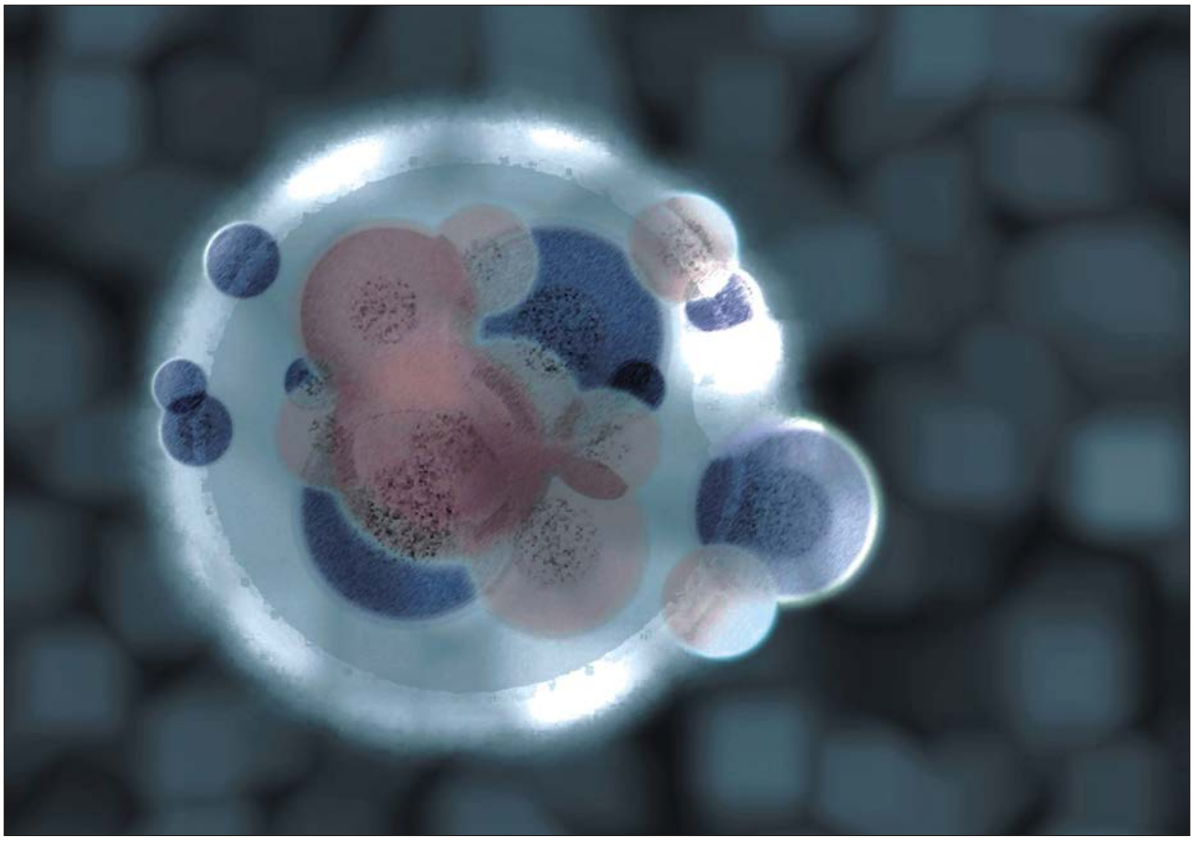

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Research Article
Effect of Plant Growth Regulators and Proline in Efficient Regeneration of Recalcitrant Indica Rice (*Oryza sativa* L.)

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Abstract

Background and Objective: Indica rice genotypes are recalcitrant in nature to *in vitro* callus initiation and regeneration which causes less amenable to genetic modification in comparison with japonica rice. The present study was aimed to establish suitable callus initiation and regeneration system for 4 Indica rice cultivars i.e., Swarna, Kasalath, Lonabalam and Biron. **Methodology:** Murashige and Skoog (MS) media supplemented with different concentrations (0.0, 0.5, 1.0, 1.5, 2, 2.5, 3.0 mg L^{-1}) of 2,4-dichlorophenoxyacetic acid (2,4-D) to prepare callus induction media (CIM) for callus initiation. In addition, L-proline in combination with 6-benzylaminopurine and 2,4-Dichlorophenoxyacetic acid (2,4-D), also implemented to investigate callus formation due to use amino acid as a supplement. Afterward, MS media fortified with different shoot and root inducing hormone at different concentration for regeneration purpose. **Results:** The results showed that Kasalath has highest callus induction frequency 76.47±2.12% at 1.0 mg L^{-1} 2,4-D concentration whereas Swarna, Lonabalam and Biron callus induction frequency were 67.87±2.08 , 36.84±0.70 and 63.15±1.41% at 2.5, 2.0 and 1.0 mg L^{-1} 2,4-D concentration, respectively. Addition of proline showed better callus induction frequency for Kasalath cultivar which is 86.66%. After subculture of primary calli, callus was inoculated into shoot induction media (SIM) and highest shoot induction frequency was observed in Lonabalam, 63.63±1.52% at 0.5 mg L^{-1} NAA, 2.0 mg L^{-1} BA, 3.0 mg L^{-1} KIN hormonal concentration. Duration of Shoot initiation was observed at 32 days, 27 days, 24 days and 33 days' interval for Swarna, Kasalath, Lonabalam and Biron. Regenerated plantlets were then inoculated into rooting media for root induction. After root formation plantlets were transferred to the pots for acclimatization. **Conclusion:** Among different Indica rice, Swarna and Kasalath showed better callus frequency whereas Lonabalam was found better responsive to regeneration media. Besides conventional hormonal effect, L-proline has been proved effective only for Kasalath genotype for callus formulation.

