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Investigating the *in vitro* regeneration potential of selected local rice cultivars in Bangladesh

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ABSTRACT

The study was conducted to establish a suitable protocol for *in vitro* regeneration of local rice (*Oryza sativa*) cultivars grown in greater Sylhet region in Bangladesh for further genetic improvement through biotechnological manipulation. To figure out the optimum medium for high frequency callus induction and shoot regeneration, dehusked seeds of *O. sativa* cv. Lakhai were cultivated on Murashige and Skoog (MS) medium strengthened with several concentrations of 2,4-Dichlorophenoxy acetic acid (2,4-D), α-Napthalene acetic acid (NAA) and 6-Benzylaminopurine (BA). In response to plant growth regulators, the ranges of callus and shoot induction frequency of cv. Lakhai were 13.33% to 100% and 6.66 to 93.33%, respectively. The highest frequency (100%) of callus initiation was found in MS medium supplemented with 3 mg L^{-1} NAA and 1.0 mg L^{-1} BA and the lowest callus initiation (13.33%) was noticed in MS medium with 1.0 mg L^{-1} NAA. The highest shoot regeneration (93.33%) was found in MS medium comprised with 0.5 mg L^{-1} NAA and 3.0 mg L^{-1} BA. Thereafter, the callus and shoot regeneration potentials of five selected local cultivars including cv. Lakhai were evaluated using the most responsive medium standardized to cultivar Lakhai. The cv. Lakhai showed the greatest shoot regeneration frequency (93.33%) and the lowest frequency (53.33 %) was observed in cv. Maloti. MS media fortified with 0.1-0.2 mg L^{-1} NAA gave the maximum frequency (100%) for root initiation. When the plantlets were grown enough with root, they were acclimatized in pot soil and grown up to maturity stage in field conditions. Thus, the developed protocol for callus induction and regeneration of rice plantlet can be implemented for various biotechnological practices related to genetic improvement of Bangladeshi local rice cultivars.

Keywords: Plant growth regulators, tissue culture, local rice, organogenesis, genotype

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1 Introduction

Rice (*Oryza sativa* L.) is the staple food for more than two billion people in Asia (Sasaki, 2005; Hien et al., 2007; Hadiarto and Tran, 2011), belongs to the Poaceae family and it is the second most widely cultivated cereal in the world, after wheat (Pazuki and Sohani, 2013; Sandhu and Kumar, 2017). About 90% of rice is grown and consumed in south and southeast Asia (Rumana, 2017). Bangladesh ranks fourth in both area coverage by rice production and total rice production and also ranks 6th position in the rice production of per hectare in the world (Jahan et al., 2018). Mainly 2 types of rice cultivars are cultivated in Bangladesh such as local rice and modern high-yielding varieties (MVs). Sylhet, the eastern part of Bangladesh, is very rich in local rice cultivars because of its diversified agro-ecological environment providing suitable environment for growing rice cultivars with wide genetic variations (Jahan et al., 2018).

Though plenty of local rice cultivars are available in greater Sylhet region, still farmers are facing obstacles for cultivating due to poor yielding potentials of these local rice cultivars.

To meet the ever-increasing demand of rice in Bangladesh for food security, the MVs and hybrid rice varieties cover most of the overall cultivable area of our country (Rumana, 2017). Therefore, our local cultivars were given less attention to improve their yield capability. In spite of the introduction of many MVs, some local varieties are still famous in farmers field due to their particular qualities like good taste, resistantcy to pest and diseases, adaptation capacity to local climatic condition, requirement of lesser amount of fertilizers and water etc. In addition, local rice cultivars have been playing a vital role as parent material because they are most adaptive to our environment. Till date 270 local rice varieties were used as parents in hybridization program of Bangladesh Rice Research Institute (BRRI) and 12 varieties were released by utilizing them as one of the parent (BRRI, 2019).

