Research in Plant Biology, 3(5): 21-27, 2013 ISSN : 2231-5101
www.resplantbiol.com

Regular Article

Morphological and Histological Observation of Embryogenic Calli Derived from Immature Embryo of BRRI Dhan28 (*Oryza sativa* L.) Variety

Md. Mahmudul Islam, Md. Enamul Haque, Shah Md. Mahbub Alam, Md. Asadul Islam, Md. Khalekuzzaman, Biswanath Sikdar*

Department of Genetic Engineering and Biotechnology, Faculty of Life and Earth Science, University of Rajshahi, Rajshahi-6205, Bangladesh
*Corresponding author Email: bsikdar2004@yahoo.com

Somatic embryogenesis is the most common method for regeneration in rice. *In vitro* studies of indica rice (*Oryza sativa* L.) variety BRRI dhan28 was used for obtaining embryogenic calli from immature embryo culture on Murashige & Skoog medium supplemented with 2.5 mg/l dichlorophenoxyacetic acid (2,4-D) and 0.5 mg/l naphthaleneacetic acid (NAA) showed the highest percentage (91%) of callus induction. In this combination 80% embryogenic calli were formed uneven with a crisp texture, loose structure and salient multicellular structures on the surface while non embryogenic calli were compact with a smooth surface. Under microscopic observation, embryogenic cells were smaller, globular and abundant in cytoplasm with one or two big nuclei. Non embryogenic cells were little cytoplasm and few large vacuoles with no or only a small nucleus and wide intercellular spaces. Non embryogenic cells had a very low cell division capability while embryogenic cells had a high capability for cell division and continued to divide and produced somatic pro-embryos with a well–defined protodermis which could develop further through the typical globular, heart, torpedo and cotyledonary stages. Only 80% of embryogenic cells were induced high differentiation rate and developed 65 globular, 52 heart-shape, 43 torpedo and 37 cotyledonary embryos of embryogenic cells after 30 to 45 days of induction.

Key words: Rice, callus, embryogenesis, morphology, histology

Rice is the most important primary food source for more than a third of the world's population. Indica and japonica rice are the two major subspecies. Indica rice varieties calculate for 80% of the cultivated rice. Somatic embryogenesis is the most common method for regeneration in rice. Somatic embryogenesis is two types: one is direct somatic embryogenesis and another indirect somatic embryogenesis. Direct somatic embryogenesis is produced by induction of somatic embryos directly from pro-embryogenic cells from leaves, stem, microspores, protoplast without the proliferation of calli where as in indirect somatic embryos are produced from friable embryogenic calli.

Received: 20.6.2013; Revised: 18.9.2013; Accepted: 20.9.2013

Plant regeneration in rice via embryogenesis has been reported from different explants, such as root, mature embryo, leaf, immature embryo and inflorescence. Plant regeneration has also been routinely obtained from rice protoplasts have reported plant regeneration from coleoptile tissue of *Poa pratensis* L. and *Triticum aestivum* L., respectively. Somatic embryogenesis is also a more popular regeneration system used in plant transformations. Some researcher used immature seed derived calli (Dong *et al.*, 1996). Most workers that observed calli from mature seed were excellent starting material for transformation of rice by *Agrobacterium* due to their compactness. An immature embryo was the first explants used to induce embryogenic calli for rice transformation (Li *et al.*, 1993) and immature embryo has high potentiality to produce embryogenic calli obtained from those explants have high regeneration capacity.

Morphological and histological studies of callus and embryo induction of plant are important for increasing the incidence of callus production and induction of embryogenic callus. Cultured rice embryo produces two types of calli. One is embryogenic calli which consists of globular pro-embryoidal cells (Bajaj and Rajan, 1995) which produces many plants predominantly via somatic embryogenesis. The other is non embryogenic calli which consists of tabular vacuolated cells which produced few or no plants. Another important trait of plants derived from somatic embryo is their uniformity and genetic stability. In the present study, the morphology and histology of the callus and embryos produced from the immature embryo of indica rice cultivar BRRI dhan28 were systematically studied in order to clarify the origin of calli and the differences in morphological and histological characteristics between the non embryogenic callus (NEC) and embryogenic callus (EC). Embryogenic callus induction is dependent on the interaction between genotype and culture conditions. The aim of this study is to describe the development of plantlets from embryogenic calli obtained from immature embryo of indica rice variety BRRI dhan28 through histological analysis.