Key words: Recalcitrant, callus initiation, regeneration, 2,4-D, NAA, KIN, L-proline

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Asian rice (*Oryza sativa* L.) is considered one of the oldest and most important crops which is believed to be domesticated ~9000 years ago, though the contentious debate on its origin remains[1,2]. Genetic analysis has established that *Oryza sativa* is genetically differentiated into several groups, with two key groups are indica and japonica[3]. Despite these 2 major groups, large-scale studies also advocate that *O. sativa* is comprised of 5 major subpopulations[4]: Aus, indica, temperate japonica, tropical japonica and aromatic rice. Aus and indica have closer genetic kinships whereas temperate and tropical japonica is closely related to each other[4]. Half of the world population depends on rice as a staple or dietary food source[5].

Rice cultivation is the principal source of income for millions of people around the globe, especially for people living in Asia and Africa. East Asia, Southeast Asia, South and Southwest Asia are well known as a rice-producing country. Rice yield had been grown 1.8% in Asia between 2000 and 2011. Central Asia made the fastest annual yield growth progress with 4.7% . Annual growth of rice in Southeast Asia is going robustly with 2.7% whereas in South and Southwest Asia it is 2.1% and only 0.6% in East Asia[6]. So far, rice production is almost 90% and consumption rate of 87% in Asia. Besides, rice provides 30% of the calorie demands for more than 3 billion Asians. Nearly 45-70% of the calorie requirements for rice-consuming countries like Bangladesh, Cambodia, Indonesia and Vietnam. Despite the achievement so far, the human population is expected to be 8 billion by the year 2020 and almost 795 million people will people still suffering from malnutrition all over the world[7].

Poverty, food insecurity and climate change are 3 major prime global challenge which was considered for 2030 agenda for sustainable development goals by the United Nations[8]. Nevertheless, the interaction between climate change and food insecurity, more precisely agricultural production are well emphasized[9,10]. Most of the rice-producing areas, more specifically Asian and African countries, suffering from hunger, malnutrition and extreme poverty till date. For that reason, the global rice production must be ameliorating, in fact, doubled by 2030 to cope with the impending population demand. Although in the last four decades rice production has increased three-fold, the growth rate of rice yield is far below than the expected demand[11]. Moreover, climate change impact will exacerbate rice production which is about 12-14% decline by 2050 compared to the 2000 production baseline[12]. Different abiotic stresses like salinity, drought and flood harness rice yield in Asian countries. Bangladesh is prone to be among vulnerable countries in the world to saline soils[13]. In addition, among 30% cultivable coastal area, 53% are affected by salinity[14]. Furthermore, drought, flood and high temperature made a precarious region for Bangladesh to ensure food security[15]. Nevertheless, we not only practice the conventional method but also different advanced biotechnological approaches to culminate food security agenda.

Tissue culture-based conventional approach is a prerequisite for Agrobacterium-based gene transformation method. *In vitro* regeneration is achieved by treating plant parts with the different synthetic hormone, resembling natural plant producing hormone. Exogenous implementation of auxin and cytokinin hormone induce regeneration in various plants. Intermediate ratio of auxin and cytokinin induces callus formation whereas a high rate of auxin to cytokinin induces root and shoot regeneration, respectively. Since the revolutionary implication of exogenous hormone, numerous regeneration protocol has been established for economically important crops[16]. Another hormone such as brassinosteroids or abscisic acid also accountable but auxin and cytokinin have been extensively used[17,18]. Generally, callus induction media overexpressed Lateral Organ Boundaries Domain (LDB) 16, LDB 17, LDB 18 and LDB 29 transcription factors which are sufficient for callus formation in *Arabidopsis*[19]. The callus is the primary requirements for successful gene transformation. So, the protocol development of rice cultivar is needed for generating abiotic stress-tolerant crops.

