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Full Length Research Paper

The effect of plant growth regulators on optimization of tissue culture system in Malaysian upland rice

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Mature seeds of four upland rice cultivars namely Kusan, Lamsan, Selasi and Siam were assessed for callus induction and plant regeneration on different concentrations and combinations of plant growth regulators, incorporated into MS (Murashige and Skoog) basal medium. Callus induction frequency was significantly different among the cultivars, as well as among the 2,4-Dichlorophenoxyacetic acid (2,4-D) levels tested. All tested cultivars exhibited highest callus frequency at 2 mgl^{-1} 2,4-D. The incorporation of α-naphthaleneacetic acid (NAA) and kinetin (Kin) in the callus induction medium supplemented with 2 mgl^{-1} 2,4-D did not significantly improve the callus induction frequency. After two subcultures, at 24 days interval, the best response to callus induction was from cultivar Selasi, while callus browning became prominent in cultivars Kusan and Siam. Embryogenic callus placed on different regeneration media exhibited the highest regeneration frequency on medium containing 0.5 mgl^{-1} NAA + 2.0 mgl^{-1} Kin + 2.0 mgl^{-1} 6-benzylaminopurine (BAP). The maximum regeneration frequency was achieved in cultivar Selasi followed by Lamsan while Siam and Kusan exhibited poor regeneration response. Among the four upland rice cultivars evaluated, Selasi and Lamsan are two promising cultivars in terms of callus induction frequency and morphology, and regeneration ability of the embryogenic callus.

Key words: Callus induction frequency, regeneration frequency, plant growth regulators, upland rice.

INTRODUCTION

Rice (*Oryza sativa* L.) is a very important cereal crop globally. It is not only a staple food for more than two-third of the global population but also serves as a model plant in genomic studies (Bajaj and Mohanty, 2005; Tyagi and Mohanty, 2000).

Negative effect of water deficiency in agricultural production including rice has become a serious problem. Scientists believe that using new cultivars which have potential to survive with high yield may help solve or minimize the problem. Upland rice is a type of rice that is planted in dry lands and grown in rain-feeding or limited irrigation condition. Using this rice may save plenty of water and diminishes water pollution (Geng et al., 2008). However, other biotic and abiotic factors that may limit their cultivation and production need to be genetically improved via genetic transformation.

Routine tissue culture system including callus induction and regeneration is a fundamental requirement for successful genetic transformation (Li et al., 2007; Seraj et al., 1997). It is known that, callus induction and regeneration ability highly rely on genotypes, explant types, carbohydrate sources, plant growth regulators, basal salts of culture medium and culture conditions (Rueb et al., 1994). Many reports exist on the optimization of tissue culture system of rice cultivars, especially using plant growth regulators (Ge et al., 2006; Lee et al., 2002; Rashid et al., 2001; Zaidi et al., 2006; Zhu et al., 1996). However, there is no report so far on the tissue culture of

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Abbreviations: 2,4-D, 2,4-Dichlorophenoxyacetic acid; **NAA,** α-naphthalene acetic acid; **BAP,** 6- benzylaminopurine; **Kin,** kinetin.

Malaysian upland rice cultivars.

This study evaluates four Malaysian upland rice cultivars namely, Kusan, Lamsan, Selasi and Siam with respect to their callus induction and regeneration ability which could be used as a tool in genetic transformation for high yield and improved quality of the crop.

MATERIALS AND METHODS

Explant sterilization and culture establishment

Mature seeds of four cultivars of upland rice namely; Kusam, Lamsan, Selasi and Siam were dehusked and immersed in 70% ethanol for 2 min, followed by 50% Clorox (v/v) supplemented with 2 - 3 drops of Tween-20 for 30 min. Treated seeds were washed with sterile distilled water three times. The seeds were then placed on callus induction media and kept in the dark at 26 ± 2°C.

MS (Murashige and Skoog, 1962) basal medium was used for callus induction and plant regeneration. The MS medium supplemented with 500 mgl^{-1} proline, 300 mgl^{-1} casein hydrolysate, 30 g l^{-1} of sucrose and solidified with 0.3% (w/v) gelrite was designated as MS_A medium.

Callus induction

Different concentrations of 2,4-D (1, 2, 3 and 4 mgl^{-1}) were added into the MS_A medium for callus induction. Subculture was performed at 24 days interval on MS_A medium fortified with 2 mgl^{-1} 2, 4-D.