Materials and Methods

Plant material and immature embryo isolation

Seeds of indica rice variety BRRI dhan28 were collected from Bangladesh Rice Research Institute (BRRI) Regional office, Rajshahi-6205, Bangladesh. Seeds were sown in the field of Genetic Engineering and Biotechnology Department at intervals of 10 days. Immature embryos from unripe seeds were collected 12 to 15 days after anthesis from field-grown plants and surface-sterilized with $HgCl_2$ (0.1%) for 8–10 min. Then seeds were thoroughly washed three to four times in sterile distilled water. Using fine scalpels and forceps, immature embryos were excised under a binocular microscope.

Culture media and culture conditions

The basic medium (BM) was composed of MS (Murashige and Skoog, 1962) salts and organic compounds, 30g/l sucrose and 8g/l agar. The pH was adjusted to 5.6-5.8 before adding the gelling agent and media were autoclaved for 20 min at 121°C and 1.07 kg/cm^2. Then media was taken in petridishes (9 x l.5cm) with 25 ml and sealed with parafilm. The immature embryos were cultured aseptically on nutrient media in the dark at 25 ± 1°C with the scutellum side facing upwards.

Callus induction

Ten immature embryo from isolated sterilized seeds were placed individually in each petridish containing 25 ml of MS with various concentrations of 2, 4-D and NAA. The seeds were incubated in the dark at 25 ± 1^{o}C. After 15 days, only embryogenic calli were transferred to fresh callus induction

medium for development. Sub-culturing of calli was carried out once in every two weeks. The callus was observed from 2nd to 7th week. The percentage (%) of callus induction frequency (CIF) for each group was calculated as follows.

$$\text{CIF (\%)} = \frac{\text{Total number of immature embryo that produced callus}}{\text{Total number of immature embryo plated}} \times 100$$

Selection of embryogenic callus

Under microscopic observation, embryogenic callus of indica rice (*Oryza sativa* L.) variety BRRI dhan28 can be described as yellowish and granular callus, compact, greenish-yellow, granular with smaller cells and very dense cytoplasm. These types of EC were selected and used for plant conversion studies.

In order to investigate the effect of *Agrobacterium*-mediated transformation on the regeneration capacity, embryogenic calli were infected by *Agrobacterium* with *GUS* reporter gene. Additionally, non- infected embryogenic calli were included as controls.

Histological study

Histological analysis of the embryogenic calli was performed according to Boissot *et al.* (1990). Embryogenic calli were collected 10 to 45 days after cultured on callus induction medium. Then, the samples were dehydrated in a graded series of ethanol (70, 95 and 100%) for 1 h each one and embedded in paraffin wax. Samples were cut into 9-12 µm sections by hand and microtome and were stained with safranin red stain. Microscopic features of different days of calli were photographed using stereomicroscope (LABOMED-CZM6) and the photographs of the histological study were taken under 10X and 40X using a contact face microscope (B-350 OPTIKA, Italy).

Histochemical *GUS* assay

After co-cultivation, infected calli were assayed for transient *GUS* activity. Explants were dipped in histochemical reagent solution and incubated for 24-48 hours at 37 ^{o}C. Then treated explants were transferred to 95% ethanol for preservation and the explants were observed under zoom stereomicroscope (Japan, model- SDZ-TR-P).