The objectives of this study were to establish an efficient callus and regeneration system for some indica rice cultivars widely cultivated in Bangladesh. Therefore, we implied different plant growth hormone to check which suited best for callus and regeneration system. Besides, amino acid effectivity also determined with hormonal concentration.

MATERIALS AND METHODS

Plant materials: This research work was conducted at the Plant Genetic Engineering Lab, Department of Genetic Engineering and Biotechnology, Shahjalal University of Science and Technology, Sylhet between September, 2016 and June, 2018.

Mature dry seeds of four Bangladeshi indica rice cultivars (Swarna, Kasalath, Lonabalam, Biron) were used in this experiment. Among the four seeds Swarna, Kasalath and Lonabalam were collected from Bangladesh Rice Research Institute (BRRI), Gazipur, Bangladesh. Biron was obtained from Sunamganj region of Sylhet district. Before storing, seeds viability had been checked by the germination test.

Sterilization procedure: All seeds were dehulled by removing the hull from the scutellar region of the embryo. Dehulled seeds were surface sterilized with 70% ethanol (v/v) for 2 min; followed by 10 min in 0.1% (v/v) $HgCl_2$ with the addition of two drops of Tween 20. Finally, the seeds rinsed 5-6 times with gentle shaking and dried on sterile filter paper.

Callus induction media: Callus induction media composed of Murashige and Skoog (MS) basal salts and vitamins were supplemented with 30 g L^{-1} sucrose and different concentration of 2,4-dichlorophenoxyacetic acid (0.5, 1, 1.5, 2, 2.5 and 3 mg L^{-1}). The medium pH was adjusted to 5.8 and 7 g L^{-1} agar was added to each medium as a solidifying agent. Different concentrations of 2,4-D named as CIM-1, CIM-2, CIM-3, CIM-4, CIM-5 and CIM-6. CIM-0 was hormone-free media and used as control for callus initiation. For callus initiation, single seeds were inoculated per test tube. After plating sterilized seed in callus media, the cultures were incubated in the culture room at 25±3°C to white fluorescent light under 16 h photoperiods. Callus induction frequency was calculated according to the following equation:

$$\text{Callus induction frequency (\%)} = \frac{\text{No. of seeds produced calli}}{\text{No. of seeds cultured}} \times 100$$

Modified callus media: Modified callus media was designed to further analysis of amino acid and other hormone effects in combination with 2,4-D on callus initiation. Murashige and Skoog (MS) basal salts and vitamins were fortified with 30 g L^{-1} sucrose and different concentration of 2,4-D, 6-benzylaminopurine and L-proline (Table 1). As L-proline was previously reported callus initiation factor for indica rice cultivar[20,21]. Furthermore, the pH of the medium was adjusted to 5.8 and 7 g L^{-1} agar was used as a solidifying agent.

Regeneration media: For shoot initiation, shoot induction media was prepared. Shoot induction media consisted of Murashige and Skoog (MS) basal salts and vitamins, fortified with 30 g L^{-1} sucrose and different concentration of 1-Naphthaleneacetic acid (NAA), 6-Benzyladenine (BA) and Kinetin (KIN) (Table 2). The pH of the medium was adjusted 5.8 and agar used as solidifying agent of shooting medium. SIM-0 was used as hormone-free control media. Shoot induction frequency was calculated according to the following equation:

$$\text{Shoot induction frequency (\%)} = \frac{\text{No. of callus produced shoot}}{\text{No. of callus cultured}} \times 100$$

Table 1: Modified callus media composition of hormone and amino acid

Medium number	2,4-D (mg L^{-1})	BAP (mg L^{-1})	L-proline (mg L^{-1})
MCM-1	0.5	0.5	500
MCM-2	1.0	0.5	500
MCM-3	2.0	0.5	500
MCM-4	3.0	0.5	500
MCM-5	1.0	-	500
MCM-6	1.5	-	500

Table 2: Hormone composition of shoot induction media

Media number	NAA (mg L^{-1})	BA (mg L^{-1})	KIN (mg L^{-1})
SIM-0	0	0	0
SIM-1	0.2	2	0
SIM-2	0.2	0	2
SIM-3	0.5	2	1
SIM-4	0.5	1	2
SIM-5	0.5	2	3
SIM-6	1.0	2	2
SIM-7	1.0	2	1
SIM-8	1.0	1	2

Rooting media was prepared by half strength of MS salts in addition with 30 g L^{-1} sucrose. Medium pH was adjusted to 5.8 and 7 g L^{-1} agar mixed with media as a supporting agent.