Callus proliferation was further evaluated by incorporating NAA and/or Kin in the MS_A medium containing 2 mgl^{-1} 2, 4-D. The treatments were MS_A + 2 mgl^{-1} 2,4-D, MS_A + 2 mgl^{-1} 2,4-D + 0.5 mgl^{-1} NAA, MS_A + 0.5 mgl^{-1} Kin and MS_A + 0.5 mgl^{-1} NAA + 0.5 mgl^{-1} Kin.

Plant regeneration

Embryogenic calli produced on MS_A medium containing 2 mgl^{-1} 2,4-D were cultured on different regeneration media for plantlet formation. The treatments were MS_1 (MS_A + 0.5 mgl^{-1} NAA + 2.0 mgl^{-1} Kin + 1.0 mgl^{-1} BAP), MS_2 (MS_A + 0.5 mgl^{-1} NAA + 2.0 mgl^{-1} Kin + 2.0 mgl^{-1} BAP) and MS_3 (MS_A + 0.5 mgl^{-1} NAA + 1.0 mgl^{-1} Kin + 1.0 mgl^{-1} BAP) (modified from Lin and Zhang, 2005), MS_4 (MS_A + 1.0 mgl^{-1} NAA + 2.0 mgl^{-1} Kin + 1.0 mgl^{-1} BAP) (Khanna and Raina, 1998), MS_5 (MS_A + 1.0 mgl^{-1} NAA + 3.0 mgl^{-1} Kin) (Kumria et al., 2001) and MS_6 (MS_A + 1.0 mgl^{-1} NAA + 3.0 mgl^{-1} BAP) (Lee et al., 2002).

Data recorded

The frequency of callus induction and plant regeneration (%) were measured using the following formulas (Zaidi et al., 2006):

$$\text{Callus induction frequency} = \frac{\text{Number of calli}}{\text{Number of incubated seeds}} \times 100$$

$$\text{Regeneration frequency} = \frac{\text{Number of regenerated calli}}{\text{Number of incubated calli}} \times 100$$

Experimental design and statistical analysis

The experiments were arranged in a completely randomized design with four replications. Each replication per treatment contained 10 - 12 seeds for callus induction and 4 - 6 embryogenic calli for plant regeneration. Data were analyzed using the two way-factorial analysis of variance (factorial ANOVA), with genotype as one treatment and plant growth regulator concentration as the other treatment. The data were subjected to SAS 9.1 software and least significant difference (LSD) was used for comparison between the treatments means (Compton, 1994).

RESULTS

The scutellum region of the seeds swelled in 4 - 5 days and calli were produced from the four cultivars after 17 - 22 days (Figure 1a). Morphologically, two types of callus were produced; embryogenic and non embryogenic (Figures 1a and b). Embryogenic calli were nodular and compact, 1 to 2 mm in diameter, and white to lemon in color. The non-embryogenic calli were completely yellow or bright brown in color, and were more intense than the embryogenic calli.

Significant difference in callus induction frequency was observed among the cultivars as well as among the 2,4-D concentrations tested (Table 1); however the interaction between the cultivars and 2,4-D concentrations was not significant. Among the cultivars, Lamsan gave the highest callus induction frequency (84%) and Siam the poorest (39%) (Table 1). In addition, all tested cultivars reached their highest callus induction frequency at 2 mgl^{-1} 2,4-D.

The callus induction frequencies were not significantly different with the addition of NAA and Kin, either alone or in combination, into MS_A supplemented with 2,4-D (Table 2). Using merely 2, 4-D gave the highest response of 77.9% while combination of 2,4-D, NAA and Kin led to the lowest callus induction frequency of 65.8%. Meanwhile, significant response to callus induction was observed among the different cultivars. In the case of cultivars Kusan and Selasi, the response was better when 2,4-D and NAA were combined, while cultivars Lamsan and Siam performed better on medium with 2,4-D alone. Both the cultivars effect and interaction between cultivars and plant growth regulators were statistically significant. The maximum and minimum responses to callus induction were clearly shown by cultivar Lamsan (88.9%) and Siam (51.9%), respectively (Table 2).

