In vitro Propagation and Genetic Transformation System Using Immature Embryo in Elite Rice (*Oryza sativa* L.) Cultivars

Md. Mahmudul Islam¹, Zahida Yesmin Roly¹, Youngsook Lee², Md. Khalekuzzaman^{3*}

¹Department of Genetic Engineering and Biotechnology, Faculty of Life and Earth Science, University of Rajshahi, Rajshahi-6205, Bangladesh

²POSTECH-UZH Cooperative Laboratory, Department of Integrative Bioscience and Biotechnology, Pohang University of Science and Technology, Pohang, 790-874, South Korea

³Professor, Department of Genetic Engineering and Biotechnology, Faculty of Life and Earth Science, University of Rajshahi, Rajshahi-6205, Bangladesh

ABSTRACT Rice (*Oryza sativa* L.), as a cereal grain, is the most widely consumed staple food for a large part of the world's human population especially in Asia. Three indica rice (*Oryza sativa* L.) cultivars namely BRRI dhan29, BRRI dhan56 and BRRI dhan57 were used in this study to investigate the regeneration ability in rice. Regeneration potential was found to be highest (80.00%) for BRRI dhan29 and lowest (70.00%) for BRRI dhan57. Consequently, the highly regenerating indica rice cultivar BRRI dhan29 was used for genetic transformation. Embryogenic calli induced after 20 days were used for genetic transformation in the experiment. *Agrobacterium* strain LBA4404 was transformed with pBI121 binary vector which contains kanamycin resistance gene as a selectable marker gene and *GUS* as a reporter gene. Calli infected with this strain were analyzed by using 5-bromo 4-chloro 3-indolyl- D glucuronide (*X-gluc*) as a substrate. Expression of gene was determined by using the transformed shoots and roots. Stable integration and expression of *GUS* gene were also confirmed by using PCR analysis. The frequency of transformation in terms of transient *GUS* assay was found to be 35.0 ± 2 S.E. This study will provide valuable information for genetic transformation in cereal crops.

Keywords *Agrobacterium*, *GUS*, Indica Rice, Regeneration, Rice transformation

INTRODUCTION

Rice is one of the most important cereals not only in the developing countries but also in the developed countries (Toenniessen 1995). At present, rice is cultivated in 153.52 million hectare land area worldwide with overall production of about 589 million tons (FAO 2003). In Bangladesh, rice covers an area of 11 million hectare and the production is 250 million tons (BBS 2005). The global population is increasing at a rapid rate with Bangladesh being no exception. To cope with the demand of this ever increasing population rice productivity level needs to be increased. Keeping this in mind, worldwide projected requirement of rice by 2015 is 750 million tons while in Bangladesh alone the projected requirement is about 270 million tons. The main challenges in increasing rice productivity are its

susceptibility to many disease and pest, salinity and abiotic stress like drought which lead to decrease in yield of rice (Baker *et al.* 1997). Nonetheless, biotechnology is a promising tool that can be used to minimize productivity loss and thus overcome these problems.

Tissue culture techniques are being increasingly practical for clonal multiplication and *in vitro* conservation of valuable native plants which threatened with extinction (Roly *et al.* 2013). Tissue culture via somatic embryogenesis is a key step in gene transfer and plant regeneration in rice biotechnology. Many bacteria are being used for genetic transformation (Shaekh *et al.* 2013). Several gene transfer methods have been well established to introduce target DNA into the rice cell. Out of these methods, *Agrobacterium* transformation is better due to its easy, low cost and low copy number of transgene integration (Labra *et al.* 2001).

Received March 21, 2014; Revised March 28, 2014; Accepted March 29, 2014; Published March 31, 2014

*Corresponding author Md. Khalekuzzaman, kzaman63@ru.ac.bd, Tel: +880-17162448083, Fax: +880-721750064

The genetic transformation of rice can be accomplished through *Agrobacterium* co-cultivation of embryogenic calli (Hiei *et al.* 1994). In general, embryogenic calli, rather than direct tissues such as shoot spices, immature inflorescences, roots and leaves are used for genetic transformation and regeneration of rice plants. This is because the callus culture is more suitable for gene delivery and regeneration of transgenic rice plants compared with organogenesis. 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 (Islam *et al.* 2013). Therefore, proliferation of the embryogenic calli with the high regeneration capacity is a prerequisite for the successful production of transgenic rice plants.