Statistical analysis: The collected data were statistically analyzed by Microsoft Excel 2016. The data were evaluated to one-way analysis of variance (ANOVA) and significance difference at $p<0.05$ analyzed according to Tukey's HSD test. Data represented in figures are mean±standard deviations (SD) of replication.

RESULTS

Experiments were conducted on 4 rice cultivars Swarna, Kasalath, Lonabalam and Biron for callus induction and plant regeneration.

Optimization of the 2,4-D hormone on callus initiation: Four rice genotype showed the variable result in case of callus formation, hormone concentration and visible time of callus. Callus induction rate is known to depend on 2,4-D concentration and therefore evaluated on 5 different 2,4-D concentration and a hormone-free control. Among 4 varieties, callus induction frequency of Swarna was highest at CIM-5 (2.5 mg L^{-1}). At 2.5 mg L^{-1} concentration, frequency was 67.87% (Fig. 1a). On the other hand, Kasalath genotype formed the highest callus (76.47%) at CIM-2 (1 mg L^{-1}) media (Fig. 1b). Lonabalam callus frequency was low at MS fortified different 2,4-D concentration. The highest frequency of 36.84% observed at CIM-4 (2 mg L^{-1}) media (Fig. 1c). Biron callus induction frequency was 63.15% at CIM-2 (1 mg L^{-1}) media and gradually reduce as the increase of hormone concentration (Fig. 1d).

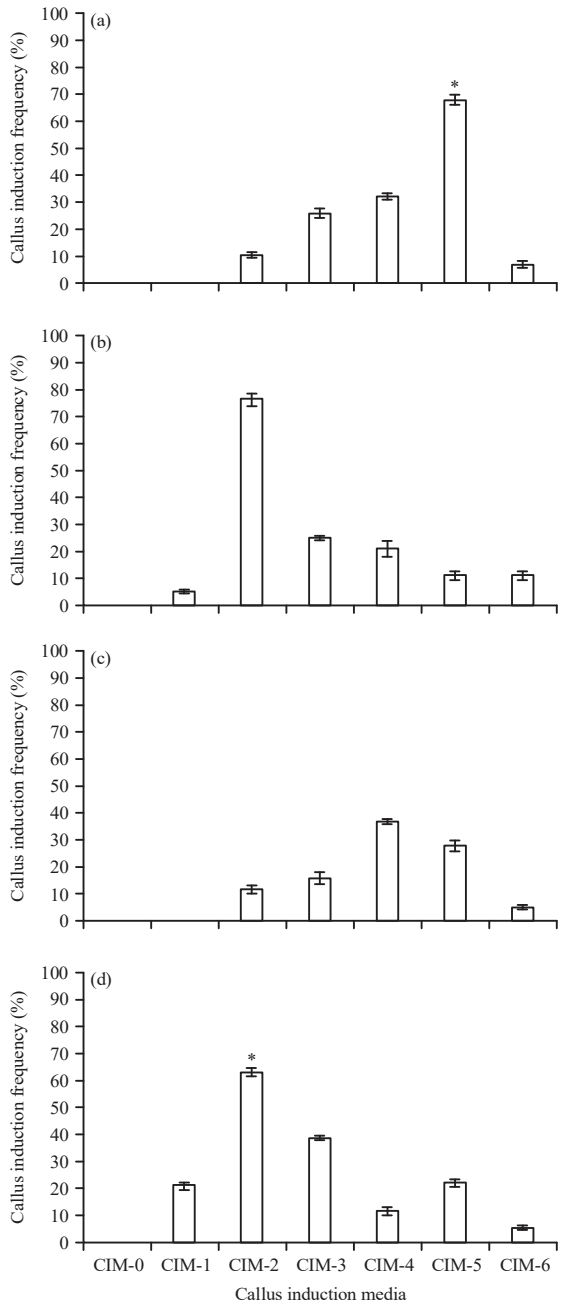

Fig. 1(a-d): Callus induction frequency (%) of four rice cultivars, (a) Swarna, (b) Kasalath, (c) Lonabalam and (d) Biron

*5% significance level according to Tukey's HSD

Callus mass on the surface of Swarna, Kasalath, Lonabalam and Biron seeds appeared within 2 weeks (Fig. 2b, e, h and k) and full callus formation observed within four weeks of incubation (Fig. 2c, f, i and l). Nevertheless, no callus observed on hormone-free media after 4 weeks of incubation (Fig. 2a, d, g and j).

