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Callus Induction and Plantlet Regeneration Systems in *Indica* Rice (*Oryza sativa* L.) Cultivar Sangyod

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Abstract

This study was conducted to determine the optimum concentrations of 2, 4-D, L-proline and casein hydrolysate (CH) for efficient callus induction and plantlet regeneration from culturing mature embryos of Sangyod, an economically important *indica* rice variety in Thailand. The highest frequency of callus induction (73.08 ± 2.65 %) was obtained from MS medium supplemented with 2 mg/L 2,4-D, 750 mg/L CH and 200 mg/L L-proline. The combination of 1 mg/L BA, 0.5 mg/L Kn and 0.5 mg/L NAA containing solidified MS medium gave the maximum mean fresh weight of callus (938.9 ± 44 mg), green spot formation (64.17 ± 7.08 %), shoot induction frequency (66.25 ± 6.80 %) and mean number of shoots/explant (6.12 ± 0.36 shoots). The greatest mean number of shoots/explant (14.93 ± 0.97 shoots) and root formation percentage (82.71 ± 3.03 %) was observed in liquified MS medium supplemented with 0.5 mg/L NAA and 1 mg/L BA.

Keywords: *Indica* rice, Sangyod, 2,4-D, L-proline, casein hydrolysate

Abbreviations: 2, 4-D = 2, 4-dichlorophenoxyacetic acid,
NAA = α-naphthaleneacetic acid,
Kn = Kinetin,
BA = 6-benzyladenine,
CH = Casein hydrolysate.

Introduction

Rice (*Oryza sativa* L.) belongs to the family Poaceae. It is the most important cereal crop in Asia where nearly 90 % of global rice production and consumption is found [1]. Global rice demand is projected to rise 26 % in the next 25 years and achieve nearly 555 million tons in 2035 [2]. Driven by both population growth and climate change, traditional plant breeding cannot meet the rising demand of rice production.

Sangyod rice is one special rice variety with a dark-red color dehusk seed, soft and aromatic when cooked, and grown in Pattalung province, Thailand for hundreds of years. Red rice had more minerals (iron), vitamins, bioactive compounds (anthocyanin, flavonoid, phenolic compounds) than white rice [3]. Pigmented rice also contains high antioxidant activity [4] that helps reduction in the risk of some chronic diseases for people such as diabetes, cancer and cardiovascular diseases.

Currently, the demand for healthier rice products are increasing globally. Providing specially rice varieties for market will increase economic profits to farmers and nutritional benefits to consumers. Research and development of efficient mass propagation tools for *Sangyod* rice variety in the future is required. Crop improvement through tissue culture techniques are being widely applied for large scale plant multiplication including rice. However, *indica* varieties are generally difficult to culture and require

a longer period as compared to *japonica* varieties [5]. Most *indica* subspecies are recalcitrant to *in vitro* response due to poor callus formation and regeneration capacity [6,7].

Successful callus induction and regeneration *in vitro* commonly depends on many factors such as genotype, types of explants, culture media, plant growth regulator (PGRs), carbon sources and culture conditions [8-11]. Genotype and culture media are the 2 key factors that determine the fate of *in vitro* raised culture [12]. In rice, plant regeneration has been obtained from different type of explants such as immature seeds [13], mature seeds [14], anther [15], leaf [16] and root [17]. Node or shoot tip has less potential for callogenesis than mature seeds [18]. In rice, mature embryos are generally applied for callus formation and efficient plant regeneration system *in vitro* cultures [14] as the best explant source for genetic transformation in comparison with other explants [19]. MS medium (Murashige and Skoog, 1962) is the most commonly used for the propagation of many plant species [20]. The plant hormones and the nitrogen source have profound impact on the response of the initial explant [21]. PGRs play a crucial role in deciding the improvement pathway of plant cells in culture media. Amino acids also offered positive impacts on rice callus growth [22,23] particularly L-proline and Casein hydrolysate (CH) were reported by several researchers [24-27]. Establishment of a highly efficient plant regeneration for *indica* varieties system are prerequisite for the application of genetic transformation technology of rice for high yield and quality improvement. Hence, optimization of plant regeneration protocols for desired genotypes is essential. To the best of our knowledge, protocols for high frequency plant regeneration are still lacking in the other pigmented rice cultivars in Thailand using embryogenic callus cultures. Moreover, development of an improvement callus induction and plant regeneration system for pigmented rice cv. Sangyod have not been reported earlier.