A lot of plants have been identified and utilized for insecticidal, genetic transformation and medicinal purpose to date (Islam *et al.* 2014). Indica rice BRRI dhan29 is a high quality cultivar that is resistant to different types of disease and pest. It is a short duration variety that matures in about 85 days. However, biotechnological studies and literature on hormonal combinations for callus induction and plant regeneration and effective procedure of *Agrobacterium*-mediated transformation of the indica rice BRRI dhan29 are still evidently lacking. Therefore, the present study was designed to investigate the different hormonal combinations in case of callus induction and plant regeneration among the three rice cultivars namely BRRI dhan29, BRRI dhan56 and BRRI dhan57, and to establish an effective *Agrobacterium*-mediated transformation procedure for highly re-generable indica rice BRRI dhan29.

MATERIALS AND METHODS

Plant materials

Immature zygotic embryo of three indica rice genotypes (*Oryza sativa* L.) namely BRRI dhan29, BRRI dhan56 and BRRI dhan57 were used as explants for *in vitro* regeneration and genetic transformation. Mature seeds of these three indica rice genotypes were collected from Bangladesh Rice Research Institute (BRRI), Regional office, Rajshahi, Bangladesh. Seeds were sown in the field of Genetic

Engineering and Biotechnology, faculty of Life and Earth Science, University of Rajshahi, Bangladesh.

Immature embryo isolation

The unripe seeds were collected 12 to 15 days after anthesis from field-grown plants and were surface-sterilized with HgCl₂ (0.5% - 0.1%) for 8 min. These seeds were then thoroughly washed four to five times in sterile distilled water. The immature embryos of the seeds were excised under a binocular microscope using fine scalpels and forceps.

Culture media and culture conditions

The basic medium (BM) was composed of Murashige and Skoog (MS) (1962), salts and organic compounds, 30g/l sucrose and 8 g/l plant agar. The pH was adjusted to 5.6-5.8 before adding the gelling agent and the media were autoclaved for 20 minutes at 121°C and 1.1 kg/cm². About 25 ml of medium was poured out into petridishes 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 embryos from isolated sterilized seeds were placed individually in each petridish containing 25 ml modified MS with various concentrations of 2,4-dichlorophenoxyacetic acid (2,4-D) and naphthalene acetic acid (NAA). The seeds were incubated in the dark at 25±2°C. Only embryogenic calli were transferred to fresh callus induction medium for multiplication. Sub-culturing of the callus 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 using the following formula:

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

Selection of embryogenic calli

Embryogenic calli of indica rice (*Oryza sativa* L.) cultivars namely BRRI dhan29, BRRI dhan56 and BRRI

dhan57 can be described as yellowish and granular callus, compact, greenish-yellow, granular with smaller cells and very dense cytoplasm callus. These types of EC were selected and used for regeneration and genetic transformation.

Regeneration

The green-colored embryogenic calli were then transferred to fresh shoot regeneration medium; MS with BAP: benzylaminopurine (17.68-22.10 $\mu\text{mol/L}$), NAA (13.26 $\mu\text{mol/L}$) and kinetin (8.84 $\mu\text{mol/L}$) and incubated under light condition. The well-developed calli with shoot primordia were sub-cultured on MS shooting regeneration medium in a test tube and incubated at 27°C under continuous light. Healthy shoots with defined stem were transferred to MS rooting medium and incubated at 28°C under continuous light. The plantlets with well-developed root system were planted in the pots containing autoclaved mud that was collected from ricefield.

Agrobacterium-mediated transformation

Bacterial strains

Agrobacterium strain LBA4404 was used with pBI121 binary vector which contains kanamycin resistance and *GUS* gene for co-cultivation in the transformation experiment.