Effects of proline and BAP on callus formation: The effects of proline and BAP to callus formation from mature seeds were investigated. Different modified callus media prepared in combination with 2,4-D for analyzing the effectivity of proline and BAP. Among 4 cultivars, the only kasalath showed a significant response on different modified media. Other rice genotype showed recalcitrant nature almost all different types of hormonal combination. Kasalath showed highest 86.66% callus induction frequency on MCM-6 (1.5 mg L^{-1} 2,4-D and 500 mg L^{-1} proline) combination. Callus induction frequency varied significantly in response to hormonal and proline combination (Fig. 3). In addition, callus size was vigorous, whitish and friable in modified callus media (Fig. 4).

Effect of plant growth regulators on shoot regeneration from callus: Different combination of cytokinin hormone was applied to induce shoot from callus. Swarna rice genotype reported highest shoot formation 41.66% at SIM-6 (1 mg L^{-1} NAA, 2 mg L^{-1} BA and 2 mg L^{-1} kinetin) composition (Fig. 5a). Kasalath shoot formation was better at different hormonal combination. At SIM-5 (0.5 mg L^{-1} NAA, 2 mg L^{-1} BA and 3 mg L^{-1} Kinetin) composition, highest 46.15% shoot initiation was observed (Fig. 5b). Similarly, Lonabalam reported the highest shoot frequency at SIM-5 composition. The shoot induction frequency was 63.63% at this composition (Fig. 5c). On the other hand, Biron cultivar has shown highest shoot formation frequency (35.72%) at SIM-4 media (Fig. 5d).

After the formation of callus, mature callus plated into shoot initiation media. Callus was observed greenish spot within 1 week of incubation period for all 4 cultivars. Average time required for complete regeneration was 32, 27, 24 and 33 days for Swarna, Kasalath, Lonabalam and Biron cultivars, respectively (Fig. 6).

Root formation on half-strength MS media: For root formation, subsequent shoot generated callus transferred into half-strength MS media. Swarna, Kasalath, Lonabalam and Biron root frequency was 30, 40, 70 and 70%, respectively (Table 3). Roots generally appeared within two weeks of incubation and at initial stage whitish in color (Fig. 7). After root formation, regenerated plantlets transferred into soil contained pot for to check their survivability on natural media.

Fig. 2(a-l): Callus proliferation stage on hormone free and hormonal media, (a, d, g, j) Swarna, Kasalath, Lonabalam and Biron genotype on hormone free media, (b, e, h, k) Formation of callus mass within two week and (c, f, I, l) Callus formation of Swarna, Kasalath, Lonabalam and Biron on hormonal media

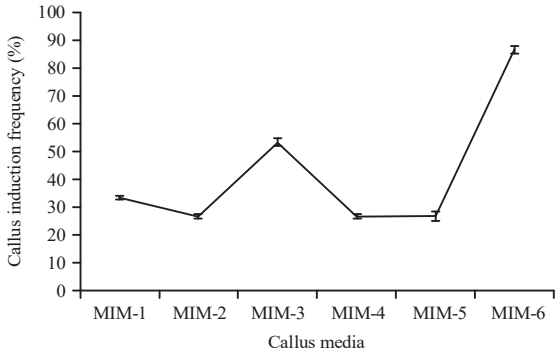

Fig. 3: Callus induction frequency of Kasalath rice cultivars on modified callus medium

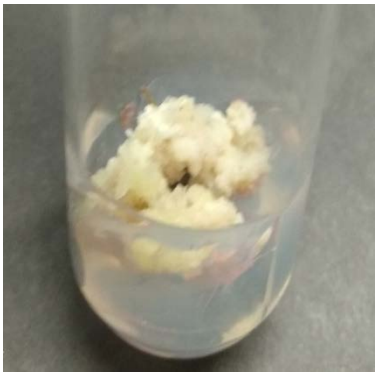

Fig. 4: Callus proliferation of Kasalath rice cultivars on modified callus medium