Results and Discussion

The choice of a suitable explant source as starting material for *in vitro* culture is one of the most important factors. Immature seeds derived calli are good source of *in vitro* regeneration due to their high totipotency (Khalequzzaman *et al.*, 2005). An immature embryo was the first explant used to induce embryogenic calli for rice transformation (Li *et al.*, 1993). Plant growth regulators have important role in callus induction. Meneses *et al.*, 2005 have worked on the effect of growth regulators on plant regeneration in rice. In the present investigation, friable pale yellowish calli **(Figure 1A-G)** were obtained with the highest callus induction percentage (91%) from swelling of scutellum **(Figure 2A)** region of rice immature zygotic embryos after culture on callus induction medium with 2.5mg/l 2, 4-D and 0.5mg/l NAA among different combinations. The results on callus induction frequency are presented in **Table 1.** The epithelial cells of the scutellum are columnar with dense cytoplasm and a prominent nucleus and nucleolus **(Figure 2B).**

Early development of somatic embryo is very important factor for regeneration of plant. In our investigation, proembryos were observed as early as five days after culture initiation **(Figure 2B)**. Meneses et al., 2005 and other investigators reported on somatic embryo development of other species

significantly longer periods for embryogenic callus formation, most of them longer than ten days and some of them reaching up to 40-60 days.

Table 1: Effect of different concentration of 2, 4-D and NAA on callus induction from immature embryo of indica rice (*Oryza sativa* L.) variety BRRI dhan28.

Treatments (mg/l)	Genotype	Days to callus initiation	% of callus formation	Texture of callus	Colour of callus	Degree of callusing
2,4-D 1.0 + NAA 1.0	BRRI dhan-28	12-17	60	C	Cr	+
2,4-D 1.5 + NAA 1.0	BRRI dhan-28	12-17	70	F	Cr	++
2,4-D 2.0 + NAA 0.5	BRRI dhan-28	12-17	85	F	Cr	+++
2,4-D 2.5 + NAA 0.5	**BRRI dhan-28**	**12-17**	**91**	**F**	**Cr**	**++++**
2,4-D 3.0 + NAA 0.5	BRRI dhan-28	12-17	80	F	Cr	++
2,4-D 3.5 + NAA 1.0	BRRI dhan-28	12-17	65	C	Cr	+

In each treatment 20 explants were used and the experiment was repeated twice. Data were collected after 12-17 days of culture (PY = Pale yellow, F = Friable, Cr = Creamy, C = Compact, ++++= Excellent, +++ = Good, ++ = Moderate, + = Poor).

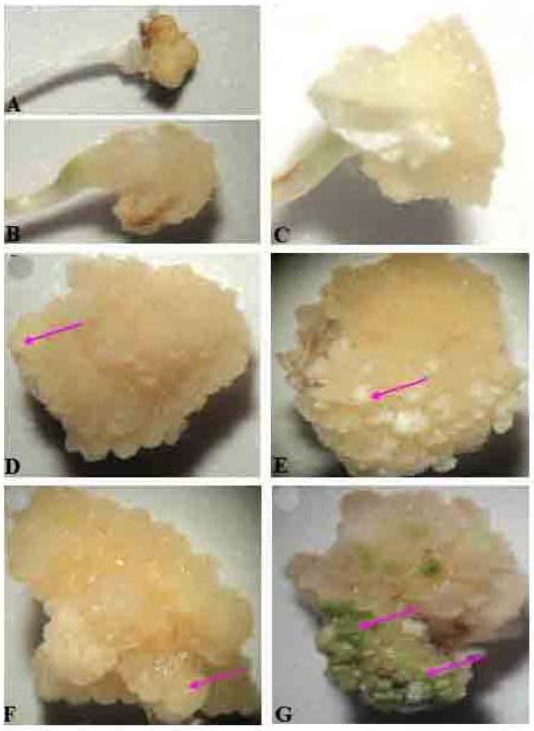

Figure 1: Different types of calli from immature embryo in indica rice variety BRRI dhan28. (A) five days old callus derived from immature embryo on callus induction medium (B) and (C) Developed proembryogenic callus (D), (E) and (F) Developed embryogenic callus with different stages of somatic embryos (arrow indicated) (G) Cotyledonary stage with meristematic region (arrow indicated)