Since subculture is obligatory in a transformation process, two cycles of subculture were performed, and obviously callus growth and browning became more evident with the subculture (Figure 1b). Among the cultivars, Selasi showed the best callus growth in both subculture cycles (Table 3). The growth of Lamsan callus was good in subculture 1, but decreased dramatically in subculture 2. Callus from cultivar Siam showed browning in both subculture cycles, while the callus of cultivar Kusan turned brown at subculture 2.

Green spots appeared from most of the calli of the four cultivars (Figure 1c) after 10 - 12 days on regenerated media. Nevertheless, no green spots were observed on calli that turned brown on the regeneration media (Figure

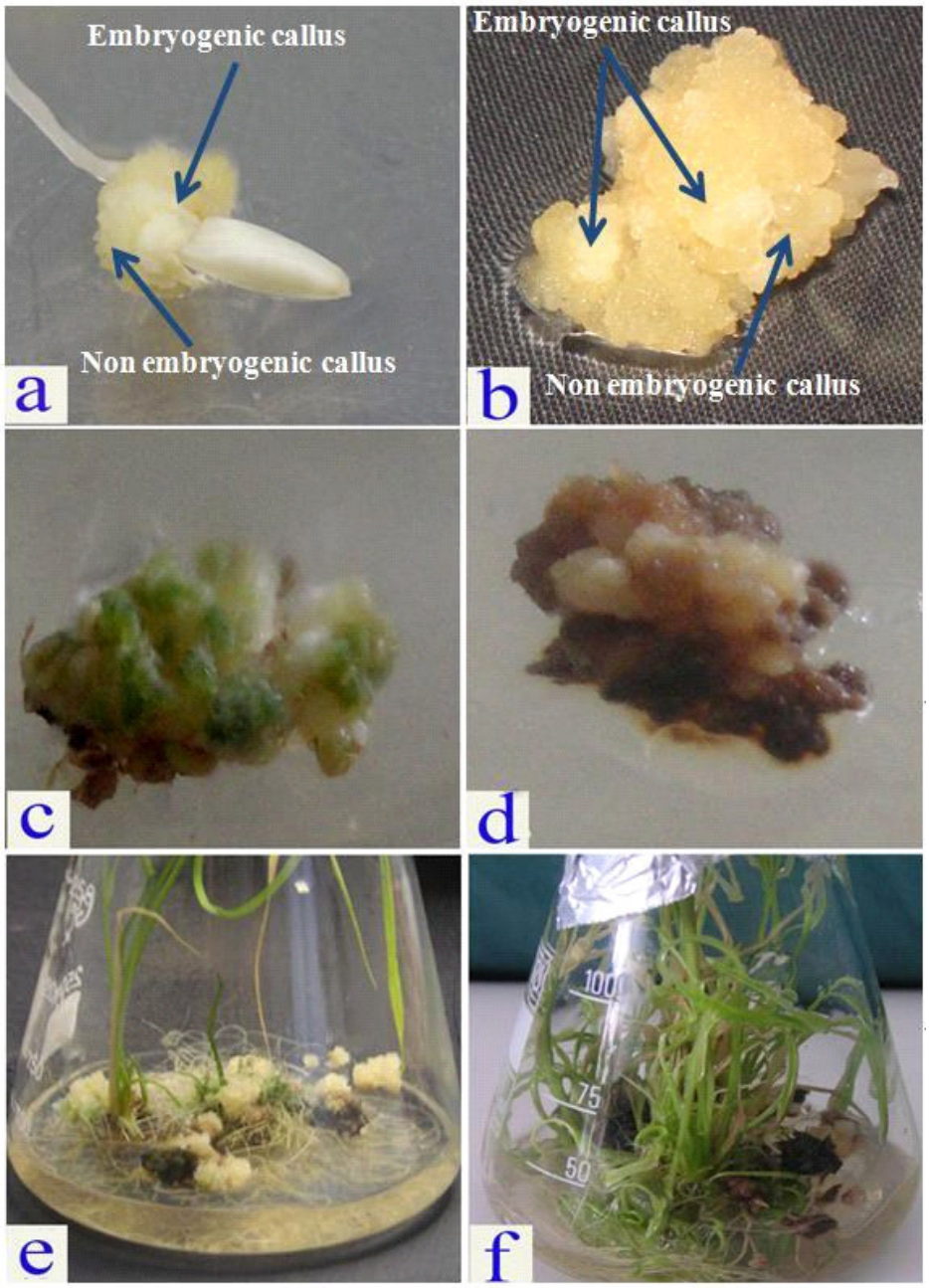

Figure 1. Callus induction and plant regeneration of Malaysian upland rice cultivars. (a) Callus induced on a mature seed after 21 days on medium with 2,4-D; (b) callus proliferation after one subculture cycle on medium with 2,4-D; (c) the appearance of green spots after 12 days on regeneration medium; (d) browning of callus on regeneration medium; (e) shoots produced on regeneration medium; and (f) the recovery of plantlets.