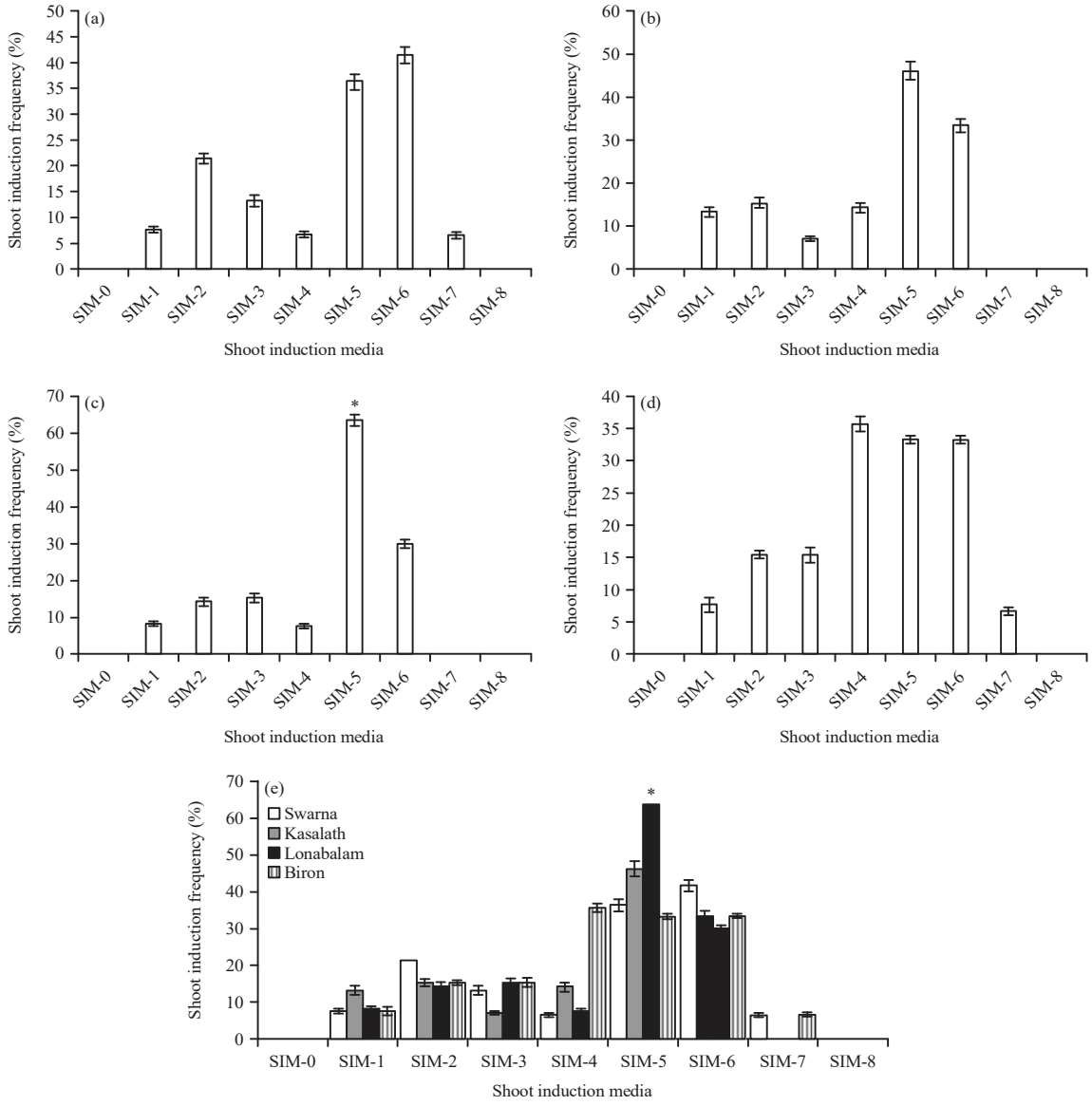

Fig. 5(a-d): Shoot induction frequency (%) of 4 rice cultivars, (a) Swarna, (b) Kasalath, (c) Lonabalam, (d) Biron and (e) Comparison of frequency among 4 varieties

*5% significance level according to Tukey's HSD

Table 3: Effect of root initiation on half-strength MS basal medium

Cultivar name	Number of plantlets inoculated in each media	Number of root response in half-strength MS media	Number of root response in MS media	Root induction (%) in half-strength MS media
Swarna	10	3	0	30
Kasalath	10	4	0	40
Lonabalam	10	7	0	70
Biron	10	7	0	70

DISCUSSION

Mature seed provides some advantage over inflorescences and immature embryos for *in vitro* regeneration to produce rice plants. As inflorescences and immature embryos are available only for a limited period in a year because of photoperiodic sensitivity of the rice genotypes and problem associated with rice isolation and sterilization of immature embryos. In addition, regenerated calli produced from scutellar tissue of mature seed are an excellent source of

