International Research Journal of Biological Sciences _____ ISSN 2278-3202
Vol. **2(11),** 29-36, November **(2013)** *Int. Res. J. Biological Sci.*

In Vitro Regeneration and Screening for Salt Tolerance in Rice (*Oryza sativa* L.)

K.M.A. Zinnah, Nayem Zobayer, Saif U. Sikdar, Lutfun Nahar Liza, Md. Al Nayem Chowdhury and M. Ashrafuzzaman*
Dept. of Genetic Engineering and Biotechnology, School of life sciences, Shahjalal University of Sci. and Tech., Sylhet-3114, BANGLADESH

Available online at: www.isca.in, www.isca.me
Received 23rd May 2013, revised 3rd August 2013, accepted 22nd October 2013

Abstract

The study was conducted to obtain salt tolerant genotype of BRRI Dhan 38 and Chini Kanai (local variety) rice varieties through somaclonal variation. Different concentration and combinations of growth regulators were supplemented to MS medium to observe the callus induction and plantlet regeneration ability of mature rice seeds. On the other hand, the calli were transferred to the best regeneration medium at different concentrations of NaCl to check the inherent capacity of calli to regenerate on medium under salt stress condition. Maximum percentage of callus induction was observed in MS medium supplemented with 5 mg/l 2,4-D for BRRI Dhan 38 and 3 mg/l for Chini Kanai. Calli derived from the different concentrations of 2, 4-D were cultured on MS medium supplemented with 1 mg/l NAA, 2 mg/L BA and various concentration of Kinetin for plantlet regeneration. It was observed that MS media supplemented with 2 mg/l of kinetin in combination with 1 mg/l NAA and 2 mg/l BA produced highest percentage of callus for BRRI Dhan 38 (80%) and Chini Kanai (60%) respectively. Plant regeneration of BRRI dhan 38 was 80% at 0 mM NaCl, but decreased to 20% at 100 mM NaCl. There was 0% plant regeneration at 150 mM NaCl for BRRI 38 and Chini Kanai respectively. In Chini Kanai plant regeneration on the no-stress medium was 60%. At 150 mM it decreased to 20% and there was no regeneration at 200 mM NaCl. It indicates that Chini Kanai is more salt tolerant then BRRI Dhan 38.

Keywords: *Oryza sativa,* plant growth regulators, salt tolerency.

Introduction

Rice (*Oryza sativa* L.) is the most important food crop and around half the world's population eats rice every day and about 70% of the world's poor depend on rice as their major source of food energy[1-2]. The demand for rice is continuously growing with the increasing population, thus genetic improvements of important rice varieties have been targeted. Agricultural genetics is one of the easier parts of the solution. Bangladesh is a densely populated country of the world and here the probability of increasing cultivable land in nearly zero. The only alternative, therefore, is to increase productivity using suitable biotechnological approaches. The application of biotechnology in combination with conventional breeding methods may help to increase food production properly. Efficient plant regeneration through *in vitro* micropropagation is very essential for the successful utilization of biotechnology in rice crop improvement[3]. The identification and screening of useful cultivars for embryogenic callus formation and subsequent plant regeneration through *in vitro* system is a vital step in rice genetic improvement programme[4-5]. In rice, *in vitro* plant regeneration from scutellum has been reported by Wijesekera[6]. The use of mature seed embryos has distinct advantage over other explants as staring material for *in vitro* regeneration.

Mature embryo from dry seed has been commonly used as primary explants for callus induction in regeneration process[7]. The use of mature embryos in monocotyledons is easy for the manipulation in tissue culture but the low regeneration efficiency has been reported by Sharma[8]. The callus induction and plant regeneration frequencies of explants are influenced by various factors such as the culture methods, the media and the culture conditions[9-12]. The efficient protocols of rice regeneration should be specifically developed for the particular explants and varieties.

Saline soils are one of the major biotic stresses that adversely affect the overall metabolic activities and cause plant demise[13]. Production capabilities of certain crops are reported to be reduced in saline conditions[14]. It has been estimated that over 2 million acres of agricultural land is lost from production each year due to the occurrence of high Na^+ and Cl^- levels in soils, so called salinization. In Bangladesh, the coastal area covers about 20% of the country and over thirty percent of the net cultivable area. It extends inside up to 150 km from the coast. Out of 2.85 million hectares of the coastal and offshore areas about 0.83 millions hectares are arable lands, which cover over 30% of the total cultivable lands of Bangladesh. Agricultural land use in these areas is very poor, which is roughly 50% of the country's average[15].

In recent years tissue culture techniques are being used as a useful tool to elucidate the mechanism involved in salt tolerance

by using *in vitro* selected salt tolerant cell lines[16-17]. Besides, these lines have been used to regenerate salt tolerant plants[18-20].