1d). About 15 - 20 days after the appearance of the green spots, shoot and root regeneration occurred simultaneously producing plantlets (Figures 1e and f).

Different combinations of plant growth regulators incorporated into MS_A (designated as MS_1, MS_2, MS_3, MS_4, MS_5 and MS_6) were tested for the optimization of the regeneration system. Both the cultivars effect and the plant growth regulators effect were significantly different (Table 4). In addition, the two-way ANOVA confirmed an interaction between growth regulator combinations and cultivars. The regeneration frequency varied from 0.0 up to 70.0% among the different cultivars and media (Table 4). With respect to the plant growth regulator combinations, the highest (35.9%) and lowest (16.0%) regeneration frequency occurred on MS_2 (MS_A + 0.5 mgl^{-1} NAA + 2.0 mgl^{-1} Kin +2.0 mgl^{-1} BAP) and MS_1 (MS_A + 0.5 mgl^{-1} NAA

Table 1. Effect of 2,4-D on callus induction frequency (%) of four Malaysian upland rice cultivars after 24 days of culture.

2,4-D (mgl^{-1})	Cultivar				
	Kusan	**Lamsan**	**Selasi**	**Siam**	**Means**
1	49.5a	73.7b	58.3b	43.7a	56.3BC
2	56.2a	100a	91.7a	47.9a	73.9A
3	43.7a	89.6ab	79.2ab	41.7a	63.5B
4	52.0a	72.7b	58.1ab	22.9a	51.5C
Means	50.4C	84.0A	71.8B	39.0D	
P value of cultivars effects	0.0001**				
P value of plant growth regulators	0.0001**				
P value of cultivars × plant growth regulators	0.0817				

*,**Significant at 0.05 and 0.01 level, respectively; means with the same letter are not significantly different at 0.05 probability level using LSD; small alphabets (a, b, c,.....) refer to differences based on separate analysis of variation for each genotype; capital alphabets (A, B, C,.....) refer to differences among cultivars and plant growth regulators concentrations in a factorial analysis.

Table 2. Effect of NAA and Kin incorporated into MS_A medium fortified with 2 mgl^{-1} 2,4-D. on callus induction frequency (%) of four Malaysian upland rice cultivars after 24 days of culture.

2,4-D + NAA + Kin (mgl^{-1})	Cultivar				
	Kusan	**Lamsan**	**Selasi**	**Siam**	**Means**
2+0+0	68.8a	93.7a	77.1a	72.1a	77.9A
2+0.5+0	72.9a	89.0a	81.2a	38.6b	70.4A
2+0+0.5	52.1a	87.3a	68.7a	60.4ab	67.5A
2+0.5+0.5	72.5a	85.4a	68.8a	36.7a	65.8A
Means	66.9B	88.9A	74.00B	51.9C	
P value of cultivars effects	0.0001**				
P value of plant growth regulators	0.0775				
P value of cultivars × plant growth regulators	0.0351*				

*,**Significant at 0.05 and 0.01 level respectively; means with the same letter are not significantly different at 0.05 probability level using LSD; small alphabets (a, b, c, ...) refer to differences based on separate analysis of variation for each genotype; capital alphabets (A, B, C, ...) refer to differences among cultivars and plant growth regulators concentrations in a factorial analysis.

+ 2.0 mgl^{-1} Kin + 1.0 mgl^{-1} BAP), respectively. Based on the cultivars effect, Lamsan exhibited the highest regeneration response of 38.1% while Kusan showed the lowest at 0.80%. Meanwhile, the best regeneration response was from Selasi on MS_2 at an average of 70.0%, followed by Lamsan at 63.7% also on MS_2. The regeneration response of Kusan was zero on all the growth regulator combinations, except on MS_6 (Table 4).

