and source of the explants [14-23]. Salinity is the main abiotic stress that has been addressed by in vitro selection, but applications to other stresses such as heat and drought have been reported [24]. These techniques are considered to be an important complement to classical plant breeding methods. In vitro selection for tolerance to abiotic stress depends on the development of efficient and reliable callus induction and plant regeneration systems. Hence this experiment was carried out to screen the indica varieties BRRI 28 and BRRI 29 for their inherent tolerance against drought stress.

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2. Materials and methods

The study was conducted in the Plant Genetic Engineering Laboratory, Department of Genetic Engineering and Biotechnology, Shahjalal University of Science and Technology (SUST), Sylhet, Bangladesh. The detail of materials used and analytical methods employed during this study is given below:

Mature seeds of two indica rice (*Oryza sativa*) varieties BRRI 28 and BRRI 29 were used for callus induction, screening and plant regeneration experiments. For callus induction, the mature seeds are surface-sterilized and placed on the appropriate medium for that cultivar. At the end of 21 days – referred to as a "passage" – the original explants and its entire associated callus are transferred to a fresh medium. After the second passage, embryogenic callus is isolated and transferred again to a fresh medium. Selected healthy seeds were manually dehusked. Seeds were placed in autoclaved beaker and washed with sterile distilled water. The seeds were then kept in 70% ethanol for 3-5 minute, followed by washing with autoclaved distilled water for several times. The seeds were then placed on the sterilized petridish having sterile filter papers with the help of forceps to remove excess water. Surface sterilized seeds of two varieties were inoculated on solid MS Murashige and Skoog Medium (1962) media containing growth regulator 2,4-D in a laminar airflow cabinet. In each test tube one seed was inoculated. Before inoculation and also in between the work, autoclaved scalpels and forceps were again sterilized by burning in fire. The cultures were incubated in culture room at 25 ± 3ºC, to white florescent light under 16 hour's photoperiods. The basal medium MS was used for callus induction. The proposed medium was supplemented with various concentration of growth hormone 2, 4- D (0.5-2.5 mg/l). The pH of the media was adjusted to 5.8 before autoclaving. After inoculation, the surface sterilized seeds of two varieties were transferred and maintained in an environmentally controlled growth room for 2-3 weeks for callus induction and growth. The cultures were positioned away from continuous light provided by general electric white florescent tubes. Temperature was maintained at (25 ± 3)ºC thought the growth period. Callus quality replicated three times and twelve test tubes with twelve seeds were used per replication for each genotype. All the calli originated from a single seed was considered as one. Frequency of callus induction was calculated according to the following formula: Callus induction frequency (%) $= \frac{\text{No.of seeds produced calli}}{\text{No.of seeds cultured}} \times 100$

Before transferring the callus on mannitol containing media, the weight of callus were adjusted to 100 mg. The proposed MS medium containing different concentration of mannitol was supplemented with growth hormone 2, 4- D (2 mg/l). The pH of the media was adjusted to 5.8 ± 2 before autoclaving. After inoculation, the callus of two varieties were transferred and maintained in an environmentally controlled growth room for 4 weeks to create variation on callus weight. The cultures were positioned away from continuous light provided by general electric white florescent tubes. For plant regeneration, calli were then inoculated on regeneration media. The pH of media was adjusted to 5.8 ± 2 before autoclaving. The culture was performed at (25 ± 3)ºC under a cycle of 16 hours light/8 hours dark for 4 weeks. Rooting was initiated on half strength MS medium. After which the frequencies of plant regeneration were calculated, based on the appearance of shoots. Plant regeneration from plated calli was calculated with the following formula: Plant regeneration (%) = $\frac{\text{No.of calli produced plants}}{\text{No.of calli plated}} \times 100$

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3. Results

3.1 Callus Induction

Mature dehusked rice seeds were used for callus initiation and after 8 - 10 days, callus began to form at the scutellum of the cultured seed in all the media. Both embryogenic and non-embryogenic calli were initiated. Embryogenic calli were found to be yellow to white, dry, compact and nodular.

