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Influence of Nutrient Composition and Plant Growth Regulators on Callus Induction and Plant Regeneration in Glutinous Rice (*Oryza sativa* L.)

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Abstract: The potential for callus induction and regeneration depends on nutrient composition and plant growth regulators. The aim of the present study was to investigate the effect of nutrient composition and plant growth regulators on callus induction and plant regeneration in the glutinous rice cultivar Khunvang. The effect of 2,4-D concentrations (1, 2, 3, 4 and 5 mg L^{-1}) on callus induction and growth were investigated. The results revealed that the highest percentage of callus induction (97%) was observed in MS medium supplemented with 5 mg L^{-1} 2,4-D under 16 h Photoperiod. The effects of casein hydrolysate concentrations of casein hydrolysate (0, 300, 500, 700 and 900 mg L^{-1}) and proline (0, 300, 500, 700 and 900 mg L^{-1}) on callus induction and growth of Khunvang were also observed. The results indicated that the increasing casein hydrolysate and proline concentrations did not show a significant effect on callus growth. However, proline concentration of 900 mg L^{-1} yielded 85.67% of callus growth.

Key words: Glutinous rice, 2,4-dicholorophenoxyacetic acid (2,4-D), casein hydrolysate, proline, plant growth regulators

INTRODUCTION

Rice (*Oryza sativa* L.) is a staple food source for people in Thailand. In particular, glutinous rice is favorable for people in the north and northeast regions of Thailand. Micropropagation of rice is considered as an important technique to produce sufficient food supplies. It is an efficient system for improving rice through explants and has been necessary for the manipulation of many plants in callus production and its subsequent regeneration (Saharan *et al*., 2004). A success in micropropagation depends largely on the genotype of plant, type and physiological status of the explants, composition and concentration of the basal salt and organic components and plant growth regulators in the culture medium (Ge *et al*., 2006). A number of studies on enhancement of callus induction and plant regeneration through different explants have been reported in many rice varieties (Ge *et al*., 2006; Lin and Jhang, 2005; Ali *et al*., 2004). However, to the best of our knowledge, there is no report on the glutinous rice cultivar Khunvang. Therefore, the aim of the present study was to optimize the condition for callus induction and plant regeneration for mature seeds of the rice cultivar Khunvang.

MATERIALS AND METHODS

Effect of Clorox® on sterilization of mature seeds: The rice cultivar Khunvang was taken from the Faculty of Agriculture of Khon Kaen University. Mature seeds were collected in paper bags and kept at 10°C. Seeds were washed thoroughly in tap water and surface-sterilized with 70% ethanol for 5 min, followed by 50% Clorox® (commercial bleach) for 30 min. They were then washed three times in sterile distilled water and surface-dried using tissue papers. They were then washed 3 times in sterile distilled water and surface-dried using tissue papers. Sterilized seeds were cultured on modified MS mediums (Murashige and Skoog, 1962) containing 30 g L^{-1} of sucrose supplemented with 1 mg L^{-1} of 2,4-D

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and 2 g L^{-1} of gellen gum. Cultures were incubated in the culture room at 28°C under white illumination with a 16 h photoperiod of 2,000 lux. Seed sterilization (%), Seed germination (%) and Callus induction frequency (%) were determined after 7 days using the equation:

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

Effect of 2,4-D on callus induction: Sterilized seeds were cultured on modified Murashige and Skoog (MS) medium (Murashige and Skoog, 1962), containing 30 g L^{-1} sucrose, 2 g L^{-1} gellen gum and different 2,4-D concentrations (0, 1, 2, 3, 4 and 5 mg L^{-1}). Cultures were maintained in the culture room at 28°C under different illuminations (16-h photoperiod or in the dark). Callus induction frequency (%) was determined after 30 days of culture using the equation:

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

Effect of casein hydrolysate and proline on callus proliferation: To observe the effect of casein hydrolysate and proline on callus proliferation, two experiments were included in the present study. In the first experiment, 0.5 g of embryogenic calli was transferred to MS medium supplemented with 30 g L^{-1} sucrose, 1 mg L^{-1} 2,4-D and different concentrations of casein hydrolysate (0, 300, 500, 700 and 900 mg L^{-1}). In another experiment, 0.5 g of embryogenic calli was transferred to the same medium supplemented with different concentrations of proline (0, 300, 500, 700 and 900 mg L^{-1}). The cultures were maintained in the culture room at 28°C under white illumination with a 16-h photoperiod of 2,000 lux. Percentages of callus induction and callus growth were recorded after 30 days of culture.