Agrobacterium strain culture and infection

Agrobacterium strain LBA4404 (pIG121) was cultured on liquid LB medium containing kanamycin (442 $\mu\text{mol/L}$) and agar (8 g/l) for 3 days at 27°C in the dark. The bacteria were collected and suspended in medium containing acetosyringone (100, 200, 400, 600 and 1,000 rpm). For *Agrobacterium* infection, the density of the bacteria was adjusted ($\text{OD}_{600} = 1.2, 1.1, 0.9, 0.8, \text{ and } 0.6$) and the rice calli were immersed in a bacterial suspension for 25 minutes. Excess bacteria were removed by blotting the calli on filter paper. The calli were transferred to petridish containing MS medium. The plates were sealed with parafilm to prevent evaporation of the medium and subjected to 3 days of co-cultivation at 25±2°C in the dark. Calli were then washed twice with sterile water to remove *Agrobacterium*. The co-cultured calli were blotted dry on filter paper and plated on MS medium supplemented with

kanamycin (442 $\mu\text{mol/L}$). The plates were sealed with surgical tape and incubated at 25±2°C using a 16 h light. Proliferating kanamycin resistant calli were transferred to the same fresh medium.

Regeneration

Proliferating kanamycin-resistant calli were transferred to the same fresh medium. Additionally, non-infected embryogenic calli were included as controls. The number of shoots and *in vitro* plants per embryogenic calli were determined after ten weeks of culture on regeneration medium which consisted of MS mineral salts supplemented with 17.68 $\mu\text{mol/L}$ 6- benzylaminopurine (BAP), 13.26 $\mu\text{mol/L}$ naphthaleneacetic acid (NAA) and 8.84 $\mu\text{mol/L}$ kinetin, 30 g/l sucrose and 6 g/l agar. The explants were cultured in the dark at 26±1°C. The percentage of calli with shoots and regeneration rate were calculated using the following formula:

$$\text{Embryogenic calli with shoots (\%)} = \frac{\text{Number of calli with shoots}}{\text{Total of embryogenic calli}} \times 100$$

$$\text{Regeneration (\%)} = \frac{\text{Number of } in vitro \text{ plants}}{\text{Total of embryogenic calli}} \times 100$$

Assay for *GUS* gene expression

After 10 days, the *Agrobacteria* were eliminated and the co-cultured calli were incubated overnight in *X-gluc* solution at 37°C and the β -glucuronidase activity was determined histochemically. The tissue containing β - showed blue color which confirms the expression of *GUS* gene. In the same way, the transformed shoot and root were also used for confirmation of the transformation of the *GUS* gene.

PCR analysis of transformants

Genomic DNA was isolated from transformed calli. PCR analyses were carried out by using two *GUS* primers, forward CCTGTAGAAACCCCAACCCG 3' and reverse 5' TGGCTGTGACGCACAGTTCA 3' for the amplification of *GUS* gene. The reaction mixture (20 μl) of PCR was composed of 1.0 μl DNA template, 2.0 μl 10x buffer, 1.0 μl (2.5mM) dNTPs, 2.0 μl (25 mM) MgCl_2 , 1.0 μl of each primer (F/R), 0.4 μl *Taq* DNA polymerase and 13 μl double

distilled water. The amplification reaction was carried out at 94°C for 4 minutes followed by 25 cycles at 94°C for 1 minute, 56°C for 45 seconds and 72°C for 1 minute. After the final cycle, the reactions were maintained at 72°C for 5 minutes before completion. Finally, PCR products were analyzed on 1% agarose gel with 0.5x TBE buffer.