On the other hand, non-embryogenic calli appeared to be watery, light yellow and nonnodular. After 3 weeks, the first subculture was carried out and calli were removed from seed and transferred onto fresh media.

In case of BRRI 28, callus induction frequency was found to be relatively the lowest (18.50%) at 0.5 mg/l of 2,4-D and a peak frequency (85.25%) was observed at a higher concentration of 2 mg/l. On the other hand, callus induction frequency of BRRI 29 was found to be relatively the lowest (16.75%) at 0.5 mg/l of 2,4-D and the highest frequency (70.50%) was observed at a higher concentration of 2 mg/l.

But at 2.5 mg/L of 2,4-D again a downward induction rate was obtained (Table 1) that the presence of 2,4-D in the culture medium was critical for callus induction of rice from mature seeds.

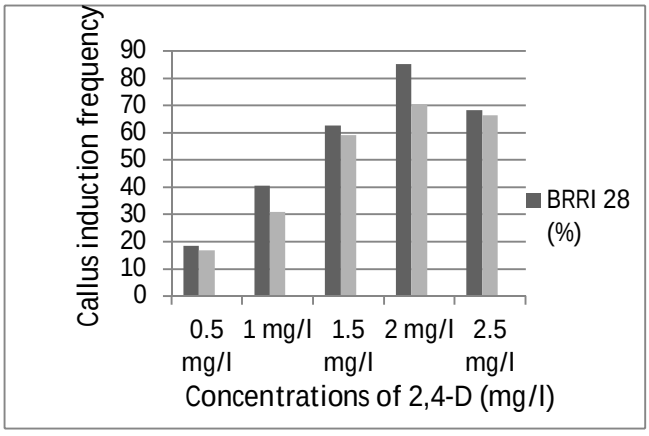

Figure 1: Effects of different concentration of 2,4-D on callus induction of BRRI 28 and BRRI 29

The rate of callus induction frequency in this case varied from 16.75% to 85.25%.Maximum calli (85.25%) were produced from the combinations of BRRI 28 with 2 mg/l of 2,4 –D. Minimum calli (16.75%) were produced from the combination of BRRI 29 with 0.5 mg/l of 2,4-D.

3.2 Variation of callus weight after four weeks of mannitol treatment

The overall purpose of the experiment was to screen the rice cultivars BRRI 28 and BRRI 29 for drought tolerance under in vitro conditions. Callus induction from mature seeds was assessed; further, the response of calli to elevated levels of mannitol was recorded as fresh weight. The response of the regenerating callus to mannitol stress was also observed. The cultivar BRRI 28 was less responsive than BRRI 29, which appears to be best suited for *in vitro* tissue culture.

Table 1: Callus weight of BRRI 28 after treating with different concentrations of mannitol

Variety	Mannitol concentration (gm/l)	Callus weight(mg) before treating with mannitol	Callus weight(mg) after four weeks of mannitol treatment
BRRI 28	0 gm/l	100 mg	197.50 mg
BRRI 28	10 gm/l	100 mg	185.75 mg
BRRI 28	20 gm/l	100 mg	122.25 mg
BRRI 28	30 gm/l	100 mg	90.50 mg
BRRI 28	40 gm/l	100 mg	77.25 mg
BRRI 29	0 gm/l	100 mg	196.00 mg
BRRI 29	10 gm/l	100 mg	188.50mg
BRRI 29	20 gm/l	100 mg	121.50 mg
BRRI 29	30 gm/l	100 mg	92.25 mg
BRRI 29	40 gm/l	100 mg	80.50 mg

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3.3 Callus proliferation response to mannitol stress

The major aim of this experiment was to check the inherent capacity of two rice cultivars BRRI 28 and BRRI 29 to mannitol stress. Both of this cultivars showed a similar response under mannitol stress. In vitro screening of control calli of rice cultivars BRRI 28 and BRRI29 for drought tolerance was carried out by growing embryogenic calli on callus induction medium supplemented with 0, 10, 20, 30, 40 mg/l for two weeks. Approximately 100 mg of one month old embryogenic callus was exposed to each mannitol concentration. Calli on control medium ex hibited normal proliferation. As the mannitol concentration in the medium increased, there was a decrease in callus fresh weight (Figure 4). At 20 gm/l mannitol the fresh weight was 122.25 mg in rice cultivar BRRI 28 compared to 197.50 mg at 0 gm/l and 125.25 mg in BRRI 29 compared to 196 mg at 0 mg/l mannitol.