Effect of plant growth regulators on plant regeneration: Two experiments were included in order to observe the effect of plant growth regulators on plant regeneration. In the first experiment, 0.5 g of embryogenic calli was transferred to MS medium containing 30 mg L^{-1} sucrose and different NAA concentrations (0, 0.5, 1.0 and 1.5 mg L^{-1}). In another experiment, 0.5 g of embryogenic calli was transferred to the same medium supplemented with different 2 iP concentrations (0, 0.5, 1.0, 1.5 and 2.0 mg L^{-1}). The cultures were maintained in the culture room at 28°C under white illumination with a 16 h photoperiod of 2,000 lux. Plant regeneration frequency (%) was recorded after 45 days of culture using the equation:

$$\text{Plant regeneration frequency (\%)} = \frac{\text{No. of calli producing plants (shoot bud formation)}}{\text{Total No. of calli}} \times 100$$

Data analysis: Treatments were arranged in a Completely Randomized Design (CRD) with 20 replicates. Collected data were analyzed using Analysis of variance method (ANOVA). Mean values of all parameters were separated according to Duncan at 0.05 level of probability using the Statistical package for social sciences v17.0 software (SPSS Inc. IL, USA).

RESULTS

Effect of Clorox® on sterilization of mature seeds: Sterilized mature seeds were treated with different concentrations of Clorox® for 30 minutes. The highest seed sterilization percentage of 100 was obtained in the presence of 90% Clorox®, but seed germination was not observed (Table 1). A decrease in percent seed sterilization was observed with the decreasing Clorox® concentration. The addition of 30, 50 and 70% Clorox® in the mediums gave rise to 49.67, 72 and 87.33% of seed sterilization, respectively. The Clorox® concentration of 50% was suitable for seed sterilization, giving rise to the highest percent seed germination (86%) and callus induction (91.33%).

Effect of 2,4-D on callus induction: The maximum callus formation ability for synthetic varieties and inbred lines was observed in MS medium supplemented with 0, 1, 2, 3,4 and 5 mg L^{-1} 2, 4-D. The results showed that the increasing 2,4-D concentrations greatly enhanced callus induction from 77.67 to 97% , when explants were cultured under a 16-h photoperiod, but in dark condition showed that callus induction dropped to 54.67% (Table 2). Under dark conditions, the highest percentage of callus induction of 63.33% was achieved in the presence of 1 mg L^{-1} 2,4-D, whereas, callus growth percentage

Table 1: Effect of Clorox® on seed sterilization

Concentrations of Clorox® (%)	Seed sterilization (%)	Seed germination (%)	Callus induction (%)
30	49.67±3.18a	75.17±0.60c	81.33±1.86c
50	72.00±1.53b	86.00±1.15d	91.33±1.86d
70	87.33±1.45b	± 2.33b	42.00±1.15b
90	100.00± 0.00d	0.00±0.00a	0.00±0.00a

Means within columns followed by different letters are significantly different using the Duncan new multiple range test (DNMRT) at p = 0.05

