Plant Archives Vol. 16 No. 1, 2016 pp. 321-326 *ISSN 0972-5210*

CALLI INDUCTION, REGENERATION AND RAISING OF MUTANT POPULATION IN INDICA RICE CULTIVARS FOR SCREENING UNDER SALT STRESS

Apekshita Singh*, Ashwani Pareek[1] and Suman Sharma[2]

Amity Institute of Biotechnology, Amity University, Sector-125, Noida, India.
[1]School of Life Sciences, Jawaharlal Nehru University, New Delhi - 110 067, India.
[2]Ramjas College, University of Delhi, Delhi - 110 007, India.

Abstract

Rice (*Oryza sativa* L.) is one of the most important food crops in the world. The successful regeneration protocols are important for introgression of new traits and development of better cultivars. Therefore, *in vitro* callus induction and regeneration was studied in three indica rice varieties, Swarna, Pokkali and IR 64, widely grown in Indian continent. The highest percentage of calli induction and regeneration was reported in Pokkali. The calli induction varied from 80% to 89% across three varieties. The MS (Murashige & Skoog) medium supplemented with various plant growth regulators such as 2,4-D (auxin), BAP (benzyl aminopurine) has been successfully used in the study. The two factors that stimulated the large percentage of callus induction are concentrations of 2,4-D and casein hydrolysate (CH). The best conditions for the maximum regeneration in the three varieties varied from using only MS with two hormones or additional supplements just as in calli induction media. The combinations for regeneration were MS + BAP + NAA; MS + CH + BAP + proline; MS + BAP + NAA + kinetin, respectively in Pokkali, Swarna and IR 64. The percentage of regeneration varied from 70 to 90%. The present study also analysed the effect of gamma irradiation and thereby raised a mutant population in cv IR 64. Rice mutant population showed variation for different characters, which can be utilized for forward genetics approach. Calli and field mutants were also screened for salt stress.

Key words : Rice cultivars, *in vitro* calli induction, regeneration, mutation, salt stress.

Introduction

Rice (*Oryza sativa* L.) one of the most important staple food crops in the world, is consumed by more than one third of the world population. It is an annual grass belonging to family Poaceae. There are two varieties of rice –*indica* and *japonica*. The recent years have witnessed research in rice biotechnology to produce transgenic rice plants with improved yield and quality, increased resistance to biotic and abiotic stresses and value added grains such as golden rice (Lee *et al*., 2002).

There is an ever increasing demand of rice in the growing population in India and China (IRRI, 2014). Rice covers half of the arable land used for agriculture in many Asian countries. It is estimated that rice production has to be increased by at least 50% by 2025. The most viable option is to increase the yield potential of cultivars as well as better agronomic characters. Recent advancement in biotechnology has enhanced the introgression of new and potentially beneficial genes into cultivated varieties. Routine transformation in rice is difficult and it is genotype specific. Therefore, successful regeneration in recalcitrant plants is important biotechnological advancement to raise new varieties with better tolerance to stresses.

The chosen material in the present study are three *indica* rice varieties IR64, Pokkali and Swarna. IR 64 is grown in the tropics. It is an elite variety carrying many valuable agronomic traits related to yield, plant architecture, grain quality, and good taste. It is a moderately salt sensitive variety. Pokkali rice, with a Geographical Indication registration, is known for its salt-resistant genes and is cultivated in the water-logged coastal regions of Kerala, India.

Rice has also established itself as a model crop (Shimamoto *et al*., 2002). The availability and screening of large mutagenesis population is prerequisite for genetic

Author for correspondence : E-mail: asingh20@amity.edu

variability. These lines can be selected for better tolerance to abiotic and biotic stresses.

A lot of efforts for mutation induction have been done in rice in order to raise large populations of improved cultivars (Sanjeev *et al*., 1998 and Siddiqui and Singh, 2010). There are many methods to induce genetic variability by mutagenesis like gamma irradiation, EMS (ethyl methane sulphonate), sodium azide etc. Induction of mutations with radiation has been the most frequently used method for directly developed mutant varieties (Ahloowalia *et al*., 2004).