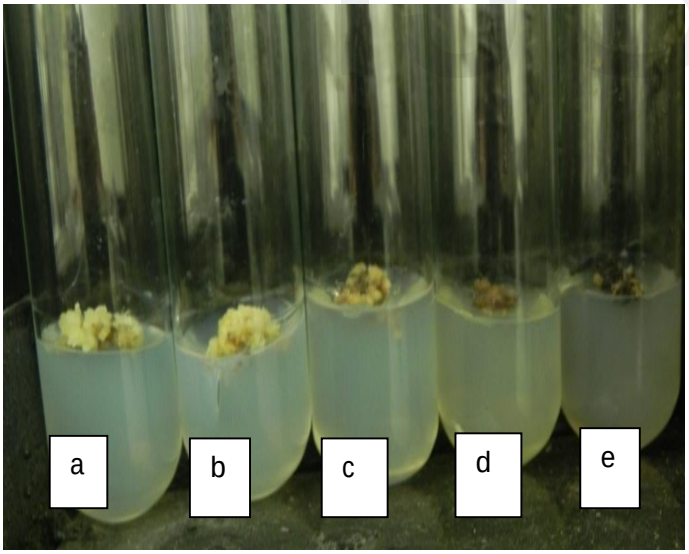

Figure 2: In vitro screening of BRRI 28 rice callus for drought stress using different concentrations of mannitol (a) Control, (b) 10 gm/l, (c) 20 gm/l, (d) 30 gm/l, (e) 40 gm/l

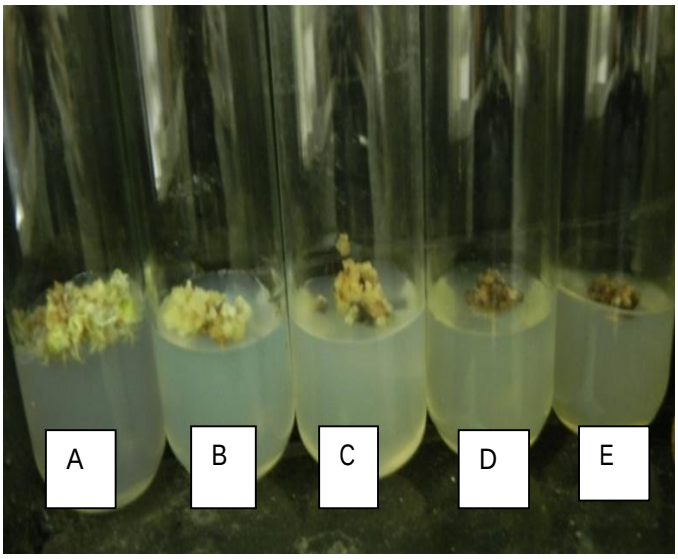

Figure 3: In vitro screening of BRRI 29 rice callus for drought stress using different concentrations of mannitol (A) Control, (B) 10 gm/l, (C) 20 gm/l, (D) 30 gm/l, (E) 40 gm/l