The objectives of this study were to define the optimum concentrations of 2,4-D, L-proline and CH for callus induction from culturing mature seeds of the *indica* rice variety Sangyod and establish a high plantlet regeneration protocol for applying gene transformation.

Materials and methods

Plant material and sterilization

Mature seeds of *indica* rice cultivar Sangyod were used as an explant source. They were obtained from the Department of Plant Science, Faculty of Natural Resources, Prince of Songkla University, Thailand. The seeds were dehusked, washed with running tap water for 20 min, then surface sterilized with 70 % (v/v) ethanol for 1 min, and immersed in 20 % (v/v) Clorox (commercial bleach) containing 0.05 - 0.1 ml of a wetting agent "Tween-20" on an orbital shaker at 100 rpm for 10 min. Finally, the seeds were rinsed with sterile distilled water 5 times in a laminar air flow hood before blotting dry on autoclaved tissue paper. Sterile seeds were then cultured on callus induction medium (CIM).

Callus induction

Three experiments were carried out to optimize the concentration of 2,4-D, Casein hydrolysate and L- proline to find out the best medium for callus induction of *Sangyod* rice.

Experiment 1: Disinfected seeds were cultured on CIM which was an MS basal medium supplemented with different concentrations (0 - 4 mg/L) of 2,4-D in combination with 1000 mg/L CH and 100 mg/L L- proline.

Experiment 2: Disinfected explants were inoculated on CIM fortified with 2,4-D at the best concentration obtained from experiment 1, 100 mg/L L-proline and various concentrations (100, 250, 500, 750 and 1000 mg/L) of CH.

Experiment 3: Finally, the influence of different concentrations of L-proline on callus induction was investigated. Disinfected seeds were cultured on CIM with the best concentration of 2,4-D from experiment 1, CH from experiment 2 and different concentrations (0, 50, 100, 200 and 300 mg/L) of L-proline.

The CIM from each experiment was composed of 3 % (w/v) sucrose and solidified with 0.75 % (w/v) agar, the pH of the culture medium was adjusted to 5.7 before autoclaving at 121 °C, 1.07 kg/ cm^2 for 20 min, culture bottles were sealed by Parafilm. All cultures were maintained at 26 ± 2 °C in the

culture room under 14 h photoperiod with irradiance of 25 μmol/m^2/s provided by cool white fluorescent tubes. After 4 weeks of culture, the frequency of callus induction, morphology and mean fresh weight of callus were recorded and statistically compared.

Callus proliferation and shoot formation

The calluses (0.1 gram fresh weight) obtained from the most suitable medium of previous experiments were transferred to a regeneration medium (RM) which was MS supplemented with various concentrations of PGRs (NAA, BA and Kn) as shown in **Table 4**. The medium was supplemented with 3 % (w/v) sucrose and adjusted to pH 5.7 prior to addition of 0.75 % (w/v) agar and autoclaving at 121 °C, 1.07 kg/cm^2 for 20 min. The cultures were maintained under the same conditions as mentioned in previous experiments. The calluses were sub-cultured to fresh medium with the same composition every 2 weeks for 10 weeks. At the end of the culture period, the mean fresh weight of callus, the percentage of green spots (GS) formation, the percentage of shoots formation and mean number of shoots per callus were determined.

Multiple shoot formation and root induction

Shoot tips at approximately 5 mm in length were transferred to liquified RM with different concentrations (0.5, 1.0 mg/L) of BA or Kn alone or in combination with 0.5 mg/L NAA (**Table 5**). All PGRs containing RM were supplemented with 30 g/L sucrose, adjusted to pH 5.7 prior to autoclaving at 121 °C, 1.07 kg/ cm^2 for 20 min. All cultures were incubated on a rotary shaker at 100 rpm under 14 h photoperiod in the culture room in order to optimize plantlet regeneration. Mean number of shoots per cultured shoot tip and the percentage of roots induction were recorded after 4 weeks of culture.

Statistical analysis

All the tissue culture experiments of callus induction, shoot proliferation, and root induction were analyzed in completely randomized design (CRD) with 8 replicates per treatment (24 explants per replication). Data were tested by using one-way analysis of variance (ANOVA) and the significant differences among means were separated by Duncan's multiple range test (DMRT) ($p \leq 0.01$) using the program R statistical package version 2.14.