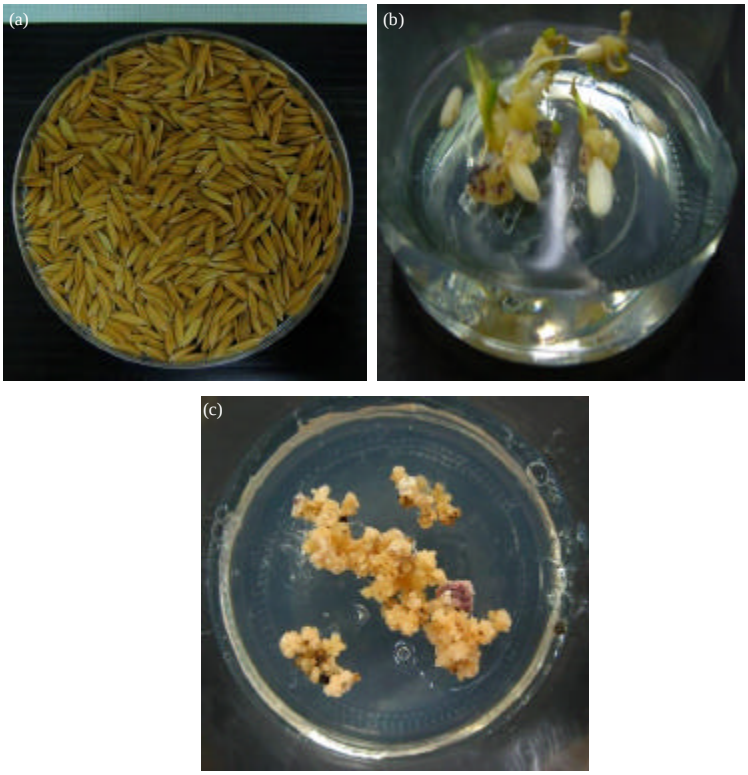

Fig. 1(a-c): (a) Mature seeds of Khunvang, (b) Callus formation after 7 days of culture, (c) Callus formation after 30 days of culture

Table 2: Effect of different concentrations of 2,4-D and illumination on the percentage of Khunvang callus induction (%) and callus growth (%)

	Illuminations					
	16/8 h photoperiod			Darkness		
Concentrations of 2,4-D (mg L^{-1})	Callus induction (%)	Callus growth (%)	Appearance	Callus induction (%)	Callus growth (%)	Appearance
0	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	-	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	-
1	77.67 ± 1.45^{b}	90.67 ± 3.48^{c}	Yellow, compact	63.33 ± 1.76^{c}	73.00 ± 2.08^{c}	White, friable
2	86.33 ± 2.33^{c}	86.00 ± 1.15^{c}	Yellow, compact	75.33 ± 2.73^{d}	81.33 ± 1.86^{d}	White, friable
3	91.00 ± 1.00^{d}	75.00 ± 2.87^{b}	Yellow, compact	66.00 ± 1.53^{c}	68.67 ± 2.03^{c}	White, friable
4	95.67 ± 1.20^{e}	74.00 ± 2.08^{b}	Yellowish brown	66.33 ± 4.48^{c}	55.67 ± 2.33^{b}	White, friable
5	97.00 ± 1.15^{e}	74.00 ± 2.31^{b}	Brown	54.67 ± 2.60^{b}	54.33 ± 4.70^{b}	White, friable

Means within columns followed by different letters are significantly different using the Duncan new multiple range test (DNMRT) at $p = 0.05$

dropped to 54.33% when the concentrations 5 mg L^{-1}. Figure 1 shows green and purple spots observed on calli. On the other hand, explants cultured on the induction medium showed callus formation in response to different 2,4-D concentrations after 7 and 30 days of culture. Calli cultured on 16/8 h photoperiod showed that callus morphology was relatively compact as compared to that grown under 24 h darkness condition (Fig. 2).

Effect of casein hydrolysate and proline on callus proliferation: An increase in concentrations of casein hydrolysate had no significant effect on callus induction and callus growth (Table 3). The highest callus growth recorded is 74% using 300 mg L^{-1} casein hydrolysate, but no significant different in medium without casein hydrolysate. Whereas, medium supplemented 900 mg L^{-1} casein hydrolysate is the highest callus weight 0.62 g (Table 3). The different

Fig. 2(a-b): Characteristics of callus induction of Khunvang; (a) Callus formation on MS medium+ 0,1,2,3,4 and 5 mg L^{-1} 2,4-D under a 16 h photoperiod, (b) Callus formation on MS medium+0, 1, 2, 3, 4 and 5 mg L^{-1} 2,4-D in the dark

