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# Improved plantlet regeneration systems in *Indica* rice (*Oryza sativa* L.) landrace *Hom Kra Dang Ngah*

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**Abstract** In order to improve the plantlet regeneration systems from embryogenic calli in *indica* rice landrace *Hom Kra Dang Ngah*, the effect of culture media and plant phytohormones was investigated. Different culture media, such as MS, N6, ARDA supplemented with different concentrations of auxin [α-naphthalene acetic acid (NAA)] and cytokinins [kinetin (Kn) and 6-benzyladenine (6-BA)] were examined. In addition, different types of carbohydrates as carbon source and concentrations of phytagel were tested. The results showed that ARDA medium supplemented with 0.5 mg/L NAA in combination with 1.0 mg/L 6-BA, 2.0 mg/L Kn, 82mM sorbitol and 1g/L casein hydrolysate, solidified with 0.3% phytagel gave the highest responsive for plantlet regeneration at 75%. For root initiation experiment, the result indicated MS medium supplemented with 0.1 mg/L IAA gave the highest number of roots. All *in vitro* regenerated plantlets were morphological and physiologically healthy and normal. A 100% of survival rate of plantlets was obtained after being transferred to soil for one month.

**Keywords:** *Indica* rice, *Oryza sativa*, *Hom Kra Dang Ngah*, Embryogenic calli, Plantlet regeneration system

## Introduction

Rice (*Oryza sativa* L.) is a major staple food and one of the most important crops in the worldwide. More than half of the world's population depends on rice for its major daily source of energy and protein. With the increase in population and reduction of arable land, together with the biotic and abiotic stress rice production is under great pressure. Breeding new rice varieties using genetic engineering techniques is one of the ways to increase rice yield and improve the total rice production. In recent year, genetic transformation technology as a key technique for improvement desirable traits of rice has made a great progress. However, the transformation of *indica* rice is

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still difficult due to a low induction rate of embryogenic callus and plantlet regeneration after transformation. Many *indica* rices are particularly genotype-dependent.

The regeneration of calli is the most difficult problem in genetic transformation process. The physiological activity of calli will be decreased with the selection and differentiation. A large number of browning calli appeared, which lead to cell die and difficult differentiation. So, the decrement of browning calli is directly promoted the frequency of in vitro differentiation. In order to solve this problem, addition of ascorbic acid in culture medium was recommended. In addition, the presence of mannitol in subculture medium followed by regular transfer calli to fresh medium of the same component could amend browning of the callus (Reddy, 1986). Ye *et al*. (2001) reported that macro-elements of N6 in combination with MS micro-elements are helpful to calli differentiation. Reducing quantity of sucrose in differentiation medium, pulsing sorbitol and addition of zeatin to culture medium enhance the percentage of calli differentiation in indica rice. L-proline, as an organic nitrogen and osmotic regulatory, can have a positive impact on calli and regeneration ability (Shahsavari, 2010).

Although there has been some success in plant regeneration from rice tissue culture, the protocol developed is not applicable to all the cultivars of rice. Therefore, the present study was carried out to develop a high frequency regeneration system in *indica* rice, landrace *Hom Kra Dang Ngha* in order to use as a tool for further efficient gene transformation.

## Materials and methods

### *Plant material and explants preparation*

Mature seeds of *Oryza sativa* L. landrace *Hom Kra Dang Ngah* were used as explant source. Mature and healthy seeds were selected, dehusked, and surface sterilized by the protocol described by Zhang and Te-chato (2012). Sterile seeds were sown on MS (Murashige and Skoog, 1962) medium with different plant growth regulators (PGR) for callus induction.

### *Callus induction and proliferation*

Sterile mature seeds were cultured on solidified MS medium for callus induction. The medium were supplemented with 2 mg/L 2,4-D, 1mg/L NAA, 1mg/L 6-BA and 0.5mg/L Kn containing 3% sucrose and 1g/L casein hydrolysate (CH). After that the medium was solidified with 0.75 % agar-agar powder. The pH of the medium was adjusted to 5.7 prior to autoclaving at

121$^{o}$C, 1.07 kg/cm$^{2}$ for 15 min. All cultures were placed in the culture room at 27±2 $^{o}$C, under 16 hour photoperiod. After 4 weeks of culture, the frequency of callus induction was recorded.