Material and Methods

This experiment was conducted in the plant genetic engineering laboratory of the Department of Genetic Engineering and Biotechnology, Shahjalal University of Science and Technology (SUST), Sylhet, Bangladesh. During this field grown seeds of aromatic rice (*Oryza sativa* L.) variety were used for callus induction, plant regeneration and salt screening.

Explant source: Mature seeds were used as explants in this experiment. The rice variety *(Oryza sativa*) BRRI 38 and CHINI KANAI were collected from the Bangladesh Rice Research Institute (BRRI) and Paikgasa, Khulna respectively.

Explant Sterilization: The mature embryo was sterilized by following the standard explants sterilization protocol, previously described by different researchers[21-22]. The explants were thoroughly washed with distilled water for three times. Then, the explants were washed with 70% ethanol (for 2 to 3 min). In the laminar air flow cabinet, the explants were treated with 0.1% $HgCl_2$ with the addition of few drops of Tween-20 for inner surface sterilization (for 4 to 6 min). Finally, the explants were washed with sterile distilled water for several times to remove all the sterilizing agents.

Callus induction media: The basal medium MS was used for callus induction[23]. The proposed medium was supplemented with concentration of growth hormone 2,4-D (0, 1, 2. 3,4,5 and 6 mg/L). The pH of the media was adjusted to 5.8 ± 2 before autoclaving. After inoculation, the surface sterilized seeds of two rice varities were transferred and maintained in an environmentally controlled growth room for 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˚C thought the growth period. Callus induction frequency for two varieties was recorded 7–9 days after inoculation. Callus quality was recorded at 2-3 weeks after inoculation in two rice varities for all treatments. The callus contained both embryogenic (white to light yellow in color, compact and friable) as well as non-embryogenic (mucilagenious and smooth) parts. Subculture was carried out once in every two weeks with transfer of only the vigorously growing portions of calli. The embryo calli were induced from the scutellar tissues of mature seeds, excised and used in later experiments for regeneration after subculturing (to exploit the full potential of cell growth) for 3 weeks on the same media used for callus induction. Experiments were 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.

Regeneration Media: For plant regeneration, the embryogenic part of calli was cut into small pieces by removing non embryogenic part. Calli were then inoculated on regeneration media with different combination and concentrations of hormones (Tab.2.8.). The MS basal medium was supplemented with 3% sucrose and kinetin (1, 2, 3 and 4 mg/L) while maintaining 1 mg/L of NAA and 2 mg/l of BA as constant. The pH of media was adjusted to 5.8 ± 2 before autoclaving. Sixteen calli were inoculated on the regeneration media and the culture was performed at 25 ± 3ºC under a cycle of 16 hours light/8 hours dark for 4 weeks, after which the frequencies of plant regeneration were calculated., based on the appearance of shoots.

Media for screening salt tolerance: Four-week-old callus was divided into pieces of 100 mg. These pieces were transferred onto the same medium those were used for plant regeneration, supplemented with different NaCl concentrations, such as, 0, 50, 100, 150 and 200 mM, for salt stress responses. At the end of the four-week period, the callus was taken for growth analysis.