The various experiments were undertaken to study the potential of different varieties for calli induction and subsequently regeneration. Salinity is a major constraint to irrigated rice production. It causes osmotic stress and ion toxicity that can alter plant metabolism eliciting many physiological changes (Zhu, 2001). Therefore, in present study a mutant rice population was also raised for screening and selection in response to salt stress.

Materials and Methods

The seeds of *Oryza sativa* cultivars IR 64, Swarna, Pokkali were obtained from IARI, Pusa, New Delhi and CSSRI, Karnal, Haryana, India.

Standardisation of *in vitro* callus induction

Oryza sativa L. ssp indica cvs IR 64, Swarna and Pokkali were selected for carrying out *in vitro* culture studies. Initially, attempts were made to standardize the protocols for raising callus from the scutellum of the seeds of both the varieties. Dehusked seeds were surface sterilized with 10% bleach for 20 min and then washed with distilled water five times and rinsed in 70% ethylalcohol. The seeds were plated on the callus induction medium.

Different media with various components and 2,4-D concentrations were tried in all the three varieties. The calli induction could be observed in the different media, but the percentage and the retention capacity was not good. Finally, MS + casein hydrolysate (300mg/l) + BAP (0.5mg/l) + 2,4-D (3mg/l) was selected as the best medium for callus induction in IR64. After 12-15 days the primary embryogenic calli were subcultured on fresh medium.

In Swarna variety, initially the calli growth was not very good in media supplemented with casein hydrolysate and 2,4-D. For rice cv Swarna, MS medium supplemented with casein hydrolysate (200mg/l), proline (300mg/l), 2,4-D (2.5 mg/l) was selected for callus induction. Similarly, rice cv. Pokkali was able to form calli in MS medium supplemented with 2,4-D (2mg/l) + CH(200mg/l). After seven days, primary embryogenic calli were subcultured on fresh medium.

Standardisation of regeneration in different cultivars

Initially, regeneration media for all the varieties were tried only with the combination of two hormones BAP and NAA in the basal MS media. Finally, a large number of calli showed green spots on MS + BAP (1.5 mg/l) + NAA (0.5 mg/l) + kinetin (1mg/l) + maltose (30g/l) in IR64. The same medium was tried for regeneration of irradiated calli in IR 64.

Similarly, Swarna callus was able to show higher regeneration potential on MS medium supplemented with casein hydrolysate (300mg/l), proline (500mg/l), 1.5mg/l BAP. Rice var. Pokkali showed regeneration in MS + NAA (1.5mg/l) + BAP (2mg/l) medium.

Standardisation of gamma irradiation

Initially, dosimetry test was carried out to determine the rate of gamma radiation emission from Co-60 chamber (School of Life Sciences, JNU, New Delhi). The rate of gamma irradiation was calculated to be 10 rads per sec.

***In vitro* gamma irradiation and their subsequent regeneration**

For gamma irradiation of rice variety IR64 calli, the doses of 10, 20, 50, 100 and 120 Gy were selected (approx 1200 calli per dose). The IR 64 calli at different doses of gamma rays were irradiated (10, 20, 50, 100 Gy) and regenerated in the same medium as that for non irradiated calli (table 1, fig. 2). The salt treatment in media was given at 50mM NaCl and 100mM NaCl.

Field plantation after irradiation of variety IR64

Husked rice seeds (500 seeds per dose) were irradiated at 50, 75, 100 and 120 Gy (100 rads = 1 Gy) and the seeds were planted in the field. The M1 (mutated) plants were harvested in the month of November to get M2 seeds. The M2 plants were further grown in IARI, Pusa. The initial screening of M3 seeds was done in laboratory conditions in hydroponic cultures for relative tolerance to salinity. Further screening is also being carried out. The parameters that are being studied include shoot length, root length, Na^+/K^+ ratio, fresh weight etc.

Screening of M3 population

The seeds were given salt stress at the rate of EC = 12ds/m in hydroponics for 5 days in Yoshida medium (Yoshida *et al*., 1976) and various readings for fresh weight, root length, shoot length and Na^+/K^+ ratios were measured and plotted for different M3 lines.