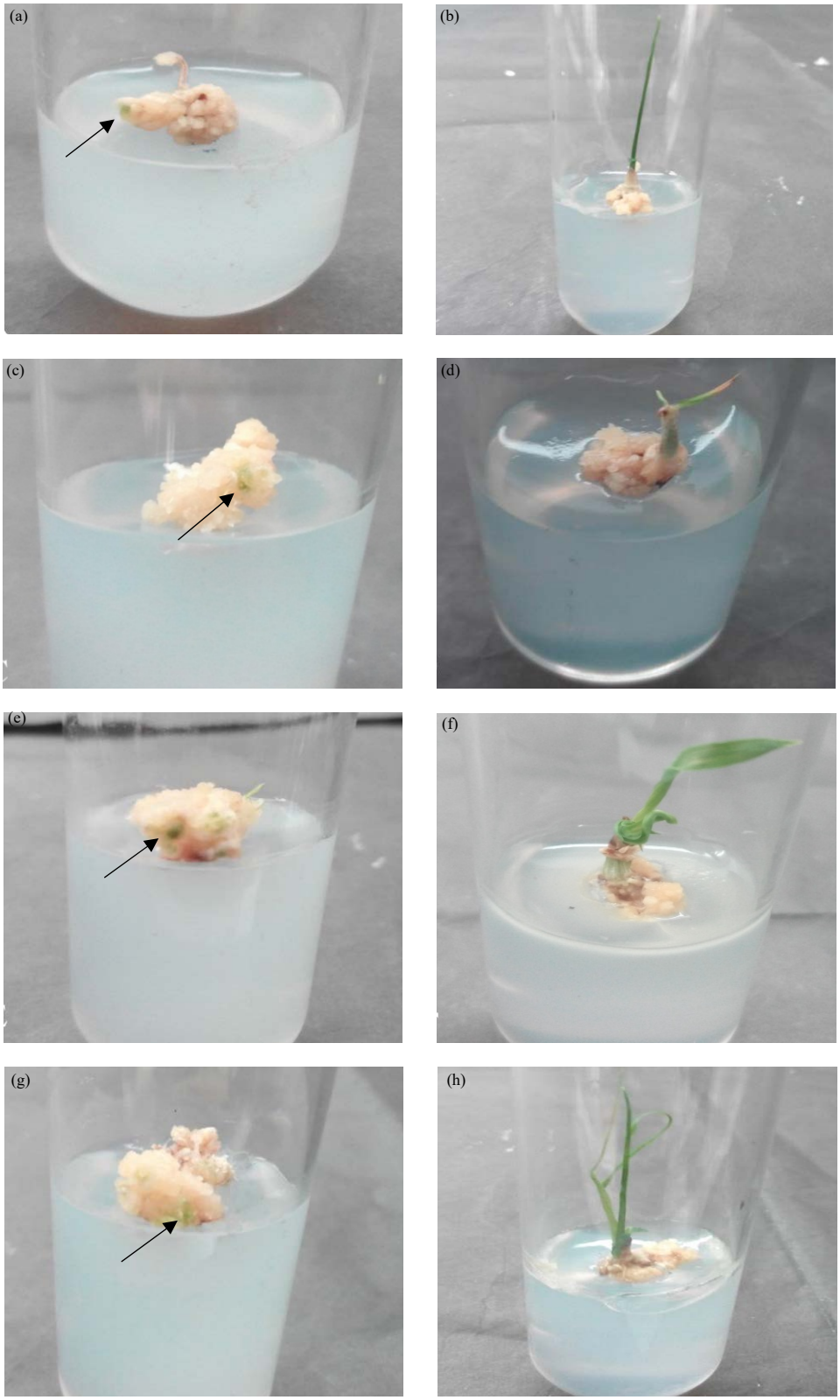

Fig. 6(a-h): Shoot proliferation of 4 rice cultivars, (a, c, e, f) Greenish color on callus with (↗) sign of Swarna, Kasalath, Lonabalam and Biron and (b, d, f, h) Shoot formation of those 4 varieties

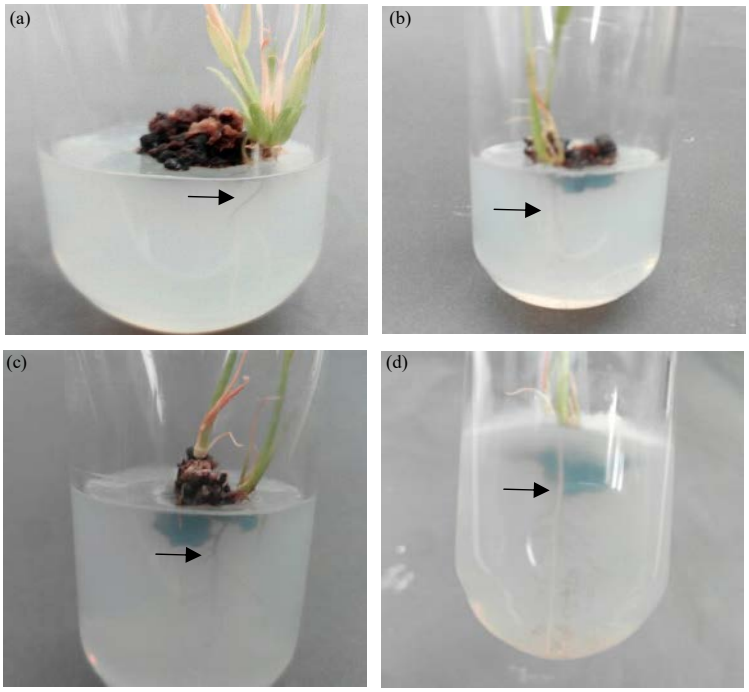

Fig. 7(a-d): Multiple shoot and root formation of 4 rice genotype, (a) Swarna, (b) Kasalath, (c) Lonabalam and (d) Biron
Arrow sign indicates root proliferation on half strength MS media of Swarna, Kasalath, Lonabalam and Biron

regeneration and transformation application[22]. The present study also designed to use the mature seed of rice and find out the reproducible result on regeneration capability of rice genotype.