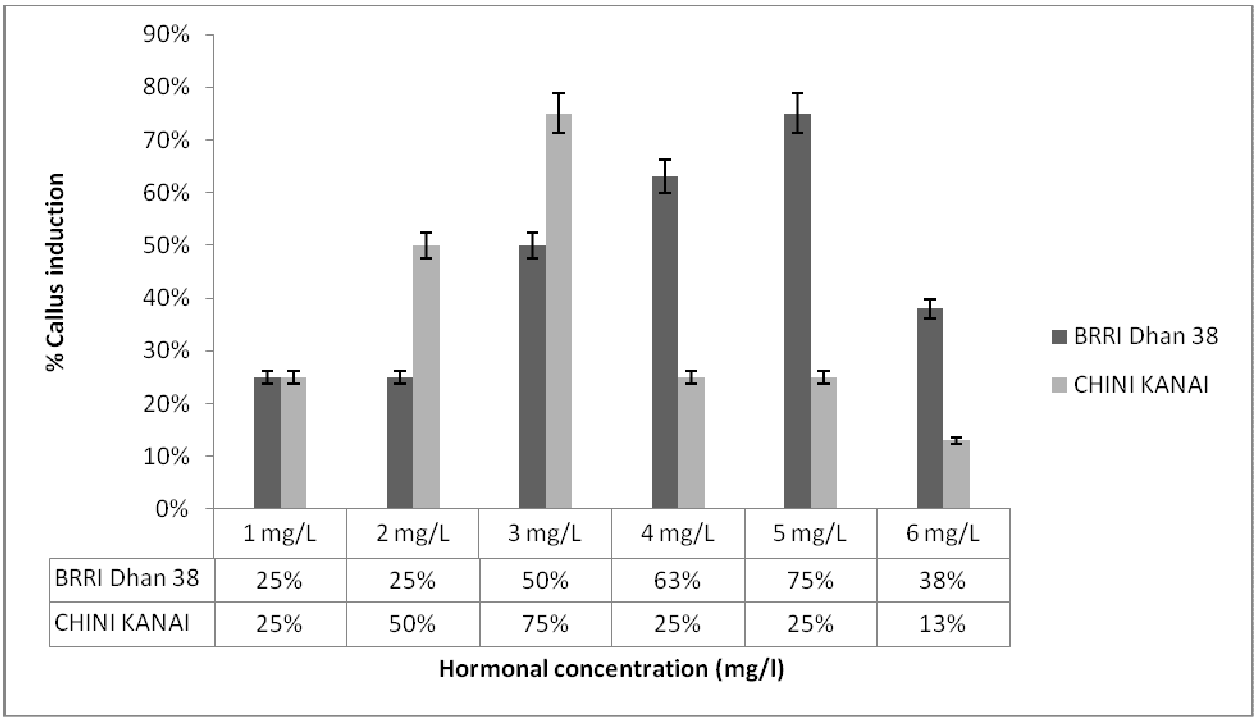
Results and Discussion

Callus Induction: Effect of 2, 4-D on callus induction: Mature dehusked rice embryos of two varieties viz. BRRI Dhan 38 and Chini Kanai (local variety) were used for callus initiation. MS medium supplemented with different concentration of 2,4-D hormone were employed for callus induction. Callus initiation was started at 5^{th} to 8^{th} days after transferring the caryopses to culture tubes and their incubation. The final data on callus induction was recorded after three weeks of inoculation. It was noticed that MS media supplemented with 3.0 mg/L and 5.0 mg/L 2,4-D produced highest percentage of callus, 75 % for Chini Kanai(Local variety) and BRRI Dhan 38, respectively (figure 1).

On the other hand, MS media supplemented with 1.0 mg/L 2,4-D produced lowest percentage of callus, 13% for Chini Kanai (Local variety) and 25% for BRRI Dhan 38 respectively. The colors of the callus were yellowish to white and the texture of them were friable (figure 2, figure 3) Subculture of subsequent callus regenerating media was showed in figure 4 and 5.

Plantlet regeneration: Effect of cytokinins and auxins on plantlet regeneration: When the dehusked rice embryos were cultured on callus inducing medium, soft friable callus was formed within 3 – 4 weeks of culture. The produced calli of convenient size were transferred on MS medium supplemented with different combination of growth regulators auxins and cytokinins. In this experiment calli derived from the different concentrations of 2, 4-D (1.0, 2.0, 3.0, 4.0 mg/L) were cultured on MS medium supplemented with 1 mg/L NAA, 2 mg/L BA and various concentration of Kinetin (0.0,1.0, 2.0, 3.0, 4.0 mg/L) for plantlet regeneration. Effect of Kinetin and BA on regeneration also been described by several authors in case of different types of plant[24-26].

It was observed that MS media supplemented with 0.0 mg/L of kinetin in combination with 1.0 mg/L NAA, 2mg/L BA produced lowest percentage of regeneration was observed, which was 20% for BRRI Dhan 38 and Chini Kanai respectively (figure 6). On the other hand, MS media supplemented with 2.0 mg/L of kinetin in combination with 1.0 mg/L NAA, 2mg/L BA produced highest percentage of regenerated plant, about 80% for BRRI Dhan 38 and 60% for Chini Kanai respectively (figure 6). Effect of Plant regeneration on Chini Kanai and BRRI Dhan 38 have been shown in figure 7 and 8.



	1 mg/L	2 mg/L	3 mg/L	4 mg/L	5 mg/L	6 mg/L
BRRI Dhan 38	25%	25%	50%	63%	75%	38%
CHINI KANAI	25%	50%	75%	25%	25%	13%

Figure-1
Effect of 2, 4-D on callus induction

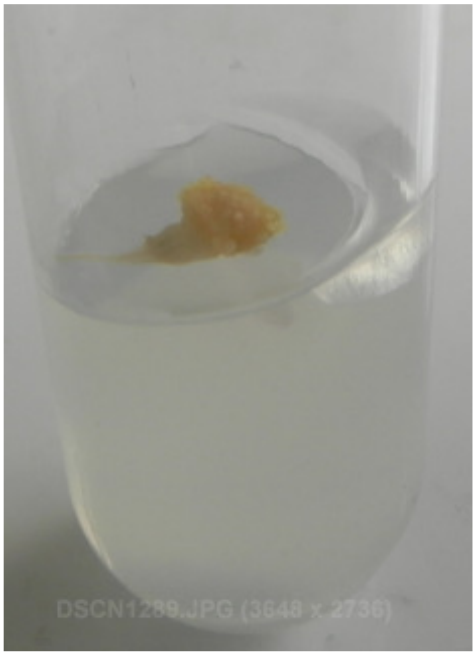


Figure-2
Callus initiation of BRRI Dhan 38 (MS+5.0 mg/L 2,4-D)