Low productivity of local rice associated with numerous traits may be improved using conventional and modern approaches (such as tissue culture and genetic engineering) by manipulating genes of interest. There are many conventional breeding methods available for improving rice varieties, but these methods need long time. To develop an upgraded rice variety, different *in vitro* methods are known to be more efficient methods than conventional methods (Mostafiz and Wagiran, 2018; Repalli et al., 2019). However, the main obstacle for breeding improvement process of local rice is the shortage of proper tissue culture protocol. Previous studies showed the result that the *indica* rice recalcitrance had lower callusing and regeneration capabilities in comparison with *japonica* subspecies in several *in vitro* tissue culture conditions (Yaqoob et al., 2016). Eventually, within *indica* subspecies, a significant variation was also found with *in vitro* culture response in different genotypes (Repalli et al., 2019). Several attempts were made throughout the years to develop tissue culture protocols for callus and shoot induction of rice by employing leaf culture (Boissot et al., 1990), anther culture (Faruque et al., 1998; Asaduzzaman et al., 2003), root culture (John and Prathapasenan, 1999), protoplast culture (Li and Murai, 1990) and dehusked grain culture (Ella and Zapata, 1991; Rudra et al., 2013; Mostafiz and Wagiran, 2018; Repalli et al., 2019) to develop novel rice varieties by using somaclonal variation. Although a few reports were found on *in vitro* regeneration of local rice grown in Bangladesh (Jubair et al., 2008; Rudra et al., 2013; Chakraborty et al., 2018), but shoot regeneration frequencies were lower. Therefore, the objective of the present study was to evaluate the *in vitro* regeneration potentials of some selected local rice varieties and to find out the most appropriate concentration and combinations of plant growth regulators for callus and shoot induction important for gene transformation technique to develop high yielding variety.

2 Materials and Methods

2.1 Plant material and experimental site

The experiments were conducted at the Tissue Culture Laboratory of the Department of Genetics and Plant Breeding, Sylhet Agricultural University, Sylhet, Bangladesh. Seeds of five local rice cultivars *viz.*, Lakhai, Maloti, Muktasail, Nagrasail and Rata were collected from greater Sylhet region of Bangladesh. To standardize the plant regeneration protocol of rice, cv. Lakhai was used and other cultivars were utilized to observe their plantlet regeneration capability. After dehusking, the seeds of five local rice cultivars were surface sterilized in laminar airflow cabinet as follows. Seeds were washed for 3 times with sterilized distilled water (SDW). Then the seeds were washed with 70% ethanol (MERCK, Germany) for two minutes with continuous shaking and then washed with SDW for 2 times. After that seeds were washed with 10% Chlorox (Sodium hypochlorite, The Clorox Company, Oakland, USA) solution during ten minutes with gentle-shaking and rinsed with SDW for 3 times. Then the seeds were placed in sterilized Petridish containing filter paper to dry.

2.2 Culture of explant

The sterilized seeds were used as explant for regeneration and placed on callus induction media (Fig. 1a). This process was done for each variety and the entire procedure was executed in laminar airflow cabinet. The callus and shoot induction media contained MS (Murashige and Skoog, 1962) salts and vitamins supplemented with various concentrations of 2,4-D (96%, Duchefa Biochemie, the Netherlands), NAA (98%, Duchefa Biochemie, the Netherlands) and BA (99%, Duchefa Biochemie, the Netherlands), 30 g L^{-1} sucrose and 10 g L^{-1} Phytoagar (Tables 1 and 2). Before autoclaving pH of all media were adjusted to 5.8. Matured calli were transferred to shoot regeneration media. The regenerated shoots were excised carefully and transferred to rooting media (MS, half-strength MS and MS + 0.1-0.4 mg L^{-1} NAA). Then the shoots (3-4 cm in height) with enough roots were taken out from the culture vessel and roughly washed by running tap water for removing all hanger-on culture medium. After that the plantlets with well-established root were transferred to the pot soil for hardening. Pots having garden soil and cow dung in 2:1 ratio were taken into plastic containers for growing the regenerated plantlets at *in vivo* condition. Then, the pots having the rice plantlets were coated

immediately by glassware (beaker) to avoid evapotranspiration. To lower the environmental hazards, the pots were put into the controlled environment condition. After ten to twelve days, the rice plantlets were shifted to field conditions until maturity.