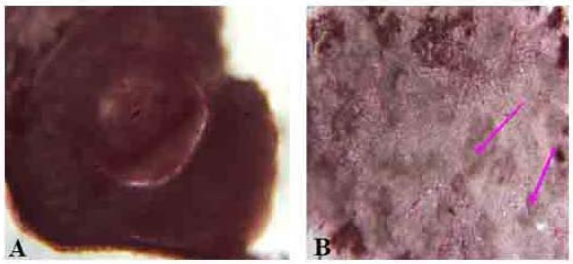

Figure 2: Somatic embryogenesis in indica rice (*Oryza sativa*) variety BRRI dhan28. (A) For showing epithelial cells, transverse section of the scutellum of immature embryo (B) Proembrygenic cells observed after 5 days on culture initiation (arrow).

The morphological and histological observation indicates that somatic embryo formation occurs both directly and indirectly from proembryonic cell complexes and directly from single cell. Somatic embryos may derive from one cell or a group of cells. In our histological observation, somatic embryos derived from peripheral region of the embryogenic calli. Similar reports were published by many researchers (Jane-Ho and Vasil, 1983) in sugarcane and (Schwendiman *et al.*, 1988) in oil palm. In the histological observation, embryogenic and non-embryogenic cells were found. Embryogenic cells were small cells with dense cytoplasm.

They had a large nucleus, a prominent nucleolus and small vacuole. Non-embryogenic cells were large cells with large vacuoles. Our results confirmed in the previous observations in sugarcane (Falco *et al.*, 1996) and maize (Fransz and Schel, 1991a).

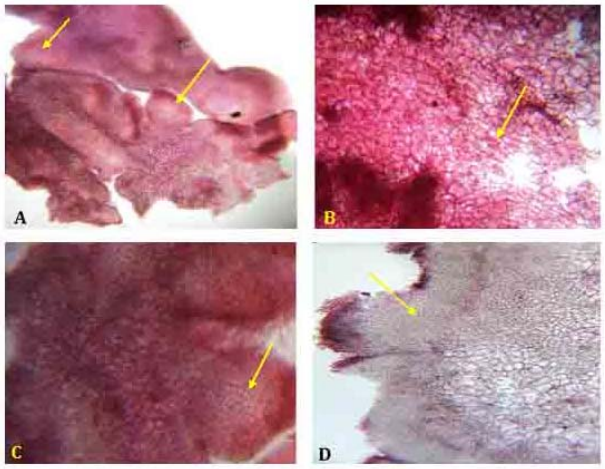

Figure 3: Histology analysis of somatic embryogenesis (A) Embryogenic cells with small vacuole and small intercellular spaces (arrow indicated) (B) Non-embryogenic cells with large vacuole and large intercellular spaces (arrow indicated) (C) Embryogenic cells showing isodiametric (arrow indicated) (D) The embryogenic cells began to divide in the peripheral regions.

Developments of somatic embryos were studied by the histological observation of 10 days old calli **(Figure 3).** The results on induction somatic embryogenesis frequency are presented in **Table 2.** In observation calli were produced two types of cells: embryogenic and non embryogenic cells. The embryogenic cells **(Figure 3A)** consisted of small and isodiametric cells **(Figure 3C)** with a dense cytoplasm, a very prominent nucleus and a nucleolus. The non embryogenic cells consisted of large cells with large vacuole were examined **(Figure 3B)**. The embryogenic cells began to divide **(Figure 3D)** and developed a clump of proembryogenic cells in the peripheral region of the calli **(Figure 4A).** These proembryogenic cells increased in size which showed a protodermis **(Figure 4B)** and developed in different shape of somatic embryo such as globular, heart, torpedo, coteledonary shape **(Table 3)**. A mass globular somatic embryo **(Figure 5A)** was formed by a series of coordinated division of proembryogenic cells after 15 days of culture. These globular somatic embryos had no connection with mother tissues **(Figure 5B)**. The globular somatic embryos turned into heart shape somatic embryos **(Figure 5C)** with dense cytoplasm and prominent nucleus and gradually into torpedo shaped **(Figure 5D)** and cotyledonary shaped somatic embryos **(Figure 5E)**. The cotyledonary shaped somatic embryos developed meristematic region and produced plantlets **(Figure 5F)** after 45 days on regeneration medium. Similar results were observed in *Helianthus smithii* Heiser (Laparra *et al.*, 1997) and *Cicer arietinum* L. (Shri and Davis, 1992).