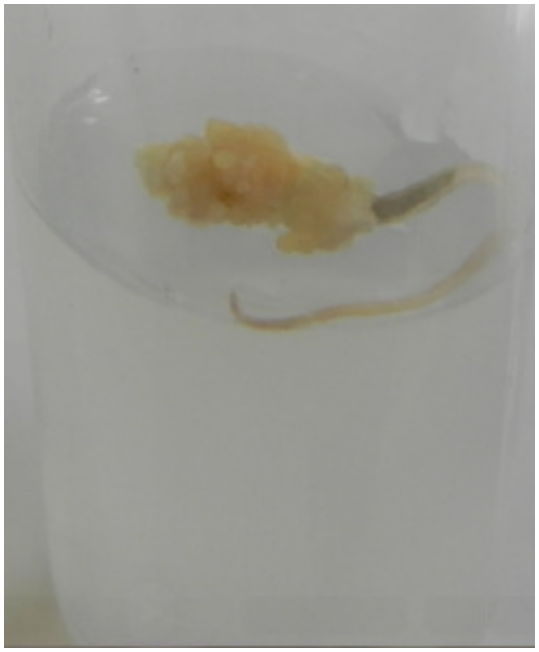


Figure-3
Callus initiation of Chini kanai (MS+3.0 mg/L 2,4-D)

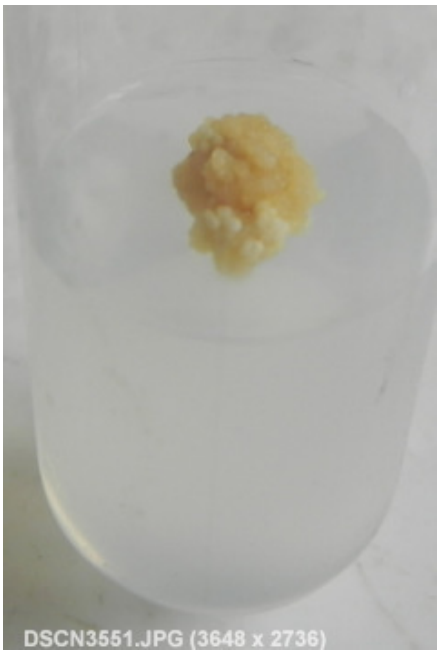
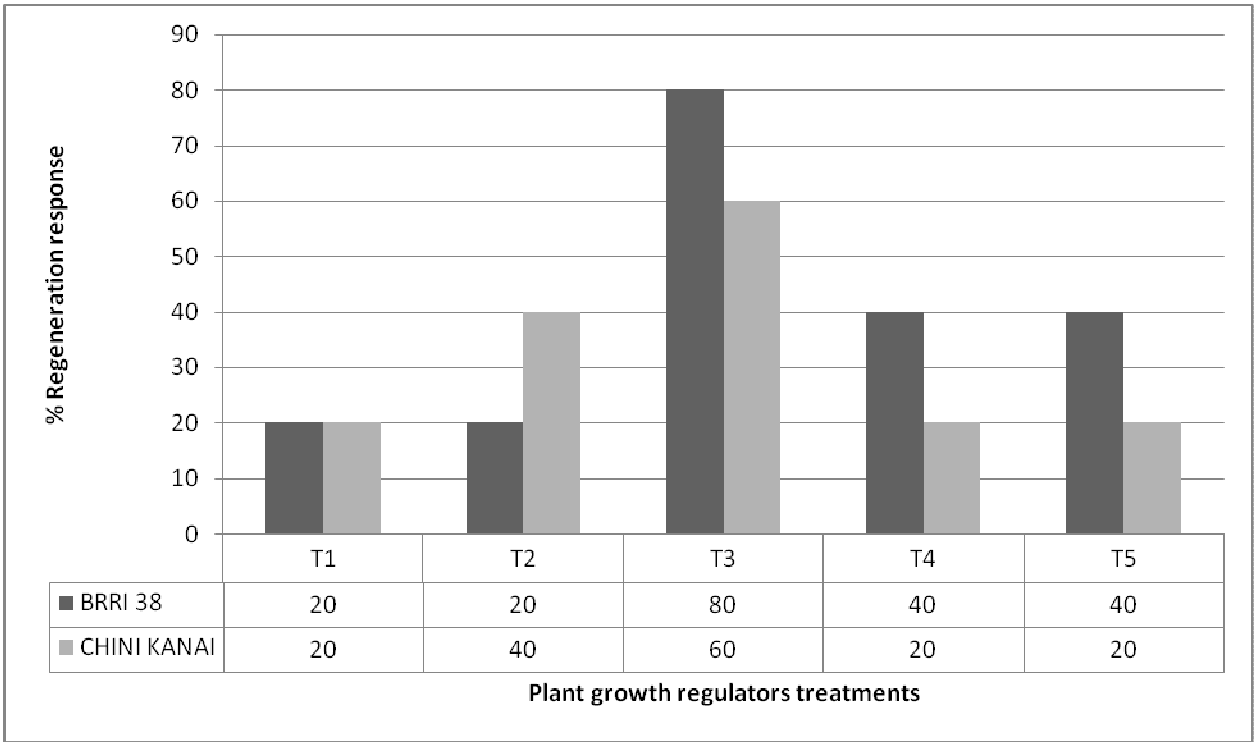