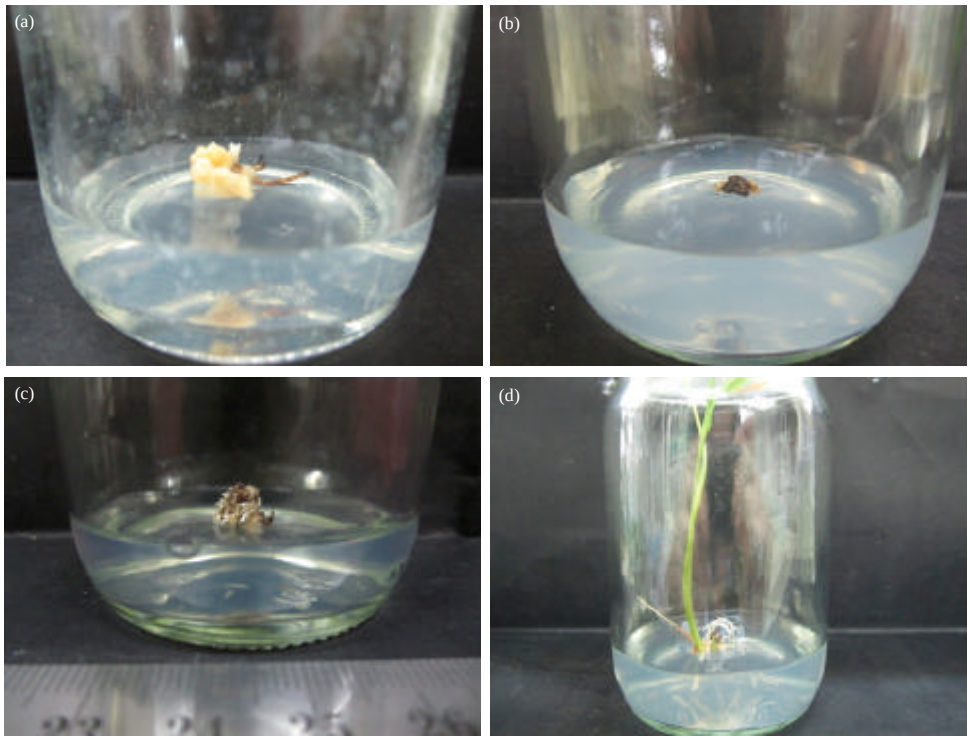

Fig. 3(a-d): Characteristics of callus regeneration of Khunvang; (a) 0 day old calli, (b) 7 day old calli, (c) 14 day old rooted shoots, (d) 30 day old fully regenerated plants

concentrations of proline give a significant effect on callus growth and callus weight of Khunvang at 900 mg L^{-1} proline but it did not gave a significant result on lower proline concentration in media. The maximum callus growth percentage of 85.67% was achieved in the presence of 900 mg L^{-1} proline but callus weight did not showed a significant result.

Effect of plant growth regulators on plant regeneration: 2 iP 2 mg L^{-1} and NAA 0.5 mg L^{-1} gave highest plant regeneration frequency of 17.5% (Table 4, Fig. 3). Low concentration of 2 iP resulted in the formation of green spots in some callus clumps, but they failed to develop into whole plantlets. On the other hand, calli cultured in mediums containing only NAA failed to develop into shoots.

Table 3: Effect of different concentrations of casein hydrolysate and proline on the percentage of Khunvang callus growth (%) and callus weight (g)

Concentrations (mg L^{-1})	Callus growth (%)	Callus weight (g)
Casein hydrolysate 0	75.33±2.91[d]	0.23±0.01[a]
300	74.00±2.08[d]	0.36±0.01[b]
500	62.67±1.45[c]	0.41±0.01[c]
700	51.67±1.67[b]	0.54±0.02[d]
900	37.33±1.45[a]	0.62±0.01[e]
Proline 0	81.00±2.08[b]	0.23±0.12[b]
300	74.00±2.08[a]	0.21±0.05[b]
500	75.00±1.53[a]	0.22±0.12[b]
700	74.00±2.08[a]	0.21±0.01[b]
900	85.67±1.20[b]	0.12±0.01[a]

Means within columns followed by different letters are significantly different using the Duncan new multiple range test (DNMRT) at p = 0.05