Results and discussion

Effect of different concentrations of 2,4-D on callus induction

CIM supplemented with various concentrations of 2,4-D (0 - 4 mg/L) in combination with 1000 mg/L CH and 100 mg/L L-proline gave different frequencies of callus formation and callus morphology. Mature seeds can be selected as explant sources for callus induction and regeneration effectively as they are accessible throughout the year. The seeds swelled and clearly observed from the scutellum region after 10 days of culture under 14 h photoperiod. Based on these results (**Table 1**), the percentage of callus induction was from 17.29 to 64.38 % across various concentrations of 2,4-D after 1 month of culture. No callus initiation was obtained on the CIM without 2,4-D (control). Our result revealed that 2,4-D in the presence of CH and L-proline stimulated callus induction of *Sangyod* rice similar to those reported previously [28,29]. The concentration of 2,4-D higher than 2.0 mg/L caused a decrease in callus induction frequency. Different characteristics of callus were found in different concentrations of 2,4-D. CIM containing 2.0 mg/L 2,4-D gave the best response in callus formation (64.38 %) and desired morphology of embryogenic callus as yellowish white color with globular structure (**Table 1** and **Figure 1b**). Our results are in agreement with Shahsavari *et al.* [23] who reported that the highest percentage of callus induction was obtained from MS medium supplemented with 2.0 mg/L 2,4-D in upland rice cultivars Selasi, Kusan, Siam and Lamsan, respectively. Several researchers have shown that MS medium supplemented with 2.0 mg/L 2,4-D was better for aromatic rice KDML105, Basmati 370 [30,31]. The appearance of non-embryogenic callus consisting of a brown color, necrosis and rhizogenesis was observed for high 2,4-D containing CIM (**Table 1**). Besides, the decrease in frequency of callus formation was obtained for high concentrations of 2, 4-D (3 - 4.0 mg/L) similar to those reports from Libin *et al.*

[32] and Mohd Din *et al.* [33]. The choice and distinction of embyogenic callus is essential to obtain efficiency of plantlet regeneration [34]. 2,4-D is known as a strong synthetic auxin and popular as a growth regulator in plant tissue culture. It plays a critical role in successful callus initiation and sustainment in rice [16]. 2,4-D alone was often used for callus induction. However, several researchers have reported that supplementation of 2,4-D together with CH or L-proline enhanced the response of callus formation and proliferation rather than 2,4-D alone [35-37]. The combination of 300 mg/L L-proline and 400 mg/L CH in MS medium supplemented with 2.5 mg/L 2, 4-D and 1.0 mg/L Kn gave the highest callus induction frequency of rice cv. BRRI dhan32 [38]. Each genotype of rice requires different hormonal composition. Therefore, CIM should be modified to suit each variety of rice. The function of 2,4-D was reported to enhance the amount of callus through increasing cell division rates. Embryogenic callus initiation could be promoted through sufficient concentration of 2,4-D [39,40]. Our results also revealed that a combination of 2,4-D with amino acids (CH and L-proline) could be useful for promoting a high percentage of callus induction in *Sangyod* rice.

Table 1 Effect of concentrations of 2,4-D on callus induction from mature seed of *Sangyod* rice on CIM supplemented with 1000 mg/L CH and 100 mg/L L-proline after 4 weeks of culture.

2,4 -D (mg/L)	Percentage of callus formation (%)	Morphology of callus
0.0	0.00 ± 0.00^{e}	
1.0	30.62 ± 2.92^{c}	White, friable
1.5	45.79 ± 3.21^{b}	Yellow, friable
2.0	**64.38 ± 2.87^{a}**	**Yellowish white, nodular**
3.0	17.29 ± 2.20^{d}	Yellowish-brown, compact, little rhizogenic
4.0	18.91 ± 2.97^{d}	Brownish, compact, little necrosis

Values are means of 8 replicates ± SD. Means followed by different letters within column are significantly different ($p \leq 0.01$) by DMRT.