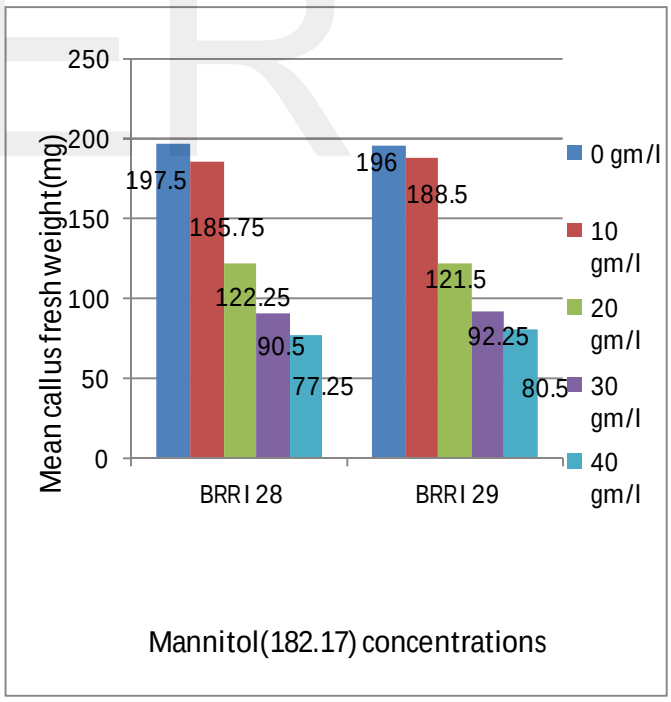

Figure 4: Comparision between BRRI 28 and BRRI 29 in response to different concentrations of mannitol

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There was a 75.25% decrease in callus fresh weight at 20 gm/l in rice cultivar BRRI 28 and a 74.50% decrease in rice cultivar BRRI 29 (Figure 4). At 0 gm/l, the callus weight was 197.5mg and 196mg whose were reduced to 185.75 mg and 188.5 mg at 10 gm/l of mannitol and finally decreased to 77.25 mg and 80.5 mg at 40 gm/l of mannitol in case of BRRI 28 and BRRI 29 respectively

3.4 Plant regeneration response to mannitol stress

To check the efficiency of embryogenic calli to regenerate in the presence of drought stress, calli we re exposed to elevated levels o f mannitol by putting mannitol in the plant regeneration medium. This experiment was performed to check the inherent capacity of calli to regenerate on medium which induced drought stress. Month old embryogenic calli were grown on plant regeneration medium supplemented with 0, 10, 20, 30, 40 gm/l for two cycles each of two weeks.

Table 2: Plant regeneration (%) of BRRI 28 at different concentrations of mannitol

Varieties	Concentration of mannitol	Plant regeneration (%)
BRRI 28	0 gm/l	42.50%
BRRI 28	10 gm/l	28.75%
BRRI 28	20 gm/l	15.25%
BRRI 28	30 gm/l	6.25%
BRRI 28	40 gm/l	0%

Table 3: Plant regeneration (%) of BRRI 29 at different concentrations of mannitol

Varieties	Concentration of mannitol	Plant regeneration (%)
BRRI 29	0 gm/l	51.75%
BRRI 29	10 gm/l	40.50%
BRRI 29	20 gm/l	21.25%
BRRI 29	30 gm/l	8.75%
BRRI 29	40 gm/l	0%

There was normal plant regeneration in the no-stress medium, but increased mannitol concentration in medium decreased percent plant regeneration in rice cultivars BRRI 28 and BRRI 29 (Figure 5). Plant regeneration of BRRI 28 was 42.5% at 0 gm/l of mannitol, but decreased to 15.25% at 20 gm/l of mannitol. There was 0% plant regeneration at 40 gm/l of mannitol.In BRRI 29 plant regeneration on the no-stress medium was 52.25% , but increased mannitol concentration in medium decreased of the per cent plant regeneration. At 20 gm/l it decreased to 20.5% and there was no regeneration at 40 gm/l of mannitol. In vitro tissue culture could be an important means of improving crop tolerance and yield through genetic transformation as well as by induced somaclonal variation. Therefore it is important to devise an efficient protocol of callus prolife ration to start in vitro selection for drought stress tolerance, and to extand opportunities for genetic manipulation of rice through tissue culture, including trying various explants and media.

The overall aim of the experiment was to screen the rice cultivars BRRI 28 and BRRI29 for drought tolerance under in vitro conditions. Callus induction from mature seeds and callus proliferation was mandatory for this experiment.