2.3 Statistical analysis

The experiment was arranged in Completely Randomized Design (CRD) with 3 replications. Frequency of callus initiation was estimated by dividing the explants number that produced callus by the total explants number. Frequency of shoot induction was determined by dividing the calli number that produced shoot buds by the total number of calli. The percentage of rooting percentage was estimated by dividing the elongated shoots number that regenerated roots under *in vitro* condition by the total elongated shoots number which were utilized for root induction. MS Excel 2010 was used to calculate the mean and standard deviation for all the treatments. Statistical analysis of the data was carried out using R analysis software (R Core Team, 2013) and significance of differences among means was evaluated by Dunkan's multiple range test (DMRT) at $P \leq 0.05$.

3 Results and Discussion

3.1 Callus induction

In the face of great importance of local rice, little information is available on the callus induction and plant regeneration method through *in vitro* culture. The present study was aimed to investigate the effect of concentrations and combinations of plant growth regulators (2,4-D, NAA and BA) on callus and shoot induction of some selected local rice grown in greater Sylhet region of Bangladesh. Plant growth regulators (PGRs) affect organogenesis response by altering various physiological processes. Callus can be cultured on both MS and N6 media (Rashid et al., 2004) and the variation in auxin and cytokinin composition can result in difference in callus induction (Chakraborty et al., 2018; Mostafiz and Wagiran, 2018). For all rice cultivars, MS media acted as proper and basic media for callus induction (Niroula et al., 2005), therefore, we used MS media supplemented with various concentrations of 2,4-D (1-4 mg L^{-1}), NAA (1-4 mg L^{-1}) and BA (1-4 mg L^{-1}). A total 24 combinations were formulated and then tested to determine suitable callus induction medium for *O. sativa* cv. Lakhai. When seeds were cultured in hormone free MS basal medium, no callus was produced and died after some days (data not shown). Previous studies showed that callus can be induced in rice using 2,4-D singly (Katiyar et al., 1999; Libin, 2012; Chakraborty et al., 2018). Therefore, in addition with NAA and BA combination we used 2,4-D and NAA singly at different concentration in MS media. Among the tested combinations, the highest frequency of callus induction (100%) of cv. Lakhai was seen in MS medium fortified with 3 mg L^{-1} NAA and 1 mg L^{-1} BA (Fig. 1b) and the least was found in MS added with 1 mg L^{-1} NAA (Table 1). Both 2,4-D and NAA singly produced a good number of light-yellowish calli, but they were fragile and produced shoot buds at low rate compared to the NAA plus BA (Table 1). This result of callus induction is consistent with the previous studies (Katiyar et al., 1999; Libin, 2012; Mannan et al., 2013). NAA combined with BA produced good quality light-greenish compact calli which further developed shoot buds in shoot induction media (Fig. 1c). The callus initiation frequency increased with the increase of NAA concentration up to 3 mg L^{-1} and further increase of BA concentration decreased the callus initiation frequency when NAA concentration remain same.

3.2 Shoot induction

To find out the appropriate shoot induction medium, matured calli (21-day-old) were transferred into shoot induction media which comprised of MS media fortified with several concentrations of 2,4-D, NAA, and NAA + BA (Table 2). From 16 different combinations of PGRs tested in *O sativa* cv. Lakhai, MS medium strengthened with 0.5 mg L^{-1} NAA and 3 mg L^{-1} BA gave the highest 93.33% shoot regeneration (Fig. 1d) while the lowest (6.66%) frequency of shoot regeneration was observed in MS medium with 2 mg L^{-1} NAA (Table 2). It was observed that when BA concentration increased up to 3 mg L^{-1} along with same NAA concentration (0.5 mg L^{-1}) showed the higher shoot regeneration frequency but further increased of NAA above 0.5 mg L^{-1} concentration decreased the shoot regeneration frequency significantly (Table 2). A significantly lower frequency of shoot regeneration was observed in media with 2,4-D or NAA alone. Combinations of NAA with BA produced significantly higher shoot regeneration frequency in cv. Lakhai. In previous studies, 0.5 mg L^{-1} NAA along with variable concentrations of BA was found to be the suitable combinations for high frequency shoot regeneration of rice which is compliant with the present study.