RESULTS

Callus induction

Three rice genotypes, namely BRRI dhan29, BRRI dhan56 and BRRI dhan57 were used in this investigation. The highest induction of callus was recorded from BRRI dhan29 at 92.0% in MS having 17.68 $\mu\text{mol/L}$ 2, 4-D + 8.84 $\mu\text{mol/L}$ NAA followed by BRRI dhan56 at 85.0% in MS having 22.1 $\mu\text{mol/L}$ 2, 4-D + 8.84 $\mu\text{mol/L}$ NAA and BRRI dhan57 (80.0%) in MS having 22.1 $\mu\text{mol/L}$ 2, 4-D + 8.84 $\mu\text{mol/L}$ NAA. Effects of subculture of calli on MS medium

with four different concentrations and combinations of 2,4-D, NAA and tryptophan on induction of somatic embryos were studied in the present study. Results further revealed that BRRI dhan29 showed the highest somatic embryogenesis rate in 20-d old calli at 76.47% in MS having +17.68 $\mu\text{mol/L}$ 2,4-D + 4.42 $\mu\text{mol/L}$ NAA+8.84 $\mu\text{mol/L}$ tryptophan followed by BRRI dhan56 with 70.0% in MS having 22.1 $\mu\text{mol/L}$ 2,4-D+8.84 $\mu\text{mol/L}$ NAA+8.84 $\mu\text{mol/L}$ tryptophan and BRRI dhan57 with 68.0% in MS having 22.1 $\mu\text{mol/L}$ 2,4-D+8.84 $\mu\text{mol/L}$ NAA+8.84 $\mu\text{mol/L}$ tryptophan.

Regeneration

The embryogenic calli of the three rice varieties were cultured on nine different regeneration media for regeneration of plantlets. BRRI dhan29 exhibited the highest regeneration rate (80.0%) of calli which formed shoots in 17.68 $\mu\text{mol/L}$ BAP+8.84 $\mu\text{mol/L}$ NAA+13.26 $\mu\text{mol/L}$ Kinetin followed by BRRI dhan56 with 75.0% rate and BRRI dhan57 at

Fig. 1. Plant regeneration of calli derived from rice cultivar BRRI dhan29 (A) 20 days old callus (B) bud initiation (C) shoot initiation (D) root initiation and (E) potting.

Fig. 2. Plant regeneration from calli-derived immature embryos in indica rice cultivar BRRI dhan56 (A) 20 days old callus (B) bud initiation (C) shoot initiation (D) root initiation and (E) potting.

70.0%. This result indicated that BRRI dhan29 had the superior regeneration capacity over BRRI dhan56 and BRRI dhan57. Different stages of rice plant regeneration were illustrated in BRRI dhan29 (Fig. 1), BRRI dhan56 (Fig. 2) and BRRI dhan57 (Fig. 3).

Genetic transformation

For *Agrobacterium*-mediated genetic transformation, the embryogenic calli were infected with *Agrobacterium* strain LBA4404 harboring the plasmid pBI121. Putative transformants harvested from selection medium were proved transgenic by histochemical *GUS*-assay.

Selection of the optimum concentration of antibiotic

Agrobacterium strain LBA4404 harboring the plasmid pBI121 contains kanamycin resistance gene (*nptII*) as the selectable marker gene. If calli explants of rice are transformed with this gene, they would be able to grow and

proliferate in the selection media under kanamycin stress. The 884 $\mu\text{mol/L}$ concentration of kanamycin was found to be suitable for successful selection. However, concentration higher than 884 $\mu\text{mol/L}$ resulted in rapid and severe browning of the cells which could be attributed to excessive stress on surviving or transformed cells.

Effects of incubation period on transformation

The effects of different incubation periods (15, 20, 25, 30, 35 and 40 minutes) in the *Agrobacterium* suspension on calli were studied at a constant optical density (OD_{600} of ~ 0.9). The optimum incubation period resulting in highest positive rate (30%) for *GUS* assay was observed in 30 minutes incubation period while the lowest rate was noted in 15 minutes incubation period (Fig. 4).

Effect of optical density (OD)

Optical density of *Agrobacterium* is an important factor

Fig. 3. Plant regeneration of calli derived from embryos in indica rice cultivar BRRI dhan57 (A) 20 days old callus (B) bud initiation (C) shoot initiation (D) root initiation and (E) potting.