Figure-4
Subculture of BRRI 38 (MS+5.0 mg/L 2,4-D)

Figure-5
Subculture of Chinikanai(MS+3mg/L 2,4D)



Here, T1= MS+ 0.0 mg/L Kinetin + 1.0 mg/L NAA+ 2.0 mg/L BA ; T2= MS+ 1.0 mg/L Kinetin + 1.0 mg/L NAA+ 2.0 mg/L BA; T3= MS+ 2.0 mg/L Kinetin + 1.0 mg/L NAA+ 2.0 mg/L BA; T4= MS+ 3.0 mg/L Kinetin + 1.0 mg/L NAA+ 2.0 mg/L BA; T5= MS+ 4.0 mg/L Kinetin + 1.0 mg/L NAA+ 2.0 mg/L BA

Figure-6
Effect of various concentration of Kinetin in combination with NAA (1.0 mg/L) and BA (2.0 mg/L) in MS medium on plantlet regeneration

Effects of salinity on callus growth: For the callus growth analysis in salt stress, 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:

$$\text{Relative growth} = \frac{(\text{Final growth} - \text{Initial growth}) \times 100\ 11100}{\text{Initial growth}}$$

Approximately, 100.00 mg of one month old embryogenic callus was exposed to each (0.00, 50.00, 100.00, 150.00 and 200.00 mM) NaCl concentration. Calli on control medium (0.00 mM NaCl) were exhibited normal proliferation. With the increment in NaCl concentration there was a gradual decrease in callus fresh weight (figure 9).

Effect of salinity on plant regeneration: 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 and 200 mM). The plant regeneration capacity was measured on the basis of plant formation. This experiment was performed to check the inherent capacity of calli to regenerate on medium which induced salt stress. Month old embryogenic calli were grown on plant regeneration medium supplemented with 0, 50, 100, 150 and 200 mM for two cycles each of two weeks. There was normal plant regeneration in the no-stress medium, but increased NaCl concentration in medium decreased percent plant regeneration in rice variety BRRI 38 and Chini Kanai (figure 11 and 12). Plant regeneration of BRRI 38 was 80% at 0 mM of NaCl, but decreased to 20% at 50mM of NaCl and 0% plant regeneration at 150 mM of NaCl. In Chini Kanai,plant regeneration on the no-stress medium was 60%, but increased NaCl concentration in medium decreased of the percent plant regeneration**.** At 100 mM it decreased to 20% and there was no regeneration at 200 mM NaCl (figure 10).

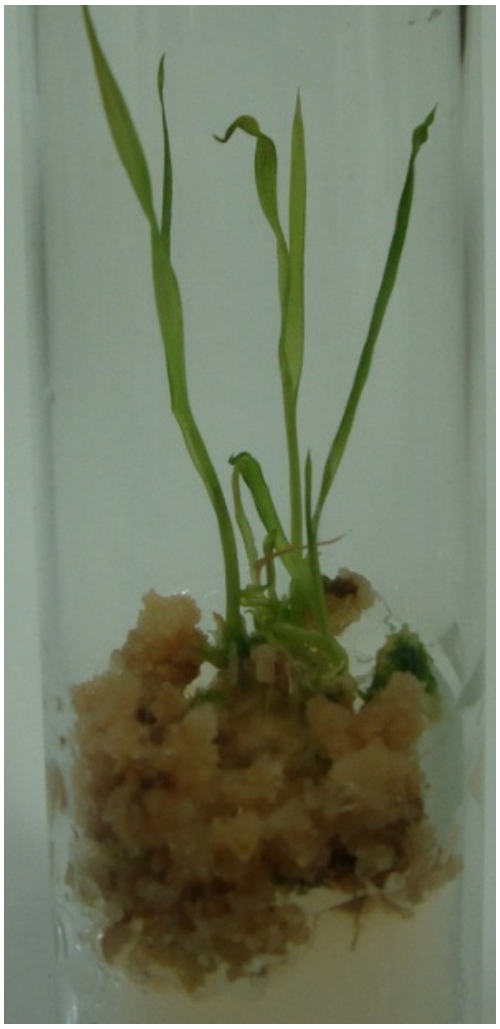


Figure-7
Plant regeneration of Chini Kanai

Figure-8
Plant regeneration of BRRI 38

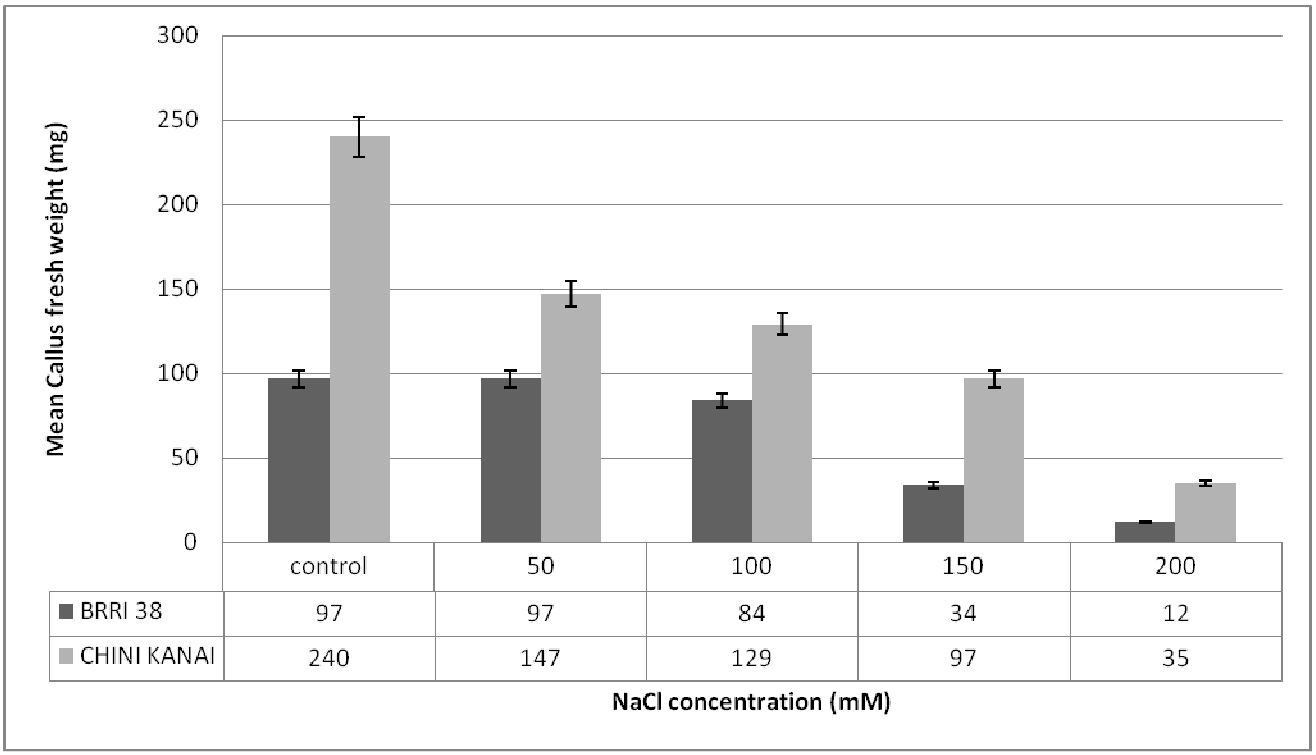


Figure-9
Comparison between callus fresh weight of BRRI 38 and Chini Kanai with increase in NaCl (mM) concentration