3.3 Root initiation

The regenerated shoots (3-4 cm) were excised and cultured into rooting media for root induction. The shoots started root formation within 4 days (Fig. 1e) and the frequency of root formation was 100% in MS or MS medium with 0.1 mg L^{-1} NAA with shortest period of time and the minimum (26.66%) was found in ½ strength MS medium (Fig. 2). The results indicated that the concentration of inorganic

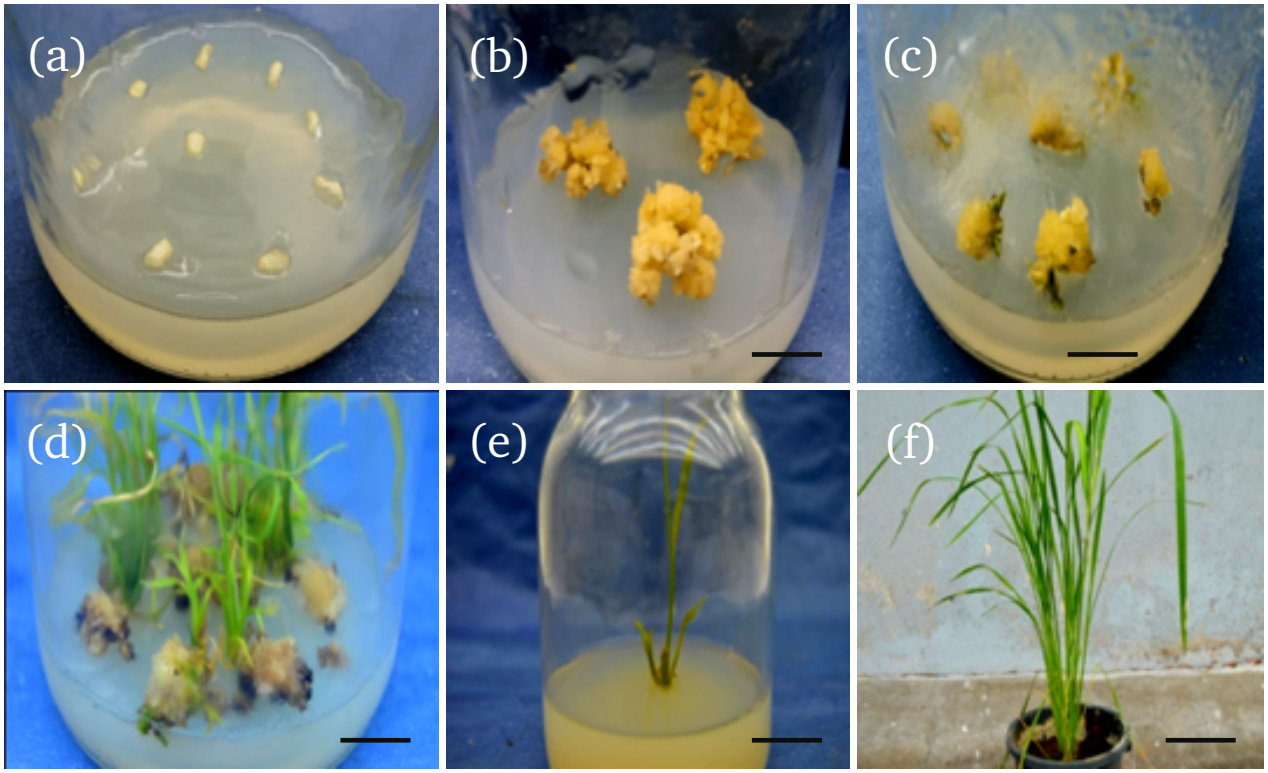

Figure 1. Plant regeneration of *Oryza sativa* cv. Lakhai. (a) Sterilized seeds on suitable callus induction medium (MS + 3 mg L^{-1} NAA + 3 mg L^{-1} BA) on the first day of culture, (b) Callus initiation at 21 days of culture on callus induction medium, (c) Shoot initiation from callus after 10 days of culture on shoot induction medium (MS + 0.5 mg L^{-1} NAA + 3.0 mg L^{-1} BA), (d) Shoot elongation and multiplication on shoot induction medium after 21 days of culture, (e) Root initiation of regenerated shoots on root induction medium (MS + 0.1 mg L^{-1} NAA), and (f) Growth of plant in pot soil at 60 days in natural condition. Scale bars indicate 1 cm (b–d), 2 cm (e), and 10 cm (f).

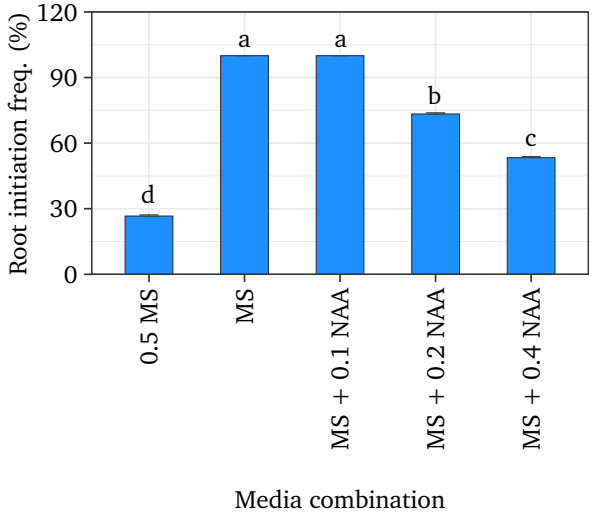

Figure 2. Influences of MS and NAA conc. on rooting of regenerated shoots of *O. sativa* cv. Lakhai genotype. Values are mean of three replicated data. Five regenerated plants were used in each replication. Vertical bar indicates standard deviation of mean. Columns with different letters designate significantly different at P = 0.05.