DISCUSSION

Mature seeds have been successfully used as explants to obtain embryogenic calli that have the ability to produce plantlets in four Malaysian upland rice cultivars. As earlier mentioned, an efficient plant regeneration system is vital for transformation experiment. Using seeds as explants has several advantages such as the availability of seeds all year round and the ease of storing seeds compared to other explants (Ge et al., 2006; Lee et al., 2002).

Significant differences in callus induction were detected among the cultivars when different concentrations of 2,4-D were used. The results of this study also showed that the presence of 2,4-D in culture medium is vital for rice callus induction from mature seeds. Absence of 2,4-D resulted in no callus formation among the tested cultivars. In most tissue culture experiments, a high auxin/cytokinin ratio is used for starting embryogenic callus formation compared to a low ratio for the regeneration of plantlets (Ge et al., 2006). The exact molecular function of plant growth regulators in tissue culture is unclear; however, it may probably be involved in the reprogramming of the expression of embryogenic genes (Ge et al., 2006).

Adding Kin and NAA to the callus induction media supplemented with 2,4-D had no significant effect on

Table 3. Morphological characteristics of callus from two subcultures of four Malaysian upland rice cultivars

Cultivar	Callus growth (subculture 1)*	Callus browning	Callus growth (subculture 2) *	Callus browning
Kusan	++ +	No	+++	Yes
Lamsan	++++	No	++	No
Selasi	++++	No	++++	No
Siam	++	Yes	++	Yes

* Indicator for callus growth in subculture 1 and 2; ++++ = very good growth; +++ = good growth; ++ = medium growth; + = low growth.

Table 4. Effect of media incorporated with different hormonal combinations on regeneration frequency of four Malaysian upland rice cultivars after 22 days of culture.

Treatment	Cultivar				
	Kusan	Lamsan	Selasi	Siam	Means
MS_1	0.00a	38.3bc	25.8cd	0.00b	16.0B
MS_2	0.00a	63.7a	70.0a	10.0b	35.9A
MS_3	0.00a	45.0b	40.0bc	10.0b	23.7B
MS_4	0.00a	43.3b	52.5ab	0.00b	23.9B
MS_5	0.00a	16.2d	50.0ab	20.0ab	21.6B
MS_6	0.50a	21.7cd	11.3d	37.5a	18.8B
Means	0.80C	38.1A	41.6A	12.9B	
P value of cultivars effects	0.0013*				
P value of media	0.0001**				
P value of cultivars × media	0.0001**				

*,**Significant at 0.05 and 0.01 level respectively; means with the same letter were not significantly different at 0.05 probability level using LSD; small alphabets (a, b, c, ...) refer to differences based on separate analysis of variation for each genotype; capital alphabets (A, B, C, ...) refer to differences among cultivars and plant growth regulators levels in factorial analysis.

callus formation. Instead, 2,4-D was found to be more effective singly for the production of embryogenic calli. The results were in agreement with that of Rashid et al. (2001) but in no harmony with some other researchers who showed 2,4-D in combination with kinetin was more effective in producing embryogenic callus (Ge et al., 2006). Generally, most researchers working on rice have used only 2,4-D for induction, proliferation and maintenance of callus either for tissue culture or for transformation experiments (Geng et al., 2008; Kumria et al., 2001; Lin and Zhang, 2005; Seraj et al., 1997).

The cultivars response to tissue culture was also dramatically different. Lamsan and Selasi showed better response to callus induction and regeneration compared to Kusan and Siam. Interestingly, Lamsan produced more calli than Selasi but the regeneration ability of Lamsan was less than Selasi, which obviously indicate that Lamsan produced more non embryogenic calli. Most calli from Kusan and Siam were non embryogenic and for this reason, including the browning of callus, led to the low regeneration ability. Furthermore, both callus induction and regeneration ability normally differs between distinctive cultivars. Some cultivars respond better than others and which seems to be genetically controlled (Li et al., 2007; Ozawa et al., 2003; TaguchiShiobara et al., 1997).

Regardless of the regeneration capacity, results from the subculture experiment showed that Kusan and Siam have no potential for transformation especially using *Agrobacterium* method, since calli from both cultivars exhibited serious browning.

Conclusion

Based on the callus induction, subculture and regeneration results, Lamsan and Selasi are the most applicable upland rice cultivars for genetic transformation to produce high yielding quality crop.

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