Table 2: Effect of different concentrations and combinations of 2, 4-D, NAA and tryptophan on induction of somatic embryos on calli derived from immature embryo of indica rice (*Oryza sativa* L.) cultivar BRRI dhan28.

Genotypes	Treatments for subculture (Mg/l)			No. of calli subcultured	No. of calli showing embryogenesis	% of somatic embryogenesis
	2,4-D	NAA	Tryptophan			
BRRI dhan 28	1.0	0.5	1.0	16	8	50.00
	1.5	0.5	1.0	17	11	64.71
	2.0	0.5	1.0	18	12	66.67
	2.5	**0.5**	**1.0**	**16**	**13**	**81.25**
	3.0	0.5	1.0	17	12	70.59
	3.5	0.5	1.0	15	9	60.00

Table 3: Somatic embryogenesis from embryo derived immature embryo of indica rice cultivar BRRI dhan28 on MS medium with varied concentrations of 2, 4-D and NAA

Treatment	Percent response	Number and type of somatic embryos			
		Globular	Heart	Torpedo	Cotyledonary
2, 4-D 1.0 + NAA 0.5	50	33	31	26	17
2, 4-D 1.5 + NAA 0.5	60	45	37	33	27
2, 4-D 2.0 + NAA 0.5	75	55	44	38	32
2, 4-D 2.5 + NAA 0.5	**80**	**65**	**52**	**43**	**37**
2, 4-D 3.0 + NAA 0.5	70	54	42	35	30
2, 4-D 3.0 + NAA 0.5	65	47	41	31	24

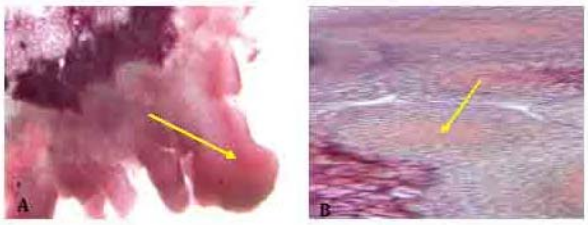

Figure 4: (A) A clump of proembryogenic cells in the peripheral region of the calli (arrow indicated) (B) Protodermic structure (arrow indicated)

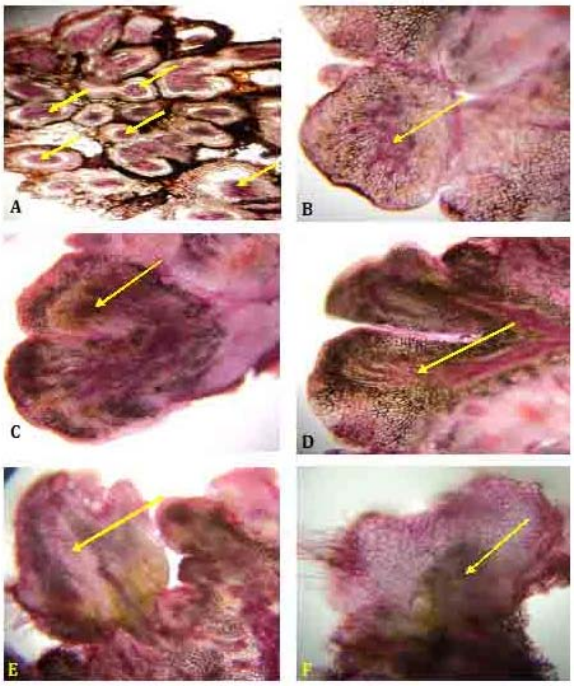

Figure 5: Somatic embryogenesis and histological aspects of somatic embryos (A) A mass of globular somatic embryos structure (B) The globular somatic embryo structure (C) Heart shape somatic embryo structure (D) Torpedo shape somatic embryo structure (E) Cotyledonary somatic embryo structure (F) Meristematic region structure