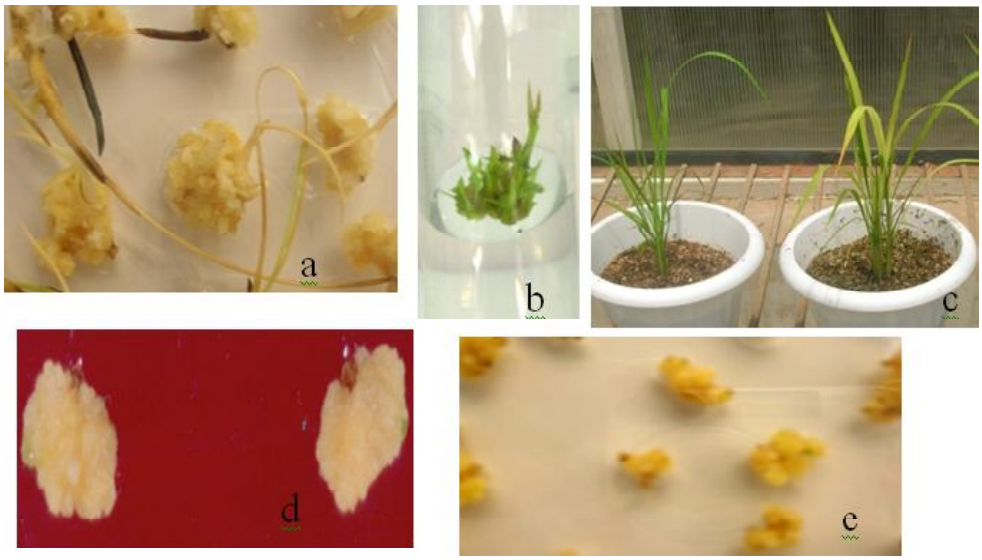

Fig. 1 : Calli in IR 64(a), Regeneration in IR 64(b), Hardeneing in IR 64(c), Calli in Swarna(d), Calli in Pokkali(e).

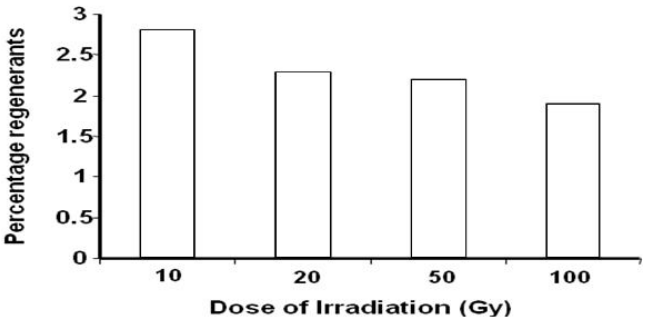

Fig. 2 : Graph showing percentage of irradiated regenerants in IR64 rice calli.

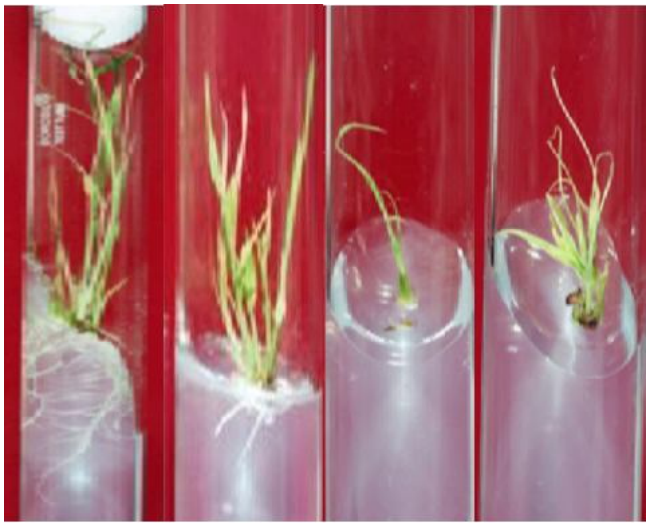

Fig. 3 : Regeneration of IR 64 at 10Gy, 20Gy, 50Gy and 100Gy of irradiation.