Table 1. Callus initiation of *O. sativa* cv. Lakhai on MS media supplemented with various concentrations of 2,4-D, NAA and BA †

Conc. of plant growth regulators (mg L^{-1})			Callus initiation freq. (%)	Nature of callus
2,4-D	NAA	BA		
1.0	–	–	36.66±1.15 de	Light-yellowish, fragile
2.0	–	–	43.33±0.58 d	Light-yellowish, fragile
3.0	–	–	66.66±0.58 c	Light-yellowish, fragile
4.0	–	–	86.66±0.58 b	Light-yellowish, fragile
–	1.0	–	13.33±0.58 h	Light-yellowish, fragile
–	2.0	–	23.33±0.58 fgh	Light-yellowish, fragile
–	3.0	–	26.66±0.58 efg	Light-yellowish, fragile
–	4.0	–	26.66±0.58 efg	Light-yellowish, fragile
–	1.0	1.0	30.00±1.00 efg	Greenish, compact
–	1.0	2.0	43.33±0.58 d	Greenish, compact
–	1.0	3.0	43.33±0.58 d	Greenish, compact
–	1.0	4.0	26.66±0.58 efg	Greenish, compact
–	2.0	1.0	36.66±1.15 de	Greenish, compact
–	2.0	2.0	36.66±0.58 de	Greenish, compact
–	2.0	3.0	53.00±0.58 cd	Greenish, compact
–	2.0	4.0	20.00±1.00 gh	Greenish, compact
–	3.0	1.0	100±0.00 a	Greenish, compact
–	3.0	2.0	93.33±0.58 ab	Greenish, compact
–	3.0	3.0	86.66±0.58 b	Greenish, compact
–	3.0	4.0	86.66±0.58 b	Greenish, compact
–	4.0	1.0	83.33±0.58 b	Greenish, compact
–	4.0	2.0	66.66±0.58 c	Greenish, compact
–	4.0	3.0	53.00±0.58 cd	Greenish, compact
–	4.0	4.0	33.33±0.58 def	Greenish, compact