The calli initiated on above medium were transferred to proliferation medium which was MS supplemented with 3% sucrose, 1 mg/L 2,4-D and 0.5mg/L NAA, 0.5mg/L 6-BA, 0.25mg/L Kn and 1g/L casein hydrolysate (CH). The medium was solidified with 0.75 % agar. After being cultured for 3 weeks the yellowish-white compact or granular embryogenic calli were selected and transferred to shoot induction medium (SIM).

### *Plantlet regeneration*

### *Effect of culture media*

To understand the effects of culture media types on plantlet regeneration, three basal culture media were used in present experiment. Those were MS, N6 (Chu *et al*., 1975), and ARDA (Te-chato and Yenchon, unpublished data) medium. All culture media were supplemented with 3% sucrose, 0.5 mg/ L NAA, 1.0 mg/L BA, 2.0 mg/L Kin and 1 g/L of CH. All three culture media were adjusted to pH 5.7 by 1N KOH and solidified with 0.75% agar. The cultures were placed in the culture room under the same conditions as mentioned in materials and methods section. After 3-4 weeks of culture, the percentage of green spots forming calli and time required for green spot formation were recorded and compared statistically.

### *Effect of phytagel*

The best result of culture medium obtained from the above experiment was selected and used for further studying the effect of phytagel on plantlet regeneration. The medium was supplemented with 3% sucrose, 0.5 mg /L NAA, 1.0 mg /L BA and 2.0 mg /L. The culture medium was adjusted to pH 5.7 by 1N KOH and solidified with different concentrations of phytagel (0.17, 0.25, 0.3 and 0.35%) in comparison with 0.75% agar-agar powder (as control treatment). Embryogenic calli obtained from CIM were transferred to various concentrations of phytagel solidified medium. After 4 weeks of culture the frequency of green spots forming callus, percentage of plantlet regeneration and a number plantlets per callus were recorded and compared statistically.

***Effect of different ratios of cytokinin (BA+Kn) to auxin (NAA)***

Different ratios of auxin and cytokinin based on their concentrations were used to find out an appropriate ratio for induction of plantlet regeneration. The experiment was consisted of 4 treatments as following:

(1) 1.0 mg /L BA+ 2.0 mg /L Kin+1.0 mg/ L NAA; ration of BA+Kn/NAA=3:1

(2) 1.5 mg /L BA+ 2.0 mg /L Kin+1.0 mg/ L NAA; ration of BA+Kn/NAA=3.5:1

(3) 1.0 mg /L BA+ 2.0 mg /L Kin+0.5 mg/ L NAA; ration *of* BA+Kn/NAA=6:1

(4) 1.5 mg /L BA+ 2.0 mg /L Kin+0.5 mg/ L NAA; ration of BA+Kn/NAA=7:1

Embryogenic calli obtained from CIM were transferred to different ratios of cytokinin (BA+Kn) to auxin (NAA) containing medium. After 4 weeks of culture the frequency of green spots forming callus, percentage of plantlet regeneration and a number plantlets per callus were recorded and compared statistically. At the same time, root formation was recorded.

***Effect of some osmotic regulatory substances and organic nitrogens***

Two osmotic regulatory substances, sorbitol and L-proline and two organic nitrogens, glutamine and casein hydrolysate were supplemented to the best resulting SIM. L-proline at concentrations 0, 500 mg/L, glutamine at 0, 300, 500 mg/L, casein hydrolysate at 1g/L and 82 mM sorbitol were added to the SIM, together with 3% sucrose and solidified with optimal concentration of phytagel from previous experiment. For PGR, 0.5 mg/ L NAA together with 1 mg /L BA and 2.0 mg /L Kin were employed. After being cultured for 4 weeks, the frequency of plantlet regeneration and a number of regenerated plantlets per callus were recorded and statistically compared.