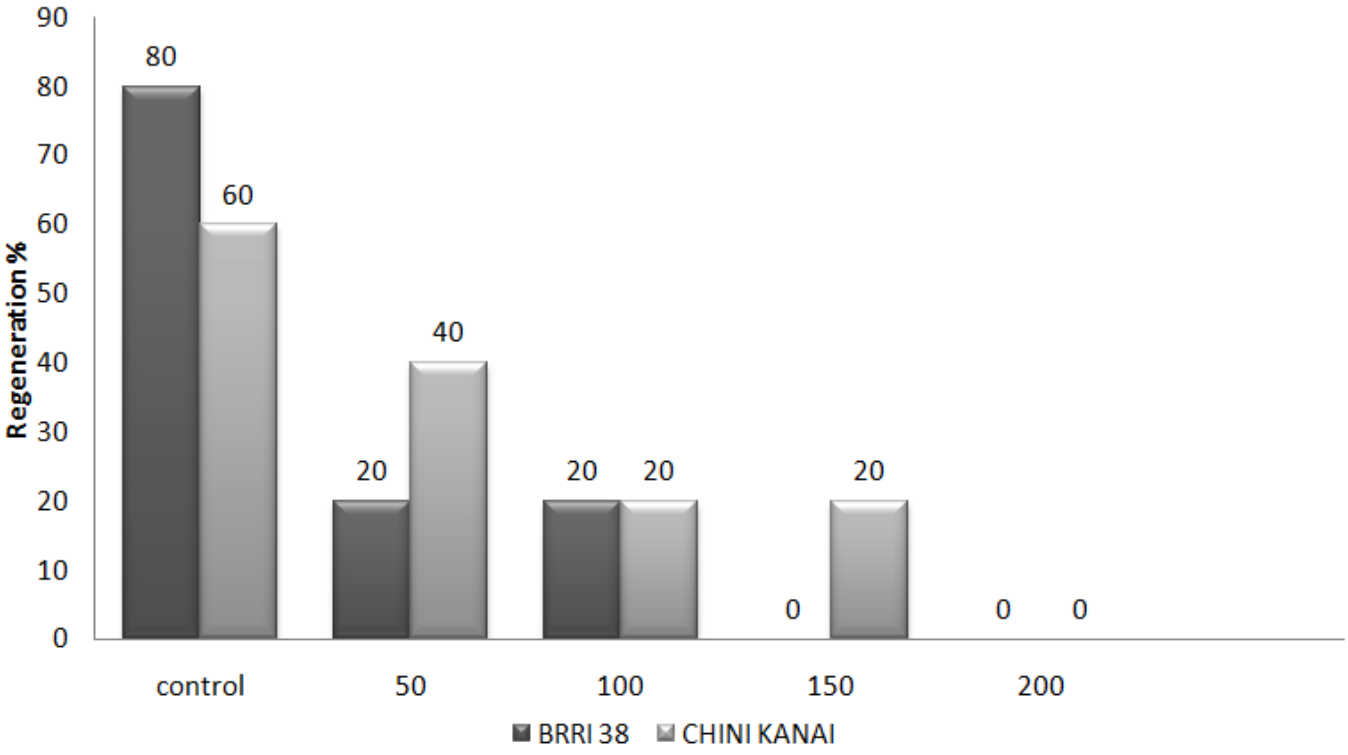


Figure-10
Regeneration (%) of two rice genotypes in NaCl treated media

Figure-11
Plant regeneration of BRRI 38 under salt condition (after 4 weeks)

Figure-12
Plant regeneration of Chini kanai undersalt stress (after 4 week)

Conclusion

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 salt stress tolerance, and to extand opportunities for genetic manipulation of rice through tissue culture, including trying various explants and media.

References

1. Khush G.S. and Virk P.S., Rice Breeding: Achievement and future strategies, *Crop Improvement,* **27,** 115-144 **(2000)**

2. Zeigler R.S., International Rice Research Institute, Los Baños, Philippines, In 5th *International Rice Blast Conference*, 5 (**2010)**

3. Hoque M.E., Ali M.S. and Karim N.H., Embryogenic callus induction and regeneration of Elite Bangladeshi Indica rice cultivars, *Plant Tissue Culture and Biotechnology*, **17(1)**, 65-70 **(2007)**

4. Hoque E.H and Mansfield J.W., Effect of genotype and explant age on callus induction and subsequent plant regeneration from root-derived callus of Indica rice genotypes, *Plant Cell Tissue & Organ Culture*, **78**, 217-223 **(2004)**

5. Islam M.M., Ahmed M. and Mahalder D., *In vitro* callus induction and plant regeneration in seed explants of rice (*Oryza sativa* L.). *Research Journal of Agriculture and Biological Sciences*, **1(1),** 72-75 **(2005)**

6. Wijesekera T.P., Iqbal M.C.M. and Bandara D.C., Plant regeneration *in vitro* by organogenesis on callus induced from mature embryos of three rice varieties (*Oryza sativa* L. spp. *indica*), *Tropical Agriculture Research*, **19**, 25-35 **(2007)**

7. Karthikeyan A., Pandian S.T.K. and Ramesh M., High frequency plant regeneration from embryogenic callus of a popular *indica* rice (*Oryza sativa* L.), *Physiology and Molecular Biology of Plants,* **15**, 371-375 **(2009)**

8. Sharma V.K., Hänsch R., Mendel R.R. and Schulze J., Mature embryo axis-based high frequency somatic embryogenesis and plant regeneration from multiple cultivars of barley (*Hordeum vulgare* L.). *Journal of Experimental Biotechnology,* **56**, 1913-1922 **(2005)**