† Data consist of three replications and 10 explants were used for each replication. The mean values were compared by DMRT. Mean ± SD followed by same letters are not significantly different at P = 0.05.

salts and NAA in the media plays an important role in root induction of regenerated shoots of cv. Lakhai. However, increased concentration of NAA (greater than 0.1 mg L^{-1}) dramatically decreased the *in vitro* rooting frequency (Fig. 2).

3.4 Establishment of plantlets

Regenerated shoots having sufficient amount roots were taken out from the culture vessels without disturbing roots. To avoid microbial infection, surplus agar around the plantlet roots was removed by washing with running tap water. Healthy well-developed rooted plantlets were transplanted into plastic pot having soil, sand and cow dung in 1:2:3 ratio. The plants were covered by glassware (beaker) for five days to maintain humidity. Then the plants were placed in acclimatization room and irrigated every alternate day with tap water. The success rate of this transfer was 100%. Panicle initiation was observed after 95 days in cv. Lakhai (Fig. 1f) and no difference was observed in the morphology of these regenerated plants compared with seed-derived control plants.

3.5 Genotypic variation

The dehusked seeds of five genotypes of local rice *viz*. Lakhai, Maloti, Muktasail, Nagrasail and Rata were placed on suitable callus induction medium (MS + 3 mg L^{-1} NAA + 1 mg L^{-1} BA). Callus initiation started around 7 days in all the genotypes and after 21 days, callus initiation frequency was 100%, 73.33%, 83.33%, 80% and 70% in Lakhai, Maloti, Muktasail, Nagrasail and Rata, respectively (Fig. 3). From the above result, cv. Lakhai showed the highest (100%) and cv. Rata showed the lowest (70%) callus initiation frequency (Fig. 3). Then the calli were transferred to the perfect shoot regeneration medium (MS + 0.5 mg L^{-1} NAA + 3 mg L^{-1} BA) to observe the frequency of shoot regeneration which was 93.33%, 53.33%, 73.33%, 76.66% and 56.66% in Lakhai, Maloti, Muktasail Nagrasail and Rata respectively (Fig. 3). It is clear that the callus and shoot induction capacity of local rice cultivars are significantly affected by genotype. In previous studies, *in vitro* regeneration potential varied among the rice genotypes (Hoque and Mansfield, 2004; Biswas and Mandal, 2007) which is in line with the present study and indicated that

Table 2. Shoot induction of O. sativa cv. Lakhai on MS media supplemented with various concentrations of 2,4-D, NAA and BA †

Conc. of plant growth regulators (mg L^{-1})			Callus initiation freq. (%)
2,4-D	NAA	BA	
1.0	–	–	13.33±0.58 def
2.0	–	–	20.00±1.00 def
3.0	–	–	33.33±1.15 cde
4.0	–	–	13.33±0.58 def
–	1.0	–	20.00±1.00 def
–	2.0	–	6.66±0.98 ef
–	3.0	–	33.33±1.15 cde
–	4.0	–	20.00±1.00 def
–	0.5	1.0	53.00±0.58 bc
–	0.5	2.0	66.66±0.58 ab
–	0.5	3.0	93.33±0.58 a
–	0.5	4.0	66.66±0.58 ab
–	1.0	1.0	33.33±1.15 cde
–	1.0	2.0	40.00±1.00 bcd
–	1.0	3.0	66.66±0.58 ab
–	1.0	4.0	46.66±1.15 bcd

† 21-d-old calli were cultured on MS media supplemented with various concentrations of 2,4-D, NAA and BA. Data consist of three replications and 10 explants were used for each replication. Mean ± SD followed by same letters are not significantly different at P = 0.05.