Results and Discussion

Callus induction and regeneration

Callus induction and regeneration in rice tissue culture depended on a number of factors, such as the genotype of the donor plants, the type and physiological status of the explant, the composition and concentration of the basal salts, organic components and plant growth regulator in the tissue culture medium (Khaleda and Al-Forkan, 2006). The different calli of IR 64, Pokkali and Swarna are shown in fig. 1. It was found that the simplest media formulations were enough for calli induction and regeneration in Pokkali. However, the elite cultivar IR 64 and Swarna cultivar needed a medium supplemented with casen hydrolysate and auxins and cytokinins. The percentage of calli induction was as high as 87% in Pokkali and as low as 80% in IR64 in best optimized media (table 1).

Recently, somatic embryogenesis from scutellar embryo of *Oryza sativa* L. variety MR 219 was published (Syaiful *et al*., 2009). They have reported that the highest percentage of embryogenic callus formation (80%) was obtained on the modified MS medium containing 4 mg/L of 2, 4-D. There are many reports of induction of calli in rice varieties and cultivars using different explants (Jain *et al*., 1996). But the seeds are the choice of explant in majority of the cases (Pravin *et al*., 2011; Wani *et al*., 2011; Puhan and Siddiqui, 2010; Verma *et al*., 2011). Pravin *et al*. (2011) reported high frequency of calli and *in vitro* regeneration for seeds in Swarna and Mahsuri varieties. They used 3% maltose, 0.5g/l each of proline, casein hydrolysate, kinetin with 0.3% gelrite. It was reported that Mahsuri regenerated better than Swarna in MS modified media. Alam *et al*. (2012) studied *in vitro* regeneration in rice BRRI dhan28, BRRI dhan 29, BRRI dhan 47, binadhan 7. The highest regeneration potential was of BRRI dhan 47 in MS + proline + 2,4-D and 0.8mg/l BAP. Upadhyaya *et al*. (2015) reported *in vitro* calli induction in Sita, Rupali, Swarna Masuri varieties with

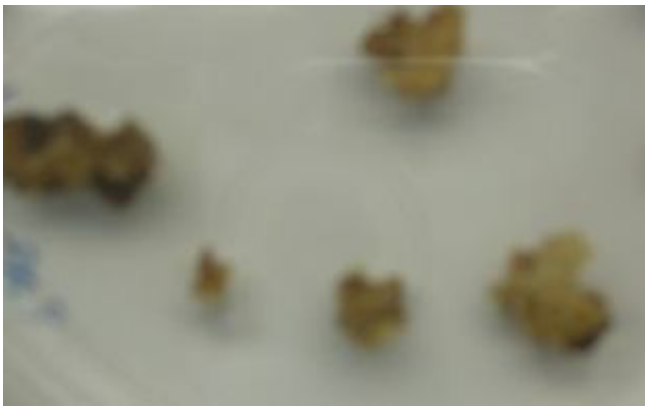

Fig. 4 : Calli (IR64) after irradiation and selection at 50mMNaCl.

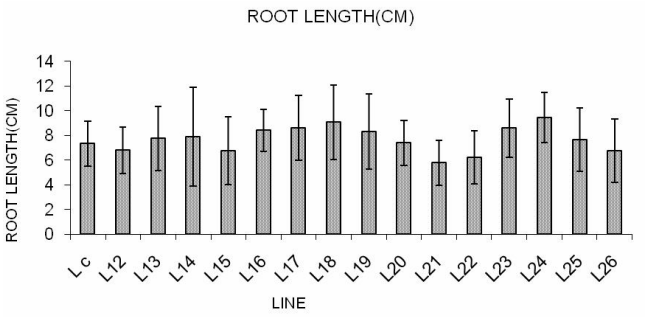

Fig. 5 : Graph showing comparative root length in M3 lines.

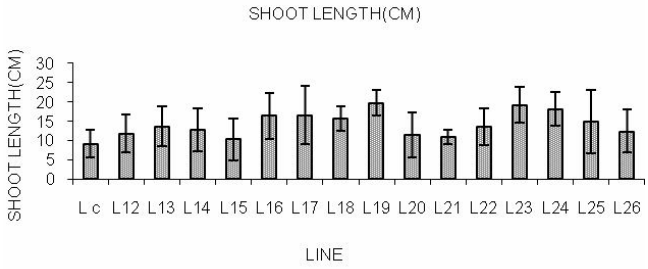

Fig. 6 : Graph showing comparative shoot length in M3 lines.

different concentrations of 2,4-D. Sita variety showed the maximum potential with 92.2%.