Effect of different concentrations of CH on callus formation

The results in **Table 2** revealed that increasing concentrations of CH promoted callus formation percentage from 25.37 % (100 mg/L) up to 68.56 % (750 mg/L) on MS medium supplemented with 2 mg/L 2,4-D and 100 mg/L L-proline. Callus induction frequency was significantly different ($p \leq 0.01$) among various concentrations of casein hydrolysate after 4 weeks of culture. The highest percentage of callus formation (68.56 ± 4.35 %) was obtained from MS medium supplemented with 750 mg/L CH. However, the response of callus induction was slightly decreased to 61.67 % when the concentration of CH was increased to 1000 mg/L. Our results are different to previous reports on rice cv. Selasi which found that the combination of, 300 or 600 mg/L CH and 500 mg/L L-proline increased the percentage of callus induction [23]. Growth of rice calluses in terms of size and quality were obtained in the presence of CH whereas the number of embryogenic calluses did not increase (**Figure 1**). The result was similar to the study reported by Raval and Chatto [25], Khaleda and Al-Forkan [35]. They reported that a high percentage of callus induction (87 %) was obtained from rice cv. HA-8 on MS medium with 2 mg/L 2,4-D and 0.6 % (w/v) CH. Moreover, NN medium (Nitsch & Nitsch, 1969) containing 2 mg/L 2,4-D and 300 mg/L CH increased callus induction in rice cv. KDML 105 [36,41]. Although our results were in contrary to the previous report of Che Radziah *et al.* [37] different concentrations of CH (300 - 1000 mg/L) did not show a significant impact on percentage of callus formation rice cv. MR 219 (72.5 to 97.5 %). It has been earlier studied that addition of amino acids such as L-proline, CH can stimulate callus formation and plantlet regeneration frequencies [10,42]. CH is an organic nitrogen source [29]. It also provides several vitamins, micronutrients, calcium and in particular a mixture of 18 amino acids and have been reported to improve callus growth in culture medium [43]. Based on this result, 750 mg/L CH was

selected due to the most suitable for induction of rice callus cultivar Sangyod and this concentration was used for the next experiment (**Table 2**).

Table 2 Effect of concentrations of CH on callus formation from mature seed of *Sangyod* rice on CIM supplemented with 2 mg/ L 2,4-D and 100 mg/ L L-proline after 4 weeks of culture.

CH (mg/ L)	Frequency of callus formation (%)
100	25.37 ± 2.76^{c}
250	36.81 ± 3.63^{bc}
500	46.67 ± 1.69^{b}
750	$\mathbf{68.56 \pm 4.35^{a}}$
1000	61.67 ± 4.29^{a}

Values are means of 8 replicates ± SD. Means followed by different letters within column are significantly different ($p \leq 0.01$) by DMRT.

Effect of different concentrations of L-proline on callus formation

Based on data shown in **Table 3**, the percentage of callus initiation (34.38 - 73.08 %) and callus fresh weight (31.3 - 67.5 mg) were obtained from MS medium supplemented with various concentrations of proline, 2 mg/L 2,4-D and 750 mg/L CH after 4 weeks of culture. Our result indicated that various concentrations of proline gave a significant effect on callus induction percentage and callus fresh weight. This finding is in approval with those earlier results reported by Chowdhury *et al.* [22], Che Radziah *et al.* [37] and Bhausaheb *et al.* [44]. The absence of L-proline in culture medium (control treatment) showed the lowest frequency of callus formation and callus fresh weight at 34.38 % and 31.3 mg, respectively. The supplement of L-proline in the culture medium at 200 mg/L yielded an optimum medium for percentage of callus induction (73.08 ± 2.65 %) and callus fresh weight (67.5 ± 7.4 mg) followed by 100 mg/L (61.67 ± 3.52 % and 66.1 ± 11.6 mg, respectively). The increment in concentration of L-proline up to 300 mg/L decreased significant the percentage of callus induction (40.21 ± 2.77 %) and callus fresh weight (39.0 ± 3.7 mg). Similar results were also reported in callus growth of some rice varieties such as Udayagiri, Pratikhya and Khandagiri [45]. The positive effects of L-proline on the response of callus induction and regeneration have been demonstrated in rice cv. Hom Kra Dang Ngah [29]. However, the highest percentage of callus formation (100 %) in rice cultivar MR 219 was observed on MS medium supplemented with 3.0 mg/L 2,4-D and 1000 mg/L L-proline [37]. In addition, NN medium (Nitsch & Nitsch, 1969) supplemented with 1.5 mg/L 2,4-D, 300 mg/L CH and 1000 mg/L L-proline was reported to be the most suitable for increasing callus formation in Supanburi 1 [36] whereas the combination of 500 mg/L CH and 500 mg/L L-proline was suggested to have a positive influence on callus formation in rice cv. HKR-46 and HKR-126 [9]. L-proline is a type of amino acid and also known as an organic nitrogen source supplied growth and development of plant cells. The addition of L-proline in the medium acts as a stress condition due to the reduction of water potential, thus, enhancing the development of embryogenic callus through the accumulation of nutritional items in cells [46]. Moreover, L-proline was recommended to act as an osmoticum, source of $NADP^{+}$ and a nitrogen storage pool, essential for rapid embryo growth [47]. Raval and Chatto [25] reported that L-proline assisted increasing embryogenic callus induction and callus growth, similar to our observation as shown in **Figure 1**. The highest percentage of embryogenic callus induction was also observed on MS medium supplemented with 3.0 mg/L 2,4-D, 2.0 mg/L, Kn and 200 mg/L L-proline in rice cv. Pratikhya and Swarna [45]. L-proline could provide a readily available nitrogen source to promote callus growth [44]. In the present study, we recommend that 200 mg/L L-proline containing MS medium together with 2 mg/L 2,4-D and 750 mg/L CH was the most effective for callus induction frequency and growth in rice cv. Sangyod (**Table 3**).