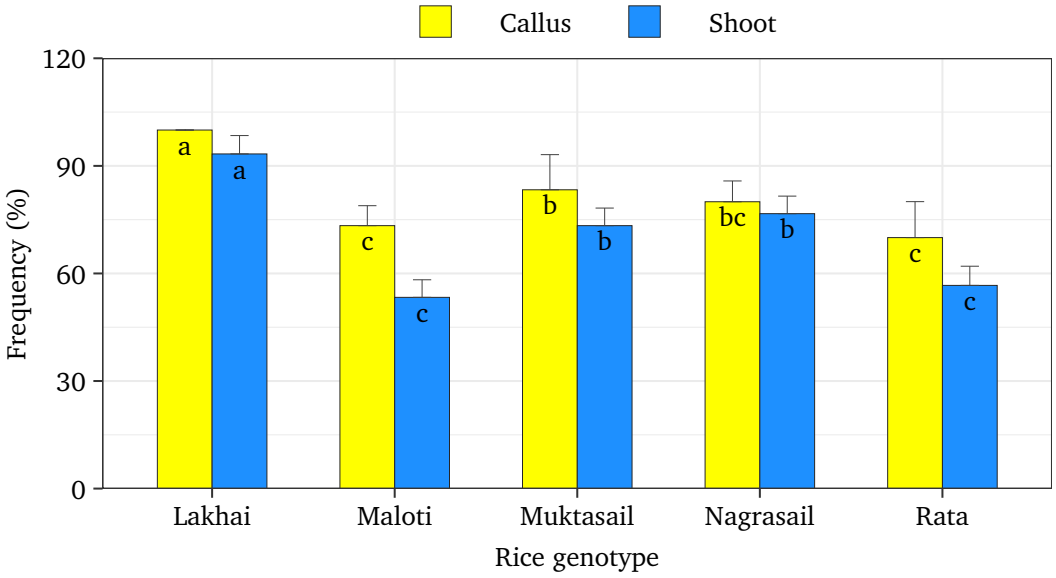

Figure 3. Influences of genotype on callus and shoot initiation of five genotypes of local varieties grown in Sylhet region of Bangladesh. Values are mean of three replicated data. Ten regenerated plants were used in each replication. Vertical bar indicates standard deviation of mean. Columns with different letters designate significantly different at P = 0.05.

regeneration is genetically controlled trait and may show different behavior. It is clear that the genotype has strong influence on the regeneration response of local rice, ranging from 53.33 to 93.33%. *In vitro* regeneration potential varied among the rice genotypes (Hoque and Mansfield, 2004; Carsono and Yoshida, 2006; Biswas and Mandal, 2007) which is in line with the present study and indicated that regeneration is genetically controlled trait and may show different behavior.

4 Conclusions

The present study was done to develop an efficient protocol for *in vitro* plant regeneration of local rice cv. Lakhai as well as to evaluate the regeneration potentials of others cultivars. The best medium for callus induction was MS medium added with 3 mg L^{-1} NAA and 1 mg L^{-1} BA. MS medium enriched with 0.5 mg L^{-1} NAA and 3 mg L^{-1} BA acted as perfect medium for high frequency shoot regeneration of local rice cv. Lakhai. The satisfactory medium for root initiation of regenerated shoots developed in tissue culture of local rice was only MS medium or MS medium enriched with 0.1 mg L^{-1} NAA. From the economic point of view, only MS medium is enough for sufficient root formation of cv. Lakhai. Among the five local genotypes, Lakhai showed the highest frequency both in callus (100%) and shoot (93.33%) regeneration while Maloti showed the lowest shoot regeneration (53.33%) frequency. The study revealed that callus induction and shoot regeneration frequency were determined by medium combinations as well as genotype of plant. Thus, this protocol established in the study will assist to upgrade the local rice variety into high yielding one by bringing some genetic manipulations through innovative method.

Conflict of Interest

The authors declare that there is no conflict of interests regarding the publication of this paper.

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