Similar to the above result, the present study showed that 2,4-D in the range of 2 to 3mg/l proved efficient for calli induction. It was observed that 2,4-D (2 mg/l) was adequate for *in vitro* regeneration in many studies such as Upadhyaya *et al*. (2015) and Alam *et al*. (2012). For regeneration, the best conditions vary in the present study. In the three genotypes, Pokkali had the highest calli induction potential and the regeneration was also high of 89%. However, the regeneration potential of Pokkali was also comparable to that of Swarna. It was observed that Swarna calli showed the best embryogenic potential with green spots appearing very early compared to the other two varieties. Pravin *et al*. (2012) also showed that the highest calli induction percentage in Swarna was 89% on modified MS medium. If the media are compared for

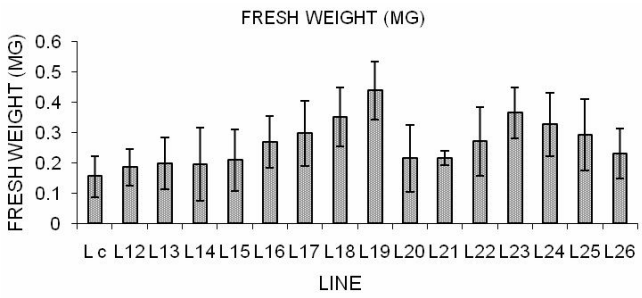

Fig. 7 : Graph showing fresh weight variation in M3 lines.

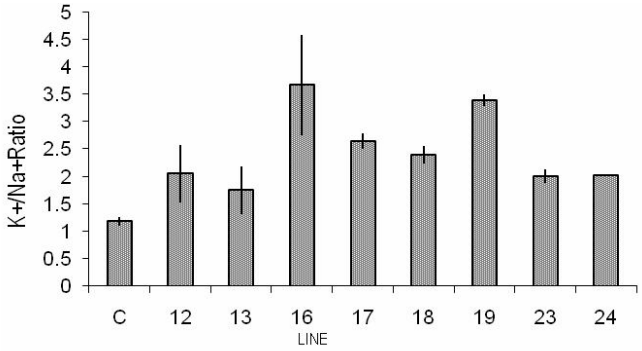

Fig. 8 : Graph showing comparative K^+, Na^+ ions ratio across M3 lines.

regeneration and calli induction, then the best potential for calli induction was in modified MS media in all. Here, the supplementation of casein hydrolysate is consistent with good growth and high regeneration potential in all varieties.

Therefore, the present study established the calli induction and regeneration protocols in all the recalcitrant varieties. The genotype variations lead to percentage regeneration from 72 to 85%. These methodologies are suitable for large scale propagation as well as for *Agrobacterium* mediated transformation. The present study also successfully regenerated a large no. of calli in IR 64, which is an elite cultivar.

Gamma irradiated field population, initial screening results and irradiated calli regeneration

In the present study, IR 64 variety of rice was used for raising a mutant population. There were two methods that were tried, one in seeds and another by calli irradiation. The irradiated calli showed regeneration in different doses

Table 1 : Effect of genotype on the calli induction and regeneration.

Genotype	Callus induction	Plant regeneration	No. of plantlets/ seed callus
IR 64	80%	72%	3.0
Swarna	85%	75%	3.0
Pokkali	90%	85%	4.0

Table 2 : Comparison of media types used in different cultivars.

S. no.	Genotype	Medium	Calli Induction	Regeneration medium	Plant regeneration
1	IR64	MS+CH(200mg/l)+2,4-D(2.5mg/l)	70%	MS + BAP(1.5mg/l) + NAA(0.5mg/l)	60%
		MS + CH(300mg/l) + 2,4-D (3mg/l) + BAP(0.5mg/l)	86%	MS + BAP(1.5mg/l) + NAA(0.5mg/l) + kinetin (1mg/l) + maltose	70%
2	Swarna	MS + CH(200mg/l) + 2,4-D(2.5mg/l)	65%	MS + CH(200mg/l) + BAP(1.5mg/l)	70%
		MS + 2, 4-D(2.5mg/l) + CH(200mg/l) + proline (300mg/l)	85%	MS + CH(300mg/l) + proline (500mg/l) + BAP (1.5mg/l)	90%
3	Pokkali	MS+2,4-D(2.5mg/l)	70%	MS+BAP (2mg/l)	75%
		MS + 2,4-D(2.5mg/l) + CH(200mg/l)	80%	MS + NAA(1.5mg/l) + BAP(2mg/l)	89%

Table 3 : Data of gamma irradiated IR 64 rice calli.