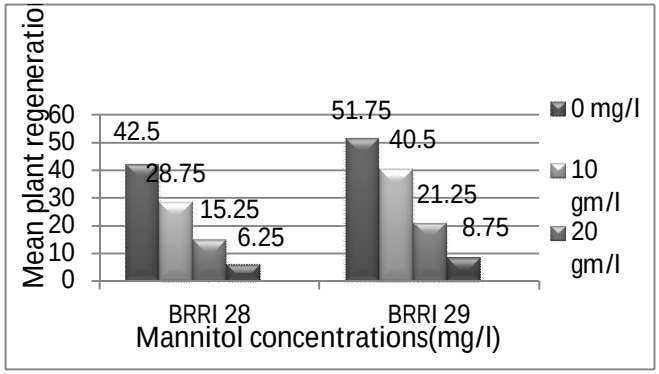

Figure 5: Decrease in mean plant regeneration per cent (%) with increase in mannitol concentrations.

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In this study, it was found that, as the mannitol concentration in the medium increased, there was a decrease in callus fresh weight. At 20 gm/l mannitol the fresh weight of callus was 122.25 mg in rice cultivar BRRI 28 compared to 197.50 mg at 0 gm/l and 125.25 mg in BRRI 29 compared to 196 mg at 0 mg/l mannitol. There was a 75.25% decrease in callus fresh weight at 20 gm/l in rice cultivar BRRI 28 and a 74.50% decrease in rice cultivar BRRI 29(Figure 3.15). At 0 gm/l , the callus weight was 197.5mg and 196mg whose were reduced to 185.75 mg and 188.5 mg at 10 gm/l of mannitol and finally decreased to 77.25 mg and 80.5 mg at 40 gm/l of mannitol in case of BRRI 28 and BRRI 29 respectively. Relevant result was made by Shabir H. Wani(2010) who used PEG instead of mannitol.

There was normal plant regeneration in the no-stress medium, but increased mannitol concentration in medium decreased percent plant regeneration in rice cultivars BRRI 28 and BRRI 29. Plant regeneration of BRRI 28 was 42.5% at 0 gm/l of mannitol, but decreased to 15.25% at 20 gm/l of mannitol. There was 0% plant regeneration at 40 gm/l of mannitol.

In BRRI 29 plant regeneration on the no-stress medium was 52.25% , but increased mannitol concentration in medium decreased of the per cent plant regeneration. At 20 gm/l it decreased to 20.5% and there was no regeneration at 40 gm/l of mannitol. On the other study, Shabir H. Wani(2010) found that, plant regeneration was 44.2% at 0% PEG (6000), but decreased to 17.6% at 1.0% PEG (6000).There was 0% plant regeneration at 2.0% PEG (6000) in PAU 201. In PR 116% plant regeneration on the no-stress medium was 54.7% , but increased PEG (6000) concentration in medium decreased of the per c e nt plant regeneration. At 1.0%t PEG (6000) it decreased to 23.7% and there was no regenerati on at 2.0% PEG (6000).

4. Conclusion

The results of this study indicate that the two rice cultivars BRRI 28 and BRRI 29 have good callus induction ability. This experiment provides an indication of the inherent tolerance of rice genotypes to drought. I think the above study will be the base line for future screening expeiments.

5. References

[1] Chaves, M.M. and M.M. Oliveira. 2004. Mechanisms underlying plant resilience to water deficits: Prospects for water-saving agriculture. J. Exp. Bot., 55: 2365–2384.

[2] Zidenga, T. (2006). Progress in Molecular Approaches to Drought Tolerance in Crop Plants, ISB News Report, http://www.isb.vt.edu/news/2006/news06.mar.htm.

[3] Sharma KK, Lavanya M. JIRCAS Working Report. 2002. Recent developments in transgenics for abiotic stress in legumes of the semi arid tropics.; p. 61-73.

[4] SAIC. 2003. Statistical Bulletin of SAARC Agricultural Data-2003. SAARC Agricultural Information Centre. Dhaka.

[5] Alam J.1997. Recent trends in agricultural development of Bangladesh: policy implications. Paper Presented in a Seminar Jointly Orgainzed by BAEA and BARC on October 7, 1997 at BARC, Farmgate, Dhaka, Bangladesh.