Embryogenic callus induction is usually established by auxin more specifically synthetic plant growth regulator 2,4-D. Different concentration of 2,4-D alone or in combination with other auxins and cytokinins reported better for callus initiation[23,24]. In the present analysis, different concentrations of 2,4-D applied to four rice cultivars and found better callus efficiency at 1-2.5 mg L^{-1} range. Swarna, Kasalath, Lonabalam and Biron showed highest callus formation at 2.5, 1, 2 and 1 mg L^{-1} of 2,4-D concentration. Without Swarna, other rice genotypes showed a reduction in callus initiation with the increase of hormone above 2 mg L^{-1}. This tendency of callus reduction also reported by Verma *et al.*[25].

Although many studies revealed the presence of synthetic auxin, 2,4-D is an important factor for successful rice callus[26,27], other researchers used 2,4-D combined with BAP[28] or NAA for better induction rate. Besides, cultured cells are generally capable of synthesizing all their essential amino acids but the addition of proline and glutamine to the medium may enhance cell growth. The use of proline and glutamine combination or separate use has been reported a positive effect on the frequency of callusing and regeneration in rice[21,23,29]. The present experiment has been done by using a different combination of 2,4-D, BAP and proline. However, only Kasalath genotype was well responsive on modified callus medium. Other rice genotype showed less significantly responsive against all three-rice genotype. Sahoo *et al.*[28] reported 90% callus induction rate of Swarna cultivar on modified callus medium and Saika and Toki[30] reported 75% induction rate of Kasalath on N6D medium. In this study, Swarna and Kasalath frequency was 67.87 and 76.47%. However, callus frequency of Kasalath was better in modified proline fortified medium which is about 86.66%.

Plant *in vitro* regeneration frequency is influenced by callus medium, shooting medium and the interaction between callus and shooting medium. Several auxins and cytokinins in the regeneration media are known to induce regeneration frequency in the recalcitrant genotype[31]. Lee *et al.*[32] proposed that highest regeneration frequency observed on MS medium containing NAA (2 mg L^{-1}) and KIN at a range of 1.0-4.0 mg L^{-1}. Regeneration mechanism may rely on the synergistic, antagonistic and additive mechanism of auxins and cytokinins that promote developmental decision toward callogenesis and shoot formation. Combination of BAP at 2 mg L^{-1} concentration with 0.5 mg L^{-1} NAA has been reported to be most effective for plantlet regeneration from callus. The combinatorial effect of BAP in combination with NAA has been reported to facilitate regeneration in rice callus culture[33]. The present study also reported that combination

of NAA (0.5 mg L^{-1}), BA (2 mg L^{-1}) and Kinetin (3 mg L^{-1}) showed effective regeneration frequency of 4 rice genotype. Rice genotypes show diverse callus induction and plant regeneration potentiality which is due to significant genotyping variation in rice[21]. Root formation mainly depended on agar and water content on media despite different hormonal effect also reported. Half strength MS media was reported different studies which had a significant effect on root formation[28,34]. The present study also implements half-strength MS media and got significant root formation of four rice genotype.

CONCLUSION

Callus initiation and regeneration frequency development of four recalcitrant rice cultivars through different hormonal and amino acid composition are effective for transformation purpose. Swarna and Kasalath showed better callus frequency whereas regeneration frequency was better of Lonabalam cultivar. As Lonabalam is moderately salt tolerant so the further enhancement of salt-tolerant gene through transformation process will increase its productivity in the coastal region. The established media concentration will ameliorate the Agrobacterium-mediated transformation process.

SIGNIFICANCE STATEMENT

This study optimizes the hormonal concentration and amino acid effectivity on Indica rice cultivars, usually cultivated in Bangladesh. As Bangladesh is vulnerable to salinity in the coastal region, new rice cultivars are indispensable in those areas. This study demonstrates the *in vitro* technique for callus formation and regeneration and thus can use this protocol for Agrobacterium-mediated gene transformation. This study will help researchers to utilize optimize hormonal concentration for bulk callus formation and regeneration, which will also facilitate Agrobacterium-mediated gene transformation.

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