Total calli	Dose of irradiation (Gy)	Calli showing regeneration	Percentage regenerants
1250	10	35	2.8
1260	20	30	2.3
1265	50	29	2.2
1256	100	25	1.9

of irradiations. However, at 120 Gy the regeneration was least and the regenerants could not be recovered further. The gamma irradiation with different doses shows an inverse relationship with regeneration of calli. As the dose of irradiation increased the percentage regenerants was found to decrease (table 1, figs. 2, 3). Similar results were obtained by Rakotoarisoa *et al*. (2008) in japonica varieties, though it was the opposite in indica rice varieties.

Few calli that grew in irradiated effect were also used to study for salt selection in 50mM and 100mM NaCl. The calli turned necrotic in 100mM NaCl (fig. 4). Under *in vitro* salt stress callus induction has been studied in rice where it declined significantly (Htew *et al*., 2011).

The M1 (mutated) plants showed preflowering than the non irradiated rice plants.

From M1 population, some off type mutants have been demarcated with features such as thick smaller grains and height visually taller than usual, in all the three doses. It was possible to visualise many off variants for such characters as dwarfness, early and late maturity, sterility, increased height, altered grain phenotypes etc. in M2 generation.

The initial screening of M3 in hydroponics under salt stress showed variations in root length, shoot length, fresh weight and N^+/K^+ ratio across few M3 lines (Figs. 5, 6, 7, 8). Serrata *et al*. (2014) raised a large mutant population for TILLING by using EMS mutagenesis in rice. Other researchers have also raised a large mutant population for screening (Krishnan *et al*. 2009 and Hirochika *et al*., 2004). Sasikala and Kalaiyarasi (2010) also analysed shoot and root length variation in six rice varieties after gamma irradiation. The high efficiency of classical mutagenesis to generate mutations has been widely documented and reflected in the release of more than 300 varieties (Maluszynski *et al*., 2000). The presence of a large number of random mutants can be used to screen promising plants for better agronomic traits.

Conclusion

Hence, the present comprehensive study has established regeneration protocols in three widely used cultivars of rice and a mutant population for reverse and forward genetic approaches. The protocols are a requisite for successful genetic transformation of rice as well as large scale propagation of any elite cultivar. Further, the mutant population will benefit the breeding programs.

Acknowledgements

The acknowledgements are due to CSSRI, India and IARI for providing the material and help in experiments. The financial support from DBT is highly acknowledged.

References

Ahloowalia, B. S., M. Maluszynski and K. Nichterlein (2004). Global Impact of mutation derived varieties. *Euphytica*, **135** : 187-204.

Alam, M. J., M. Imran, L. Hassan, M. H. Rubel and Shamsuddoha (2012). *In vitro* regeneration of high yielding indica rice (*Oryza sativa* L.) varieties. *J. of Environment Science and Natural Resources*, **5(1)** : 173-177.

Hirochika, H., E. Guiderdoni, G. An, Yi Hsing, M. Y. Euan, C. D. Han, N. Upadhyaya, S. Ramachandran, Q. Zhang and A. Pereira *et al*. (2004). Rice mutant resources for gene discovery. *Plant Molecular Biology*, **54** : 325-334.

Htwe, N. N., M. Maziah, H. C. Ling, F. Q. Zaman and A. M. Zain (2011). Response of some selected Malaysian rice genotypes to callus induction under *in vitro* salt stress.

African J. of Biotechnology, **10(3)** : 350-362.

Jain, R. K. *et al*. (1996). Optimisation of biolistic method for transient expression and production of agronomically useful transgenic Basmati rice plants. *Plant Cell Reports*, **15** : 963-968.