***Root induction and acclimatization***

The regenerated plantlets at 3-4cm in height were excised and transferred to culture tubes (25x125mm) containing 10 ml of MS basal medium without PGR or supplemented with 0.1mg/l IAA for root initiation. After vigorous roots appear, complete plantlets were transferred to clay soil containing in 12 inch plastic pots covered with plastic bottle and acclimatized in the greenhouse at 28-30℃supplied by natural light conditions until the plants were healthy enough and ready to transfer to field conditions.

### *Statistical analysis*

The experiment was designed as a randomized complete block design (RCBD) with 3 replicates per treatment. Analysis of variance (ANOVA) was applied to indicate which variety or combination was optimal and gave the best results for plantlet regeneration. Data were analyzed using the SAS program Version 6.11 (SAS Institute, Cary, NC). A probability level of 5% (p=0.05) is chosen for all statistical inferences. Means among treatment were separated by least significant difference (LSD).

## Results

### *Callus induction and proliferation*

In this study, calli produced from suitable media (obtained from previous study) were obtained after 4 weeks of inoculation. After being transferred to subculture medium for proliferation for 4 weeks, two types of callus were obtained, embryogenic and non-embryogenic ones (Fig. 1.). Calli that were creamy white, some compact, friable and globular are defined as embryogenic, which further developed into plantlets (Fig. 1 A). By contrast, the non-embryogenic calli were completely yellow or bright brown in color with soft or compact in texture (Fig. 1 B) and couldn't develop into plantlets.

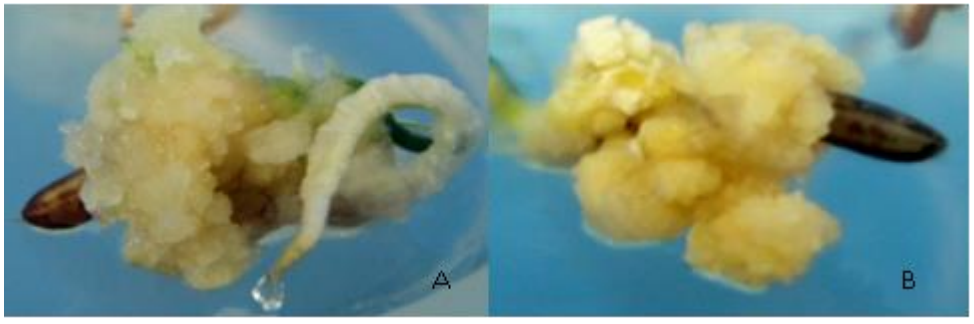


**Fig. 1.** Morphological characters of embryogenic callus and non- embryogenic callus on MS medium after being culture for 4 weeks.
A: Embryogenic callus: creamy white, some what compact, friable and globular; B: Non-embryogenic callus: completely yellow or bright brown in color with soft and compact texture.

### *Plantlet regeneration*

### *Effect of culture media on plantlet regeneration*

The embryogenic calli were transferred to different plantlet regeneration culture media for improving rice plantlet regeneration system. The calli showed different response in regeneration ability on the three different types of culture media. The earliest time for green spot formation in callus was 20 days of culture on ARDA medium followed by N6 medium. Among those three culture media ARDA gave the best results in both time and frequency for green spot formation. After 20 days of culture on plantlet regeneration media, the calli developed green spots and a maximum of 61.3% green spot produced calli were obtained on ARDA medium. Those green spots developed into shoots after 30 days of culture. In $N_6$ medium, the calli at 8.6% developed green spots after the same time of culture. However, this phenomenon was not observed on MS medium (Fig. 2.). The results indicated that the regeneration frequency (0.0–61.3%) was significantly different among the three tested culture media (Fig. 3.). With respect to medium types, the highest regeneration frequency at 61.3% took place on ARDA medium while the lowest regeneration frequency at 0% was obtained from MS medium. For this reason, ARDA medium was applied for culturing callus in the next experiments.