9. Ilahi I., Bano S., Jabeen M. and Rahim F., Micropropagation of rice (*Oryza sativa* L. cv. Swat-II) through somatic embryogenesis, *Pakistani Journal of Biotechnology*, **37**, 237-242 **(2005)**

10. Kyungsoon L., Hysung J. and Kim M., Optimazation of a mature embryo-based in vitro culture system for highfrequency somatic embryogenic callus induction and

plant regeneration from japonica rice cultivars, *Plant Cell Tissue and organ Culture,* **71**, 237-244 **(2002)**

11. Saharan V., Yadav N.R. and Chapagain B.P., High frequency plant regeneration from desiccated calli of indica rice (*Oryza sativa* L.), *African Journal of Biotechnology,* **3**, 256-259 **(2004)**

12. Rafique M.Z., Rashid H., Chaudhary M.F., Chaudhry Z. and Cheema N.M., Study on callogenesis and organogenesis in local cultivars of rice (*Oryza sativa* L.), *Pakistani Journal of Biotechnology,* **43**, 191-203 **(2011)**

13. Roychoudury A., Basu S., Sarkar S.N. and Sengupta D.N., Comparative physiological and molecular responses of a common aromatic indica rice cultivar to high salinity with non aromatic indica rice cultivars, *Plant Cell Reports*, **27**, 1395-1410 **(2008)**

14. Rani C.R., Reema C., Alka S. and Singh P.K., Salt Tolerance of *Sorghum bicolor* Cultivars during Germination and Seedling Growth, *Research Journal of Recent Sciences,* **1(3)**, 1-10 **(2012)**

15. Petersen L. and Shireen S., Soil and water salinity in the coastal area of Bangladesh, SRDI **(2001)**

16. Venkataiah P., Christopher T. and Subhash K., Selection and characterization of sodium chloride and mannitol tolerant callus lines of red pepper (*Capsicum annuum L.) Plant Physiology*, **9(2)**, 158-163 **(2004)**

17. Gu R., Liu Q., Pie D. and Jiang X., Understanding saline and osmotic tolerance of *Populus euphratica* suspended cells, *Plant Cell Tissue & Organ Culture,* **78**, 261-265 **(2004)**

18. Chen R., Gyokusen K. and Saito A., Selection, regeneration and protein profile characteristics of NaCl-Tolerant callus of *Robinia pseudoacaia* L., *Journal of Forestry Research,* **6**, 43-48 **(2001)**

19. Jaiswal R. and Singh N.P., Plant Regeneration from NaCl Tolerant Callus/Cell Lines of Chickpea, *ICPN,* **8**, 21-23 **(2001)**

20. Miki Y., Hashiba M. and Hisajima S., Establishment of salt stress tolerant rice plants through step-up NaCl treatment *in vitro*. *Biol. Plant*., **44**, 391-395 **(2001)**

21. Sikdar, Saif U., Nayem Zobayer, Fazle Azim, M. Ashrafuzzaman, and Shamsul H. Prodhan, An efficient callus initiation and direct regeneration of Stevia rebaudiana, *African Journal of Biotechnology,* **11**, 10381-10387 **(2012)**

22. Zobayer N., Prodhan S.H., Sikdar S.U., Azim F. and Ashrafuzzaman M., Study of shoot multiplication of Strawberry (*Fragaria ananassa*), *International Journal of Agricultural Research, Innovation and Technology*, **1(1&2)**, 69-72 **(2011)**

23. Murashige T. and Skoog F., A revised medium for rapid growth and bioassay with tobacco tissue culture, *Physiolgia Plantarum*, **15**, 473-479 **(1962)**

24. Dhoran V.S. and Gudadhe S.P.. Effect of Plant Growth Regulators on Seed Germination and Seedling Vigour in *Asparagus sprengeri* Regelin, *International Research Journal of Biological Sciences,* **1(7)**, 6-10 **(2012)**

25. Fazle Azim, M.M. Rahman, Shamsul H. Prodhan, Saif U. Sikdar, Nayem Zobayer and M. Ashrafuzzaman. Development of Efficient Callus initiation of Malta (*Citrus sinensis)* Through Tissue Culture, *International Journal of Agricultural Research, Innovation and Technology*, **1 (1&2)**, 64-68, **(2011)**

26. Jitendra M., Monika S., Ratan S.D., Priyanka G., Priyanka S. and Kiran D.J., Micropropagation of an Anti diabetic Plant - *Stevia rebaudiana* Bertoni, (Natural Sweetener) in Hadoti Region of South-East Rajasthan, India, *ISCA Journal of Biological Sciences,* **1(3)**, 37-42, **(2012)**