Fig. 4. Effects of different incubation periods on genetic transformation of immature embryo of BRRI dhan29 by transient *GUS* histochemical assay.

for genetic transformation. In this study, five different optical density levels were tested viz., 1.2, 1.1, 0.9, 0.8 and 0.6 (Fig. 5). The highest number of GUS-expressing calli was observed at OD 0.8-0.9. It was clearly demonstrated that the bacterial strain LBA4404 (pBI121) showed highest peak of performance in lower range of OD whereas it gradually decreased when OD value was higher.

Transformation and proof of stable integration of foreign gene

After inoculation with *Agrobacterium*, the explants were co-cultivated for 4 days on normal MS media (Fig. 6A). The explants were then sub-cultured on regeneration medium (BAP 17.68 $\mu\text{mol/L}$ + 8.84 $\mu\text{mol/L}$ NAA + 13.26 $\mu\text{mol/L}$ KIN) containing carbenicillin. With 2-3 weeks of culture, the calli developed shoot buds (Fig. 6B) and were

Fig. 5. Effects of different optical density at 600 nm on genetic transformation of immature embryo of BRRI dhan29 by transient *GUS* histochemical assay.

Fig. 6. Different stage of genetic transformation in indica rice cultivar BRRI dhan29 (A) Infected calli (B) Shoot bud development (C) shoot initiation (D) First cycle selection: Shoots of transformed calli (ii&iii) and control (i) in selection medium with 75mg/l kanamycin (E) Second cycle selection Sub-cultured of shoots after first cycle selection in medium with 100 mg/l kanamycin; transformed shoots (ii& iii) and control (i). (F) Root induction in selection medium and (G) Establishment of regenerated plants on soil.

again sub-cultured on the same medium for shoot development (Fig. 6C). After proliferation, the shoots were transferred to selection medium with kanamycin and the same hormonal combination for first (Fig. 6D) and second cycle of selection (Fig. 6E). At the same time the control plants which were subsequently cultured on selection medium showed 90% dead shoots within 21 days. Regenerated shoots harvested from selection medium were then transferred to normal MS medium for root induction (Fig. 6F), and the micro plants were transplanted in the pots (Fig. 6G).

GUS assay was performed in two stages, first, after 4 days of co-cultivation in calli and second, when the leaves and roots from putative plants were taken and incubated in *X-gluc* buffer. Indigo blue coloration was observed in calli (Fig. 7A), leaves (Fig. 7B) and roots (Fig. 7C). The *GUS* gene amplicon with a size of 880 bp was amplified in transformed plant's DNA and plasmid DNA (Fig. 7D).

DISCUSSION

Tissue culture technique is recognized as a novel means to generate genetic variability (Larkin and Scowcroft, 1981) and has been proposed as an excitant supplementary technique for plant which can accelerate the breeding programs through the use of new expended genetic variability. In Bangladesh, little systematic work has been reported in tissue culture for improvement of rice. 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. In the present study, immature embryos were used to establish an efficient and reproducible protocol

Fig. 7. Confirmation of *GUS* gene expression (A) Gus expression in transformed callus (B) *GUS* expression in leaf segment of putative plant (C) root segment of putative plant (D) Confirmation of the introduction of *GUS* gene through PCR analysis.

for callus induction, somatic embryogenesis, plantlets regeneration and genetic transformation of rice (*Oryza sativa* L.). The investigation was conducted to establish an efficient and reproducible protocol for callus induction, plantlets regeneration and *Agrobacterium*-mediated genetic transformation in rice (*Oryza sativa* L.). Three rice genotypes, namely BRRI dhan29, BRRI dhan56 and BRRI dhan57 were used in the investigation. These cultivars are high-yielding variety with fine grain quality. So this demandable cultivar has to be investigated for optimal use.

Callus induction

For induction of callus the mature seed explants of three rice genotypes were cultured on MS medium containing different concentrations of 2,4-D and NAA singly or combined to identify and select the growth regulator treatments suitable for maximum induction of callus. Days required to initiation of callus, percentage of callus formation, texture and color of callus and degree of callusing were considered as parameters for evaluating the experiment. Results indicated that of the three varieties, BRRI dhan29 had the maximum regeneration potential followed by BRRI dhan56 and dhan57. Regeneration responses of embryogenic calli derived from mature seeds were influenced with the concentrations and combinations of NAA and KIN present in the regeneration media. Similar results were reported by Pandey *et al.* (1994) and Islam *et al.* (2013) who observed a high regeneration frequency for the medium containing high levels of KIN.

Regeneration

The present findings showed differences in the callus induction frequency and regeneration of shoots among the genotypes. Genotypic variation in callus induction and subsequent plant regeneration potential in rice were reported by many researchers (Rashid *et al.* 2000). For regeneration via callus different combination of auxin and cytokinin were tried. The combinations were BAP + IBA, BAP + NAA, BAP + IAA, KIN + NAA and KIN + IAA. The results from these treatments varied upon with cultivars. The combination of BAP + IBA was the best for regeneration efficiency. The combination of 17.68 µmol/L BAP+8.84 µmol/L NAA + 13.26 µmol/L KIN showed the

highest percentage of plant regeneration. Among the three cultivars, BRRI dhan29 exhibited the best performance edging out the other two varieties. This result was in consistent with the previous reports (Islam *et al.* 2013).

Genetic Transformation

A number of experiments related to this study have been conducted before to optimize the effective use of the experimental tools involved in genetic transformation venture in plants. The ultimate objective of this study was to incorporate reporter gene (*GUS*) in rice plant. BRRI dhan29 was selected as a target variety for transformation of reporter genes. It was revealed from the results of tissue culture experiments that BRRI dhan29, showing the best callus induction and regeneration performance, proved to be the most suitable variety for genetic transformation. The highly regenerative embryogenic rice calli of BRRI dhan29 were co-cultivated with *Agrobacterium* harboring with *GUS* gene. Histochemical assay was performed on the co-cultivated rice calli according to the method described by Jefferson (1987) with some modifications. Following histochemical assay, when the infected calli were incubated with the substrate *X-glue* which was cleaved in *GUS* expressing cells resulting in deposition of a blue indigo dye, a number of blue spots appeared in the body of the rice calli. *GUS* expression indicated that the *GUS* gene harbored in bacterial cells has been transformed and integrated into the genome of rice calli, thereby confirming the gene transformation by *Agrobacterium* co-cultivation.

In finding the most suitable level of OD for the *Agrobacterium* used in this experiment, 5 different levels of OD were tested: 1.2, 1.1, 0.9, 0.8, and 0.6. The highest numbers of *GUS* expressing calli were observed at OD 0.8-0.9. The bacterial strain LBA4404 (pBI 121) showed the highest peak of performance in lower range of OD which gradually decreased in the higher OD. Using *Agrobacterium* at OD of 0.5-0.7 was coincidental with the fact that most *Agrobacterium* stains would be experiencing the early log (exponential) phase during which active cell division occurs at 0.4-0.8 OD.

So far, this is the first extensive investigation ever done in this country both in the field of tissue culture and transformation of Indica rice. Tissue culture protocols were

developed working on large numbers of rice varieties using different tissue explants. Condition for the indirect gene transfer technology by the *Agrobacterium*-mediated gene transfer method was established for rice improvement. Establishment of efficient transformation system developed in this study laid out a fundamental work for the future advancement of gene transfer technology in the country. It opens new horizons for incorporation of target genes for beneficial agronomic characters in rice as well as other commercial agricultural crops.

ACKNOWLEDGMENTS

The authors would like to express gratitude to Professor Joarder, DNA and Chromosome Research Laboratory, Department of Genetic Engineering and Biotechnology, University of Rajshahi, Rajshahi-6205, Bangladesh.