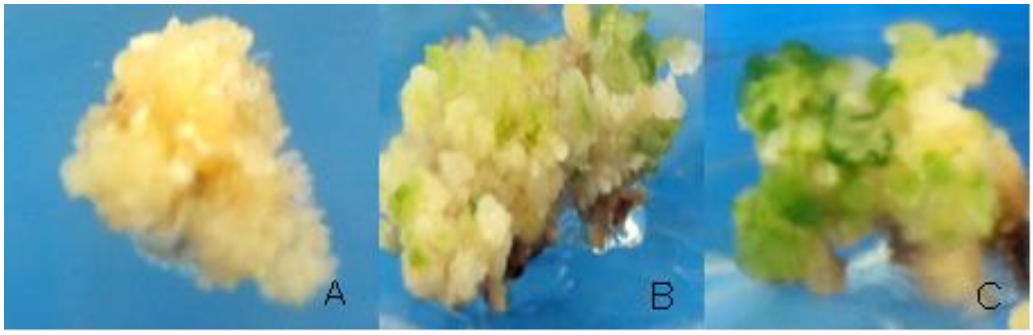


**Fig. 2.** Development of green spots in embryoenic calli cultured on different culture media for 30 days.
A: MS medium; B: $N_6$ medium; C: ARDA medium.

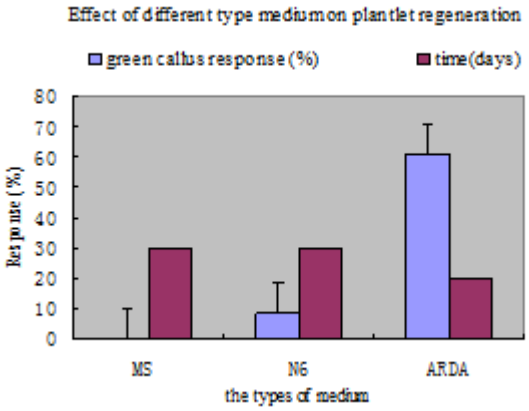


**Fig. 3.** Effect of culture media on green spot formation frequencies and time required for green spot development.

### *Effect of phytagel on plantlet regeneration*

The embryogenic calli exhibited a variable response with respect to agar powder and phytagel concentration in the plantlet regeneration medium. Firstly, the ANOVA results indicated that the frequency of green spots forming calli and percentage of plantlet regeneration on phytagel solidified medium were significant difference from those obtained on agar powder solidified one (Table 1.). Therefore, phytagel was more suitable for plantlet regeneration than agar powder. Secondly, higher strength of the phytagel (more than 0.25%) was more beneficial for the differentiation of calli and plantlet regeneration than the low concentration (0.17%). Some calli cultured on the low concentration phytagel just turned into green spots but were not able to develop into shoots. So the percentage of plantlet regeneration was as low as 8.3%. However, most of the green spots developed in a low concentration of phytagel (2.5%) containing medium and those green spots formed a large number of multiple shoots after transferring to culture medium containing higher concentration of phytagel (>0.25%). Meanwhile, most of the green spots developed in high concentration of phytagel (>0.25%) and gave a large number of multiple shoots after transferring to culture medium (Table 1.). moreover, It was observed that phytagel at concentration of 0.3% promoted the highest frequency of green spot forming calli at 100% and produced the highest number of regenerated shoots per callus at 7 shoots with a frequency of 61.0 %. Phytagel at 3.5% also gave frequency of green spot forming calli at 100% (same as that of 3%) but frequency of shoot formation was slightly higher (63.3%). Even though frequency of shoot formation was slightly higher but a number of shoots produced was slightly lower at 6 shoots per callus. The two concentrations of

phytagel, 0.3% and 0.35% exhibited non significant differences on plantlet regeneration frequency, however, significant differences were found with the other two concentrations (0.17% and 0.25%) (Table 1). So, the concentration of phytael at 0.3% was chosen and used in the following experiments.