Table 4: Effect of different concentrations of plant growth regulators on Khunvang plant regeneration frequency (%)

2 iP:NAA (mg L^{-1})	Plant regeneration frequency (%)
0:0.5	0.00±0.00[a]
0:1	0.00±0.00[a]
0:1.5	0.00±0.00[a]
0.5:0	0.00±0.00[a]
0.5 :0.5	0.00±0.00[a]
0.5 :1	0.00±0.00[a]
0.5:1.5	0.00±0.00[a]
1:0	6.67±0.88[cd]
1:0.5	8.90±0.21[d]
1:1	3.00±0.29[b]
1:1.5	1.23±1.45[ab]
2:0	13.00±2.08[e]
2:0.5	17.50±1.44[f]
2:1	12.17±1.42[e]
2:1.5	5.33±0.33[c]

Means within columns followed by different letters are significantly different using the Duncan new multiple range test (DNMRT) at p = 0.05

DISCUSSION

The critical step for this study is to obtain high numbers of calli. Calli are masses of undifferentiated cells that are a good starting material for *in vitro* manipulation (Wani *et al*., 2011). In the present study, an attempt was made to improve the numbers of calli by optimizing illuminations, concentrations of proline and casein hydrolysate. The availability of mature seeds (Jiang *et al*., 2000) makes them suitable as explants; thus, they were used in the present study. In general, 2,4-D alone was widely used for callus induction rice and concentrations used in many previous studies ranged from 1.5 to 2.0 mg L^{-1}. However, Syaiful *et al*. (2009) recommended 2,4-D concentration up to 6 mg L^{-1} for callus induction. Low 2,4-D concentration (1 mg L^{-1}) was not strong enough; therefore, it was not suitable for callus induction of Khunvang. Our results were in good agreement with many previous studies reporting that low level of 2,4-D (1 mg L^{-1}) induced root and shoot growths rather than calli at a relatively high frequency (Lee *et al*., 2002). The maximum callus induction percentage of 97% was achieved in the presence of 5 mg L^{-1} 2,4-D under 16 h photoperiod and 63.33% was achieved in the presence of 1 mg L^{-1} 2,4-D under dark conditions. Our results showed that the frequency of callus growth was lower than that of callus induction under 16 h photoperiod. This indicates that there are a number of calli that are non-prolific; hence, they do not grow.

Different 2,4-D concentrations induced a fair amount of calli and also promoted callus growth. Swollen scutellum developed into callus within 7 days under dark and 16 h photoperiod conditions. In Poaceae, the presence of green spots in cultures has been considered as predictors of potential shoot formation (Nabors *et al*., 1982). Nutritional supplements such as casein hydrolysate, proline and glutamine, have been earlier reported to enhance callusing response (Lin and Jhang, 2005). Based on the present study, it was found that no significant results were observed on the percentage of callus induction and growth using different concentrations of casein hydrolysate. Our results were in good agreement with Tyagi *et al*. (2007) who stated that although casein hydrolysate led to significant improvement in the quality of calli, there was no improvement in callusing frequency which remained approximately 50%.

Different concentrations of proline gave significant results on the percentage of callus growth. The promotive effect of proline on the frequency of callusing and regeneration has been reported by Chowdhury *et al*. (1993). Moghaddam *et al*. (2000) also stated that the presence of proline in the culture medium seems to produce a required stress condition which decreases water potential, increases the accumulation of nutritional elements in cells and finally enhances embryogenesis. So as to enhance green-plant regeneration, supplements such as proline have been used because the use of proline in the medium has been reported to be effective for the initiation and maintenance of embryogenic calli (Datta *et al*., 1992).

CONCLUSION

Optimal medium compositions were chosen on the basis that gives a high percentage of callus induction and growth. In our experiment, the highest percentage of callus induction (97%) could be achieved by culturing mature seeds of Khunvang on MS mediums supplemented with 5 mg L^{-1} 2,4-D under a 16 h photoperiod. According to the present study, different concentrations of casein hydrolysate did not have a significant effect on callus induction and growth. In contrast, proline plays a role in promoting callus growth. Therefore, addition of proline in the medium can enhance callus induction and growth of Khunvang.

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