Table 3 Effect of L-proline on callus formation from mature seed of *Sangyod* rice on MS medium supplemented with 2 mg/L 2,4-D and 750 mg/ L CH after 4 weeks of culture.

L-Proline (mg/L)	Percentage of callus formation (%)	Mean callus fresh weight (mg)
0	34.38 ± 2.31[b]	31.3 ± 5.4[b]
50	46.04 ± 4.82[b]	49.5 ± 8.1[ab]
100	61.67 ± 3.52[a]	66.1 ±11.6[a]
200	**73.08 ± 2.65[a]**	**67.5 ± 7.4[a]**
300	40.21 ± 2.77[b]	39.0 ± 3.7[ab]

Values are means of 8 replicates ± SD. Means followed by different letters within column are significantly different ($p \leq 0.01$) by DMRT.

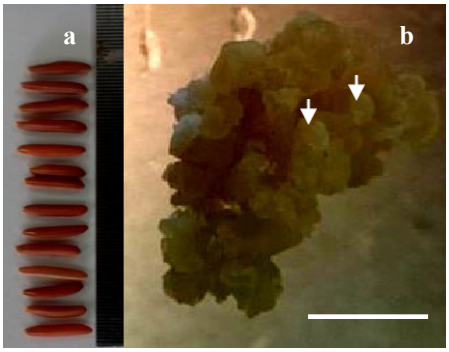

Figure 1 Morphological characteristics of callus induction of *Sangyod* rice on MS medium supplemented with 2 mg/L 2,4-D, 750 mg/L CH and 200 mg/L L-proline after 4 weeks of culture; (a) dehusked mature seeds of *Sangyod* rice, and (b) characteristic of embryogenic callus with globular shape (arrows) observed under microscope (X40) (bar = 1 cm).

Effect of various concentrations of cytokinin and auxin on regeneration efficiency

The ratio of cytokinin (BA with Kn) to auxin (NAA) is essential for *in vitro* regeneration efficiency of rice cv. Sangyod. The combinations of NAA with BA and Kn are often used for plantlet regeneration in several rice varieties from embryonic callus [48]. Both types of cytokinin and auxin affect cell cycles. Therefore, the ratio of cytokinin to auxin is a key factor in controlling many growth processes including organ regeneration from varied tissues [49]. Several kinds of interaction may be antagonistic, additive or synergistic [50]. Calluses transferred to those PGRs containing regeneration medium showed different response of development (**Table 4**). Green spots were observed from embryogenic callus after 6 weeks of culturing (**Figure 2**). Shoot induction percentage and mean number of shoots/callus varied based on the combination of PGRs which performed 2 weeks later. Green spots were produced from calluses by the process of photosynthesis of the callus when they were placed under light [51]. In Poaceae, the appearance of green spots has been recognized as predictors of shoot induction capacity [52]. Our data showed that different concentrations of PGRs gave significant influence on callus growth and shoot regeneration (**Table 4**). The combination of 1 mg/L BA, 0.5 mg/L Kn and 0.5 mg/L NAA gave the maximum mean fresh weight of callus (938.9 ± 44 mg), the highest percentage of green spot formation (64.17 ± 7.08 %), optimum shoot induction frequency (66.25 ± 6.80 %) and maximum mean number of shoots/callus (6.12 ± 0.36 shoots) which were statistically different ($p \leq 0.01$) to the other concentrations of PGRs.

A decrease in callus fresh weight and shoot regeneration frequency in *Sangyod* rice were observed from a high ratio of cytokinin (BA + Kn) to auxin (NAA) above 3:1 in **Table 4**. Conversely, in rice cv. Hom Kra Dang Ngah with higher ratios of these PGRs (6:1) had the highest percentage green spot formation (75.5 %) and plantlets regeneration (33.3 %) on MS medium supplemented with 0.5 mg/L NAA, 1.0 mg/L BA and 2.0 mg/L Kn [53]. Some authors have reported that addition of 0.5 mg/L NAA, 3 mg/L BA and 0.5 mg/L Kn gave the most suitable plantlet regeneration frequency at 80 % and mean number of shoots/explant at 3.1 shoots/callus in rice cv. Topa [54]. Our results showed that the combination of NAA and BA or Kn alone also promoted callus growth (fresh weight) and shoot regeneration in rice cv. Sangyod. Similar to the result reported in rice cv. Super Basmati on MS medium supplemented with 1 mg/L NAA and 3 mg/L Kn which gave 9.66 ± 2.0 shoots shoots/callus [55]. The response of callus growth and shoots regeneration in rice cv. Sangyod *in vitro* was affected by various interactions and concentrations of PGRs (NAA, BA and Kn). A low ratio of cytokinin to auxin was suitable for this cultivar. The different responses of variety might be due to recalcitrance and genotype-dependence in *indica* rice. Therefore, it is essential to modify the combination of PGRs base on different genotypes to increase regeneration efficiency.

Table 4 Effect of PGRs (NAA, BA and Kn) containing solidified MS medium on regeneration efficiency from mature seed of *Sangyod* rice after 6 weeks of culture.

Concentrations of PGRs (mg/L)			Mean fresh weight of callus (mg)	Percentage of GS formation (%)	Shoot induction frequency (%)	Mean number of shoots/ callus
NAA	**BA**	**Kn**				
0.5	2.0	0.0	526.0 ± 20.5^{bc}	28.33 ± 5.74^{ab}	42.50 ± 7.73^{ab}	3.13 ± 0.28^{b}
0.5	0.0	2.0	608.7 ± 18.2^{bc}	37.50 ± 13.21^{ab}	35.00 ± 9.45^{b}	2.68 ± 0.65^{b}
0.5	**1.0**	**0.5**	**938.9 ± 44.0^{a}**	**64.17 ± 7.08^{a}**	**66.25 ± 6.80^{a}**	**6.12 ± 0.36^{a}**
0.5	1.0	1.5	613.7 ± 16.2^{bc}	22.50 ± 9.59^{b}	36.25 ± 7.78^{b}	2.55 ± 0.50^{b}
0.5	1.0	2.0	653.0 ± 14.9^{b}	16.67 ± 3.56^{b}	21.25 ± 4.79^{b}	3.05 ± 0.48^{b}
1.0	1.5	1.0	571.7 ± 29.5^{bc}	40.00 ± 6.55^{ab}	38.75 ± 2.95^{b}	4.03 ± 0.42^{b}
1.0	1.5	2.0	563.3 ± 35.1^{bc}	47.50 ± 13.83^{ab}	32.50 ± 5.26^{b}	3.18 ± 0.14^{b}

Values are means of 8 replicates ± SD. Means followed by different letters within column are significantly different ($p \leq 0.01$) by DMRT.

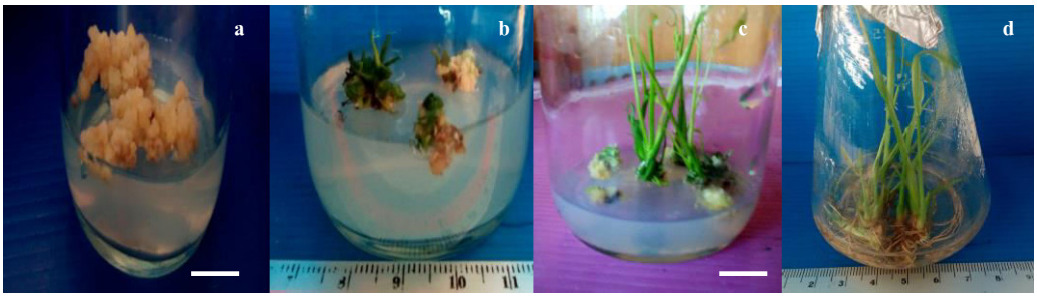

Figure 2 Morphological of callus formation and plantlets regeneration of *Sangyod* rice; (a-c) callus proliferation, green spot formation and shoot formation on solidified MS medium supplemented with 0.5 mg/L NAA, 1 mg/L BA and 0.5 mg/L KN (bar = 1 cm), and (d) shoot multiplication and root formation in liquified MS medium supplemented with 0.5 mg/L NAA and 1 mg/L BA.

Effect of different concentrations of PGRs (NAA, BA and Kn) on multiple shoot and root formation

In rice, plantlet regeneration efficiency is affected by many factors such as genotype, physiological status of the explants, PGRs and culture environments [56]. Shoot tips are needed for good explants and indicate strong dividing of the meristematic cells that might easily maintain *in vitro* regeneration [57]. A high cytokinin/auxin ratio promotes shoot induction. In contrast, a high ratio of auxin/cytokinin ratio encourages root production [58]. Based on the results in **Table 5**, multiple shoots were induced and recorded in all treatments. The greatest mean number of shoots/single shoot (14.93 ± 0.97 shoots) and percentage of root formation (82.71 ± 3.03 %) was observed in liquified MS medium supplemented with 0.5 mg/L NAA and 1 mg/L BA after 4 weeks of culture. By contrast, PGR-free MS medium gave the lowest results in all parameters. The present study showed that the combination of 0.5 mg/L NAA and 1 mg/L Kn gave the lower mean number of shoots/single shoot (10.53 ± 0.98 shoots) and the percentage root formation (65.75 ± 5.59) than the combination of BA with NAA. However, there was not significant difference when liquified MS medium in the presence of both 0.5 mg/L BA and 0.5 or 1.0 mg/L Kn was used. Several reports showed that the most suitable PGRs for plantlet regeneration in rice cv. Chiniguri was obtained on MS medium supplemented with 0.05 mg/L NAA and 5 mg/L BA [59]. High numbers of multiple shoot induction of *indica* rice variety Jaya was achieved on liquified MS medium with 5 mg/L BAP, 1 % (w/v) mannitol and 3 % sucrose [60]. Similarly, in cv. MR219, liquified MS medium containing 0.1 mg/L Kn gave a higher shoot induction efficiency than solidified MS medium. Liquified medium supplies good aeration and enhances the capacity for dissolved nutrient composition uptake by the whole surface of the explant [61]. In this study, the combination of 0.5 mg/L NAA and 1 mg/L BA containing liquified MS medium was the most effective for multiple shoot formation and root induction in rice cv. Sangyod.

Table 5 Effect of PGRs containing liquified MS medium on multiple shoot and root formation from culturing single shoot-derived plantlets of rice cv. Sangyod after 4 weeks of culture.

PGRs (mg/L)			Mean number of shoots/explant	Root Induction frequency (%)
NAA	**BA**	**Kn**		
0	0	0	4.67 ± 0.40^{c}	0.00 ± 0.00^{c}
0.5	0.0	1.0	10.53 ± 0.98^{b}	65.75 ± 5.59^{ab}
0.5	**1.0**	**0.0**	$\mathbf{14.93 \pm 0.97^{a}}$	$\mathbf{82.71 \pm 3.03^{a}}$
0.5	0.5	0.5	9.67 ± 1.00^{b}	59.52 ± 5.87^{b}
0.5	0.5	1.0	11.07 ± 1.06^{b}	53.63 ± 5.28^{b}

Values are means of 8 replicates ± SD. Means followed by different letters within column are significantly different ($p \leq 0.01$) by DMRT.

Conclusions

In the present study, an efficient embryogenic callus induction and plantlet regeneration protocol for *indica* rice cv. Sangyod was established using mature seeds. The results revealed that 2,4-D, CH, L-proline and PGRs were key factors in promoting callus induction and plantlet regeneration. The successful optimum medium for callus induction of *indica* rice cv. Sangyod was an MS medium supplemented with 2 mg/L 2,4-D, 750 mg/L CH and 200 mg/L L-proline. Highly efficient plantlet regeneration systems were established using solidified MS medium together with 1 mg/L BA, 0.5 mg/L Kn and 0.5 mg/L NAA. In addition, the combination of 0.5 mg/L NAA and 1 mg/L BA containing liquified MS medium was the most effective for the mean number of shoots/explant and root induction.

Improvement of callus formation and regeneration efficiency can be used for genetic engineering to create new varieties with desirable traits in the future.

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