**Table 1.** Effect of agar and different concentrations of phytagel on plantlet regeneration capacity

| Gelling agent (%) | Calli forming green spots(%) | Plantlet regeneration frequency (%) | No. of shoots/ callus |
|---|---|---|---|
| Agar(0.75%)(control) | 32.1b | 8.3c | 3 |
| Phytagel(0.17%) | 0.0c | 0.0d | 0 |
| Phytagel(0.25%) | 87.5a | 21.5b | 5 |
| Phytagel(0.30%) | 100a | 61.0a | 7 |
| Phytagel(0.35%) | 100a | 63.6a | 6 |
| F-test | * | * | |
| C.V.(%) | 71.1 | 96.1 | |

*Significant difference at 0.05 level.
Means of those parameters with the same letter within column are not significantly different using LSD.

### *Effect of different ratio of cytokinin (BA+Kn) to auxin (NAA)*

In this experiment the results showed that different ratios of (BA+Kn) to NAA gave the various frequencies of differentiation and plantlet regeneration. The ratio of (BA+Kn) to NAA at 6:1 gave the highest frequency of green spots forming calli and plantlets regeneration at 75.5% and 33.3%, respectively. Plantlets obtained on that ratio of PGR containing medium produced roots at the same time (Fig. 4D). Lower ratio of those PGRs at 3 or 3.5 to 1 gave the lower percentage of green spots forming calli and plantlets regeneration (Table 2.). In addition, the plantlets obtained on these PGRs containing medium didn't produce roots (Fig. 4C). The results from this study suggested that subsequent plantlet regeneration from green spots forming calli depends upon the ratio of (BA+Kn) to NAA. In this case, the ratio of (BA+Kn) to NAA at 6:1 was the most suitable for induction of green spots forming calli, plantlet regeneration and root formation.

**Table 2.** Effects of different ratio of (BA+Kn) to NAA containing ARDA medium on the response of calli after being cultured for 4 weeks

| NAA | BA mg/L | Kn | Ratio of (BA+Kn):NAA | Greenspots forming calli (%) | Plantlet Regeneration (%) | Roots |
|---|---|---|---|---|---|---|
| 0.5 | 1.0 | 2.0 | 6:1 | 75.5a | 33.3a | Yes |
| 0.5 | 1.5 | 2.0 | 7:1 | 69.3a | 32.1a | Yes |
| 1.0 | 1.0 | 2.0 | 3:1 | 45.8bc | 20.3b | No |
| 1.0 | 1.5 | 2.0 | 3.5:1 | 52.5 b | 25.4b | No |
| F-test | | | | * | * | |
| C.V.(%) | | | | 22.9 | 21.9 | |

*Significant difference at 0.05 level

Means of callus response frequency with the same letter within column are not significantly different using LSD.