Khaleda, L. and M. Al-Forkan (2006). Genetypic variability in callus induction and plant regeneration through somatic embryogenesis of five deepwater rice (*Oryza sativa* L.) cultivars of Bangladesh. *African Journal of Biotechnology*, **5(16)** : 1435-1440.

Krishnan, A., E. Guiderdoni, G. An, Y. Hsing, C. Han and M. Chul *et al*. (2009). Mutant Resources in rice for Functional Genomics of the Grasses. *Plant Physiology*, **149(1)** : 165-170.

Lee, K., H. Jeon and M. Kim (2002). Optimization of a mature embryo based in vitro culture system for high frequency somatic embryogenic callus induction and plant regeneration from japonica rice cultivars. *Plant Cell Tiss. Ogan. Cult.*, **71** : 237-244.

Maluszynski, M., K. Nichterlein, L. van Zanten and B. S. Ahloowalia (2000). Officially released mutant varieties – the FAO/IAEA Database. *Mut Breed Rev*., **12** : 1–84.

Pravin, J., D. Mahendra, T. Saluja and A. K. Saraswagi (2011). Assessment of critical factors influencing callus induction, *in vitro* regneneration and selection of bombarded indica rice genotypes. *Journal of Cell and Plant Science*, **2(2)** : 24-42.

Puhan, P. and E. A. Siddiq (2013). Protocol optimization and evaluation of rice varieties response to *in vitro* regeneration. *Advances in Biosciences and Biotechnology*, **4** : 647-653.

Rakotoarisoa, N. V., A. Andrianjaka, F. J. Zapata-Anas, R. Afza and C. Mba (2008). Inducing cold tolerance in Malagasy rice varieties IR 58614, Malady, Rojofotsy through *in vitro* mutagenesis. *Plant Mutation Reports*, **2** : 16-23.

Sanjeev, S., A. K. Richharia and A. K. Joshi (1998). An assessment of gamma ray induced mutations in rice (*Oryza sativa* L.). *Indian J. Genet*., **58** : 455-463.

Sasikala, R. and R. Kalaiyarasi (2010). Sensitivity of rice varieties to gamma irradiation. *Electronic Journal of Plant Breeding*, **1(4)** : 885-889.

Siddiqui, S. A. and S. Singh (2010). Induced genetic variability for yield and yield traits in Basmati Rice. *World J. of Agricultural Sciences*, **6(3)** : 331-337.

Serrat, X., R. Esteban, N. Guibourt, L. Moysset, S. Nogues and E. Lalanne (2014). EMS mutagenesis in mature seed derived calli as a new method for rapidly TILLING mutant populations. *Plant Methods*, **10** : 5.

Shimamoto, K. and J. Kyozuka (2002). Rice as a model for comparative genomics of plants. *Annual Review Plant Biology*, **53** : 399-419.

Syaiful, B. P., N. A. A. Siti, A. A. Maheran, M. Sariah and O. Othman (2009). Somatic embryogenesis from scutellar embryo of *Oryza sativa* L. var. MR 219. *Pertanika Journal of Tropical Agriculture Science*, **32 (2)** : 185-194.

Upadhyaya, G., M. Sen and A. Roy (2015). *In vitro* callus induction and plant regeneration of rice (*Oryza sativa* L.) var Sita, Rupali and Swarna Masuri. *Asian J. of Plant Science and Research*, **5** : 24-27.

Verma, D., R. Joshi, A. Shukla and P. Kumar (2011). Protocol for *in vitro* somatic embryogenesis and regeneration of rice (*Oryza sativa* L.). *Indian J. Exp. Biol.*, **49(12)** : 958-63.

Wani, S. H., A. A. Lone, A. Jaine, T. desilva and S. S. Gosal (2010). An efficient and reproducible method for regeneration of whole plants from mature seeds of a high yielding indica rice (*Oryza sativa* L.) variety PAU 201. *The Asian and Australian J of Plant Science and Biotechnology*, **4** : 57-61.

Yoshida, S., D. A. Forno, J. H. Cock and K. A. Gomez (1976). *Laboratory Manual for Physiological Studies of Rice*. IRRI, Manila. Philippines.

Zhu, J. K. (2001). Plant Salt Tolerance. *Trends in Plant Science*, **6(2)** : 66-71.