[6] Hasegawa, P.M., R.A. Bressan, J.K. Zhu and H.J. Bohnert. 2000. Plant cellular and molecular responses to high salinity. Ann. Rev. Plant Physiol. Mol. Biol., 51: 463-499.

International Journal of Scientific & Engineering Research, Volume 4, Issue 6, June-2013
ISSN 2229-5518

[7] Ashraf, M. 2010. Inducing drought tolerance in plants: some recent advances. Biotechnol. Adv. 28: 169-183.

[8] Castillo, E.G., T.P. Tuong, A.M. Ismail and K. Inubushi. 2007. Response to salinity in rice: Comparative effects of osmotic and ionic stresses. Plant Prod. Sci., 10: 159-170.

[9] Ashraf, M. and P.J.C. Harris. 2004. Potential biochemical indicators of salinity tolerance in plants. Plant Sci., 166: 3-16

[10] Zang, X. and S. Komatsu. 2007. A proteomics approach for identifying osmotic-stress-related proteins in rice. Phytochemical. 68: 426-437.

[11] Karp, A., H. Steel, S. Parmar, K. Jones, R. Shewry and A. Breiman, 1987. Relative stability among barley plants regenerated from cultured immature embryos. Genome, 29: 405-412.

[12] Karp, A., 1995. Somaclonal variation as a tool for crop improvement. Euphytica, 85: 295-302

[13] Ganeshan, S., Baga, M. , Harwey, B.L., Rossnag el, B.G., Scoles, G.J., Ch ibbar, R.N. (2003).

[14] Seraj, Z.I., Islam, Z., Faruque, M.O., Devi, T., Ahmed, S. (1997). Identification of the regeneration potential of embryo derived calli from various Indica rice varieties. Plant Cell Tissue and Organ Culture 48, 9 –13

[15] Mahajan, S. and N. Tuteja. 2005. Cold, salinity and drought stresses: An overview. Arch. Biochem.Biophys., 444: 139-158. vars differing in salinity resistance. Plant Growth Regulation 19, 2 218.

[16] Khalequzzaman, M., Haq, N., Hoque, ME., Aditya, TL. (20 05). Regeneration efficiency and genotypic effect of 15 Indica type Bangladeshi rice (Oryza sativa L.) landraces. Plant Tissue Culture 15, 33–42

[17] Al-Bahrany, A.M. (2002). Callus growth and proline accumulation in response to Polyethylene glycol induced osmotic stress in rice Oryza sativa L. Pakistan Journal of Biological Sciences 15, 12 94 – 1296.

[18] O'Toole, J.C. (1982). Adaptation of rice to drought prone environments. In: Drought resist anin crops, with emphasis on rice , IRRI, Los Baños.

[19] Rashid, H., Yokoi, S., Toriyama, K., Hinata, K. (1996). Tran sg enic plant production mediated by Agrobacterium in Indica rice. Plan t Cell Reports 15, 727–730.

[20] Wang, W., B. Vinocur and A. Altman. 2003. Plant responses to drought, salinity and extreme temperatures: towards genetic engineering for stress tolerance. Planta, 218: 1-14.

[21] Wang, W., B. Vinocur, O. Shoseyov and A. Altman. 2001. Biotechnology of plant osmotic stress tolerance: physiological and molecular considerations. Acta Hort ., 560: 285-292.

[22] Wani, S.H., Gosal, S.S. (2011). Introduction of Os glyII gene into Indica rice through particle bombardment for increased salinity tolerance. Biologia Plantarum (in press).

[23] Lutts, S., M. Almansouri and J.M. Kinet. 2004. Salinity and water stress have contrasting effects on the relationship between growth and cell viability during and after stress exposure in durum wheat callus. Plant Sci., 167: 9-18.

International Journal of Scientific & Engineering Research, Volume 4, Issue 6, June-2013
ISSN 2229-5518

[24] Zalc, J.M., Wicr, H.B., Kidwell, K.K., Steber, CM. (2004). Callus induction and plant regeneration from mature embryos of a diverse set of wheat genotypes. Plant Cell Tissue and OrganCulture76,277–281.