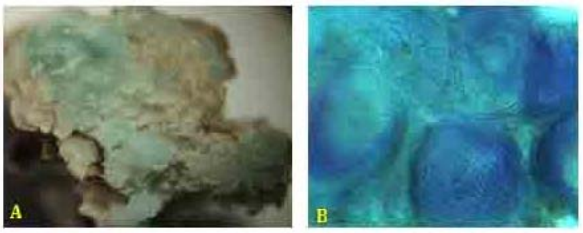

Figure 6: *GUS* expression (*Agrobacterium* mediated) in somatic embryos of 15 days old embryogenic calli of indica rice (*Oryza sativa*) variety BRRI dhan28. (A) Transform callus (B) section of transform callus.

Transformation efficiency was observed in embryogenic calli by transfer of *GUS* gene through *Agrobacterium*-mediated genetic transformation without damage of embryogenic cells **(Figure 6B)** and *GUS* expression was observed on embryogenic calli after three days of co-cultivation **(Figure 6A)**

Conclusion

Somatic embryos can be considered as a useful model system for further studies upon *in vitro* morphogenesis, and to compare plants regenerated via somatic embryogenesis for their genetic transformation, and genetic homogeneity or variability.

Acknowledgements

Authors are grateful to USDA and MoE for the financial support to establish a laboratory named "Professor Joarder DNA and Chromosome Research Lab.", Department of Genetic Engineering and Biotechnology, University of Rajshahi,

Rajshahi-6205, Bangladesh, to carry out this work.

References

Bajaj S, Rajam MV. 1995. Efficient regeneration from long term callus culture of rice by spermidine. Plant Cell Rep. 14: 717-720.

Dong J, Teng W, Bucholz WG and Hall TC. 1996. *Agrobacterium*-mediated transformation of japonica rice. Mol Breed. 2: 267-276.

Falco MC, Januzzi Mendez BM, Tulmann Neto A and Appezzato Da Gloria B. 1996. Histological characterization of *in vitro* regeneration of *Saccharum* sp. R. Bras Fisiol Veg. 8: 93-97.

Fransz PF and Schel JHN. 1991. Cyto-differentiation during the Development of Friable Embryogenic Callus of Maize Friable Embryogenic Callus of Maize *(Zea mays)*. Can J Bot. 69: 26-33.

Jane-Ho W and Vasil I K. 1983. Somatic embryogenesis in sugarcane (*Saccharum officinarum L.*): Growth and plant regeneration from embryogenic cell suspension cultures. Ann Bot. 51: 719-726.

Khalequzzaman M, Najmul H, Hoque ME and Aditya TL. 2005. Regeneration efficiency and genotypic effect of 15 *indica* type Banglsdeshi rice (*Oryza sativa* L.) landraces. Plant Tissue Cult. 15: 33-42.

Laparra H, Bronner R and Hahne G. 1997. Histological analysis of somatic embryogenesis induced in leaf explants of Helianthus smithii. Heiser Protoplasma. 196: 1–11.

Li LQ, Kochko A, Fauquet C and Benchy R. 1993. Improvement rice transformation system using thebiolistic method. Plant Cell Rep. 12: 250-255.

Meneses, Flores AD, Munoz M, Arreta G and Espinoza AM. 2005. Effect of 2,4-D hydric stress and light on *indica* rice (*Oryza sativa*) somatic embryogenesis. Rev Biol Trop. 53: 361-368.

Murashige T and Skoog F. 1962. A revised medium for rapid growth and bioassays with tobacco cultures. Physiol Plant. 15: 473-497.

Schwendiman J, Pannetier C and Michaux-Ferriere N. 1988. Histology of somatic embryogenesis from leaf explants of the oil palm *Elaeis guineensis.* Ann Bot. 62: 43-52.

Shri PV and Davis PM. 1992. Zeatin-induced shoot regeneration from immature chickpea (*Cicer arietinum* L.) cotyledons. Plant Cell Tissue and Organ Culture. 28: 45-51.