REFERENCES

- Baker BP, Zambryski B, Staskawicz, Dinesh-Kumar SP. 1997. Signaling in plant-microbe interactions. *Science* 27: 726-733.
- BBS. 2005. "Bangladesh Bureau of Statistics" <http://www.bbs.gov.bd>.
- FAO. 2003. "FAOSTAT" <http://www.fao.org>.
- Potrykus I, Datta SK, Datta K. 1990. Fertile *indica* rice plants regenerated from protoplasts isolated from microspore derived cell suspensions. *Plant Cell Rep.* 9: 253-256.
- Hiei Y, Shozo O, Komari T, Kumashiro T. 1994. Efficient transformation of rice (*Oryza sativa* L.) mediated by *Agrobacterium* and sequential analysis of the boundaries of the T-DNA. *Plant J.* 6: 271-282.
- Islam MM, Haque ME, Alam SMM, Islam MA, Khalequzzaman M, Sikdar B. 2013. Morphological and Histological Observation of Embryogenic Calli Derived from Immature Embryo of BRRI Dhan28 (*Oryza sativa* L.) Variety. *Res. Plant Biol.* 3: 21-27.
- Islam MM, Islam MZ, Shaekh MPE, Das P, Chowdhury HK, Shahik SM, Muzahid NH, Khan MA, Ekram AE. 2014. Screening of *Cleome viscosa* (L.) for Dose Mortality, Insect Repellency, Cytotoxicity and Larvicidal Activities in the Laboratory Condition. *Int. J. Sci. Eng. Res.* 5: 2201-2212.
- Jefferson RA. 1987. Assaying chimeric genes in plants: the *GUS* gene fusion system. *Plant Mol. Biol. Rep.* 5: 387-405.
- Khalequzzaman M, Najmul H, Hoque ME, Aditya TL. 2005. Regeneration efficiency and genotypic effect of 15 indica type Bangladeshi rice (*Oryza sativa* L.) landraces. *Plant Tissue Cult.* 15: 33-42.
- Labra M, Savini C, Bracale M, Pelucchi N, Colombo L, Bardini M, Sala F. 2001. Genomic changes in transgenic rice (*Oryza sativa* L.) plants produced by infecting calli with *Agrobacterium tumefaciens*. *Plant Cell Rep.* 20: 325-330.
- Larkin PJ, Scrowcroft WR. 1981. Somaclonal variation a novel source of variability from cell culture for plant improvement. *Theor. Appl. Genet.* 60: 197-214.
- Li LQ, Kochko A, Fauquet C, Benchy R. 1993. Improvement rice transformation system using the biolistic method. *Plant Cell Rep.* 12: 250-255.
- Murashige T, Skoog F. 1962. A revised medium for rapid growth and bioassays with tobacco cultures. *Physiol. Plant.* 15: 473-497.
- Pandey SK, Ramesh B, Gupta PK. 1994. Study on effect on genotype and culture medium on callus formation and plant regeneration in rice (*Oryza sativa* L.). *Indian J. Genet.* 54: 293-299.
- Peng J, Konowicz H, Hodges TK. 1992. Transgenic indica rice plants. *Theor. Appl. Genet.* 3: 855-863.
- Rashid H, Toriyama A, Qurashi K, Hinta. Malik KA. 2000. An improved method for shoot regeneration from calli of indica rice. *Pak. J. Biol. Sci.* 3: 2229-2231.
- Roly ZY, Mahmudul M, Haque ME, Islam MA, Sikdar B. 2013. *In vitro* multiple shoot regeneration from cotyledonary nodes, shoot tips and nodal segments of ridge gourd. *Disc. Biotechnol.* 4: 4-8.
- Shaekh MPE, Mondol A, Islam MM, Kabir AS, Saleh MA, Salah Uddin M, Hoque KMF, Ekram AE. 2013. Isolation, Characterization and Identification of an Antagonistic Bacterium from *Penaeus monodon*. *Int. J. Sci. Eng. Res.* 4: 254-261.
- Toenniessen GH. 1995. The Rockefeller Foundation's international program on rice biotechnology, In: D.W. Altman, K.N. Watanabe (Eds.). *Plant Biotechnology to Developing Countries*, RG Landes Company. 193-212.