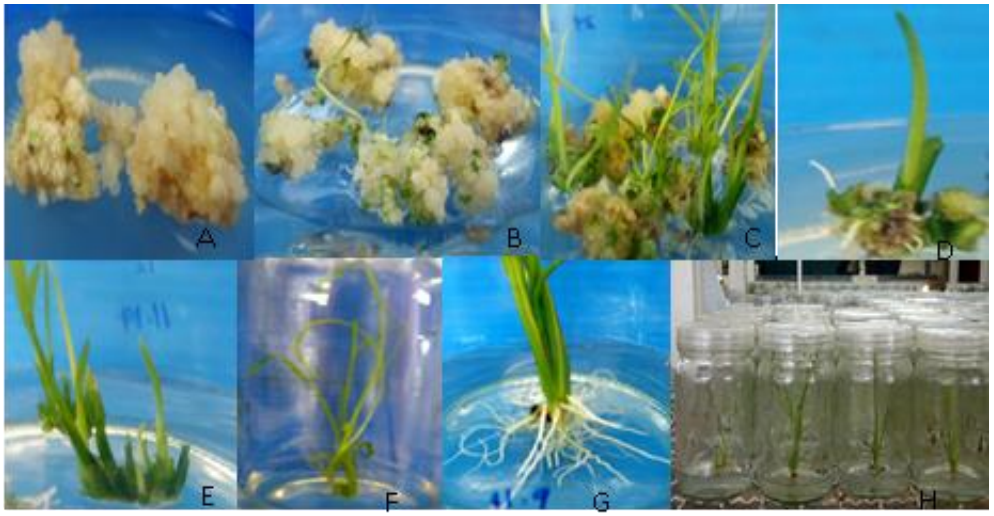


**Fig. 4.** The different stages of plantlet regeneration from somatic embryos or green spots developed in embryogenic calli after being cultured on SIM medium with 0.5 mg/L NAA, 1 mg/L BA and 2.0 mg/L Kin.

A. Proliferation of callus on CIM after 3 weeks of subculture; B. Green spot formation on SIM after 3 weeks of first round regeneration; C. Shoots developed from green spots on SIM after 3 weeks of second round regeneration; D. Shoots produced on regeneration medium supplemented with 1.5mg/L 6-BA + 0.5 mg/L NAA + 2.0 mg/L Kn, root formed after 3 weeks of regeneration culture; E & F. Shoot or cluster of shoots at 3-4 cm in height just after transfer to PGR-free MS medium for root initiation; G & H. Root formation from shoot or cluster of shoots after 3 weeks of culture on PGR-free MS medium.

### *Effect of some osmotic regulatory substances and organic nitrogens*

Organic nitrogen in term of casein hydrolysate (CH) alone at concentration of 1 g/L never promoted plantlet regeneration from green spots forming calli. Decrease in concentration of CH to 300 mg/L in combination with L-proline and glutamine at the same concentration of 500 mg/L slightly improved plantlet regeneration (27.3%). Replacement of the two organic nitrogens, L-proline and glutamine with sorbitol at concentration of 82 mM gave significant results. Sorbitol provided the highest plantlet regeneration percentage at 75% (Table 3.).

**Table 3.** Effect of osmoregulatory substances and organic nitrogen containing SIM on plantlet regeneration response after being cultured for 4 weeks

| Treatments | Cultured calli | Green spots forming calli | Plantlet regeneration (%) |
|---|---|---|---|
| asein hydrolysate (1g/L) | 28 | 20 | 0.0c |
| Casein hydrolysate (300mg/L) +L-proline (500mg/L) + glutamine (500mg/L) | 28 | 22 | 27.3b |
| Casein hydrolysate (1g/L) + sorbitol (82mM) | 28 | 26 | 75a |
| F-test | | | * |
| C.V. (%) | | | 90.9 |

*Significant difference at 0.05 level

Means of plantlet regeneration frequency with the same letter within column are not significantly different using LSD.

### *Root induction and acclimatization*

The regenerated plantlets at height of 3-4cm excised and transferred to rooting medium responded in different way. This evidence depended upon the former culture media. Shoots on ARDA medium with 0.5 mg /L NAA together with 1.5 mg /L BA with 2.0 mg /L Kin produced complete roots on PGR-free MS medium after 3 weeks of culture (Fig. 4G). On the other hand, shoots on ARDA medium supplemented with 1 mg /L NAA together with 1 mg /L BA with 2.0 mg /L Kin produced roots on MS medium with 0.1mg/L IAA (Fig. 4H). The frequency of root induction reached at 100% in both PGR-free and IAA containing MS medium.

## Discussions

Many factors affecting plantlet regeneration have been reported in tissue culture of rice species, such as medium composition, plant growth regulator (PGR), nitrogen sources, carbon source and so on. In addition, the callus induction medium and proliferation medium together with PGR are important role for plantlet regeneration.

In the present study, the regeneration systems of landrace *Hom Kra Dang Ngah* were optimized. The findings revealed that the composition of culture media had significant effect on plantlet regeneration. Among the three culture media, the ARDA medium provided a maximum percentage of plantlet regeneration. Many authors have reported the use of different culture media, such as MS, $N_6$, $B_5$, NB etc. in tissue culture of rice species (Liu *et al.*, 2005; Wang *et al.*, 2007; