Optimization for Callus Induction and Plant Regeneration from Mature Seeds of Thai Rice Variety: Nam Roo (*Oryza sativa* L.)

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Abstract The aim of this study was to develop a productive protocol for optimum callus induction and regeneration of Thai rice variety: Nam Roo (*Oryza sativa* L.). Callus was induced on either MS (Murashige and Skoog) or NB (Nitsch and Nitsch) media supplemented with various concentrations of 2,4-D (2,4-dichlorophenoxyacetic acid), 0.5 mg/l NAA (α -naphthaleneacetic acid), 1 g/l L-proline, 30 g/l sucrose and 2.6 g/l phytagel. Callus from Nam Roo seed gave its maximum mean size (391.49 mm³) and mean weight (0.3412 g.) on NB medium supplemented with 1 mg/l 2, 4-D for 4 weeks. For plant regeneration the callus was cultured on either MS or NB media containing with different concentrations of BAP (6-benzylaminopurine) and phytagel, 0.5 mg/l NAA and 30 g/l sucrose. The highest regeneration frequency (%) was observed from callus grown on MS medium composed of 2 mg/l BAP in 5.2 g/l phytagel. It took 6 weeks for the callus to regenerate into a complete plant. Finally, the plantlets were transferred into the plastic pot.

Keywords Nam Roo Rice (*Oryza sativa* L.), Callus Induction, Plant Regeneration

1. Introduction

Rice (*Oryza sativa* L.) is one of the most important cereal crops which supplies food for more than half of the world's population. However, rice yield and quality are affected by pests and diseases, as well as by environmental stress. Due to the increasing world population, the value and demand for this crop also steadily rise. Therefore, the techniques of biotechnology are very important for developing increased productivity and quality of rice. Tissue culture has played an increasingly important role in rice improvement in recent

years. Thai rice variety, Nam Roo (*Oryza sativa* L.), is non-glutinous rice and it is indigenous upland rice, it was collected from seeds of Lisu hill tribe at Chiang Dao district, Chiang Mai, Thailand. Nam Roo rice is photoperiod sensitive and resistance to blight diseases, approximately 141 cm in height and provides an average yield of 247 kg per hectare. When cooked, the grains have a light and fluffy texture [1]. The embryogenic callus can be induced from tissues of various organs such as immature and mature embryo, leaf blade and root for rice breeding, has made higher yields, improved quality of local rice. For plant regeneration from an embryogenic callus, the somatic embryo is an intermediate stage between the undifferentiated callus and seedlings. It is a differentiated and meristematic form of a somatic (callus) cell developed through a series of complex morphological and cellular changes [2-5]. So, the aim of this study was to develop a productive protocol for optimum callus induction and plant regeneration of Thai rice variety: Nam Roo (*Oryza sativa* L.)

2. Materials and Methods

2.1. Explains Preparation

Mature seeds of rice (*Oryza sativa* L.) variety Nam Roo were obtained from Samoeng Rice Research Center, Chiang Mai Province, Thailand. The de-husked seeds were surface-sterilized in sterile distilled water for 1 minute and 70% ethanol for 1 minute followed by shaking in 20% sodium hypochlorite for 30 minutes and finally rinsing three times in sterile distilled water. The sterilized seeds were dried on autoclaved filter papers.

2.2. Callus Induction

The surface-sterilized seeds were cultured on MS [6] and

NB [7] media supplemented with various concentrations of 2,4-D (Table 1), 1 mg/l NAA, 1 g/l L-proline, 30 g/l sucrose and 2.6 g/l phytigel. The pH of media was adjusted at 5.8 prior to autoclaving at 121 °C for 15 minutes. Cultured mature seeds were maintained in the dark condition at 25±2 °C. After 4 weeks of induction, the frequency of callus induction (%), mean size and mean weight of callus formation were calculated. The data were analyzed using the SPSS 20. Analysis of variance (ANOVA) was used for means comparison and Duncan's multiple range test was further analyzed when significant difference was found at P level of 0.05.

2.3. Plant Regeneration

For plant regeneration, the calli were separated two parts. Firstly, fresh callus was directly transferred to regeneration media. Secondly, the calli were dried on the sterilized petri dish and incubated under dark condition for 7 days. After that, the dry callus was cultured on regeneration media. The regeneration media were MS and NB media containing different concentrations of BAP and phytigel, 0.5 mg/l NAA and 30 g/l sucrose (Table 2). The fresh and dry calli were incubated at 25±2 °C under light condition for 6 weeks. After culture, the number of callus forming shoot and root was counted to determine plant regeneration efficiency. Then the plantlets were transferred into the plastic pot.

3. Results

3.1. Callus Induction

The experiments were made to determine the suitable type of medium (MS or NB media) and optimal concentration of 2,4-D for callus induction for Nam Roo rice. Callus was induced on MS and NB media supplemented with 1 mg/l NAA and various concentrations of 2,4-D which were 0.5, 1, 2, 3 and 5 mg/l for 4 weeks. The results showed that mature seeds were induced to callus in all media. The frequency of callus induction was 100% in all media as show in Table 1.

The induced callus was formed mostly embryogenic callus, which was creamy, dry and compact appearance. A similar was reported by Ghobeishavi et al.[8], that found embryogenic callus of Nemat and Domsiah rice varieties were yellowish white, compact and nodular. In this study, combination effects of both media (MS and NB) and concentration of 2,4-D were tested. By analysis of variances found that the growth of callus cultured on NB medium was higher than callus cultured on MS medium. Considered from means size and means weight of callus in Table 1 and Figure 1. Callus of Nam Roo rice gave its maximum mean size (391.49 mm³) and mean weight (0.3412 g) on NB medium supplemented with 1 mg/l 2,4-D (Table 1, Figure 1 and Figure 2). A similar with Rattana et al.[9] reported that the optimal concentration of 2,4-D for Pathum Thani 1 was 1 mg/l in NN medium, the average size of calli were 1.06 cm.

and the average fresh weight of calli were 0.35 g.

Figure 1. The mean size (A) and mean weight (B) of callus formation from mature seeds of Nam Roo rice that were cultured on MS or NB media supplemented with different concentrations of 2,4-D for 4 weeks.

Table 1. The frequency of callus induction (%), mean size and mean weight of callus formation from mature seeds of Nam Roo rice that were cultured on MS or NB media supplemented with various concentrations of 2,4-D for 4 weeks.

Media	Concentration of 2,4-D (mg/l)	No. of seeds	Frequency of callus induction (%)	Mean size of callus (mm ³)	Mean weight of callus (g)
MS	0.5	40	100	165.69 ^d	0.2225 ^{de}
	1	40	100	145.95 ^d	0.1987 ^c
	2	40	100	170.92 ^d	0.2405 ^{cde}
	3	40	100	152.25 ^d	0.2077 ^c
	5	40	100	157.93 ^d	0.1940 ^c
NB	0.5	40	100	352.70 ^{ab}	0.3070 ^{ab}
	1	40	100	391.49 ^a	0.3412 ^a
	2	40	100	345.23 ^{ab}	0.3172 ^{ab}
	3	40	100	310.12 ^b	0.2797 ^{bc}
	5	40	100	246.65 ^c	0.2695 ^{bcd}

Values followed by different letters indicating significant differences according to Duncan's Multiple Range Test ($p \leq 0.05$)

As though Gnanesh et al.[10] presented 1 mg/l 2,4-D induced better callogenic response in both cultivar of rice:

Karimundaga (94%) and Kanadatumba (58%). The difference in the composition of medium can result in variation in callus induction [11]. NB medium composed of nitrogen sources as N6 medium and PP medium [12] as recommended by Rueb et al. [13]. It is perhaps due to the reason that NB medium contained more nitrogen than the MS medium. And auxin is most commonly used to induce callus formation [14]. However, the optimal concentration of 2,4-D depends on the explants and genotypes of rice [15].

Figure 2. Callus induction from mature seed of Nam Roo rice. Sterilized seeds were cultured on solid MS or NB media supplemented with various concentrations of 2,4-D, 1 mg/l NAA, 1 g/l L-proline, 30 g/l sucrose and 2.6 g/l phytagel for 4 weeks.

3.2. Plant Regeneration

For plant regeneration, the fresh callus and dry callus were cultured on MS and NB media with various concentrations of BAP (1, 2 and 3 mg/l) and phytagel (2.6 and 5.2 g/l) 0.5

mg/l NAA. The green spot was appeared on callus about 14 days of transfer to regeneration media and roots were grown from the callus at the same time (Fig. 3A, 3B). The same as Sahrawat and Chand [16] that shoot bud started from nodular green spots after transferred to regeneration medium. Shoot regeneration was grown and elongated after 4 weeks (Fig. 3C, 3D). After cultured for 6 weeks, shoot and root were completely induced (Fig. 3E, 3F) and the frequency (%) of regeneration of fresh callus and dry callus obtained from media were presented in Table 2. The condition that resulted the highest frequency of plant regeneration (100%) was 2 mg/l BAP and 0.5 mg/l NAA in 5.2 g/l phytagel of dry callus. Karthikeyan et al. [17] reported that in a recent report it has been mentioned that plant regeneration was achieved through embryogenic callus on MS medium supplemented with different concentrations of BAP and NAA. Similarly, the presence of BAP and NAA were found the suitable for somatic embryo germination in rice MR219 [18]. They reported that 82.5% of the somatic embryos were grown into the seedling upon transferring the somatic embryos on the MS medium with 2 mg/l BAP and 0.5 mg/l NAA.

The addition of BAP to the regeneration medium was important for shoot induction from callus Toppo et al. [19]. The dry callus was developed to shoot more than fresh callus (Table 2). And, at result observed that shoots in 5.2 g/l phytagel were higher than 2.6 g/l phytagel. Similar to Kaur and Gosal [20] presented that the desiccation created by using agar caused increasing in regeneration frequency of sugarcane callus. Regenerated plantlets were hardened and transferred to the soil in the plastic pots within 6 weeks (Fig. 2G). From the present study, it could be concluded that callus induction frequency was found higher on NB medium. The maximum mean size and mean weight of callus induction were cultured on NB medium supplemented with 1 mg/l 2,4-D. The highest regeneration frequency (%) was grown on MS medium supplemented with of 2 mg/l BAP in 5.2 g/l phytagel. The regenerated plants had normal growth after transferred into the plastic pot. The method in this study will be useful in biotechnological approaches to improve rice through *in vitro* induced artificial mutations using radiation such as X-ray, gamma-rays and ion.

Table 2. The frequency of shoot regeneration from fresh and dry callus that cultured on MS or NB media supplemented with 0.5 mg/l NAA various concentration of 1 2 and 3 mg/l BAP, 2.6 and 5.2 g/l phytagel for 6 weeks.

Media	No. of callus	Concentration of BAP (mg/l)	Concentration of phytagel (g)	Frequency of regeneration (%)	
				Fresh callus	Dry callus
MS	12	1	2.6	0 (0/12)	0 (0/12)
	12		5.2	16.67 (2/12)	0 (0/12)
	12	2	2.6	0 (0/12)	16.67 (2/12)
	12		5.2	0 (0/12)	100 (12/12)
	12	3	2.6	0 (0/12)	16.67 (2/12)
	12		5.2	50 (6/12)	66.67 (8/12)
NB	12	1	2.6	8.33 (1/12)	8.33 (1/12)
	12		5.2	0 (0/12)	0 (0/12)
	12	2	2.6	0 (0/12)	8.33 (1/12)
	12		5.2	16.67 (2/12)	33.33 (4/12)
	12	3	2.6	0 (0/12)	0 (0/12)
	12		5.2	58.33 (7/12)	0 (0/12)

Figure 3. Characteristics of green spots on the surface of callus from Nam Roo rice after transferring to regeneration media for 2 weeks (A, B). Shoot and root regeneration of callus on MS medium supplemented with 2 mg/l BAP, 0.5 mg/l NAA and 5.2 g/l phytigel for 4 weeks (C, D). Shoot and root regeneration of callus for 6 weeks (E, F). Plantlets of Nam Roo rice from callus was transferred into the plastic pots (G)

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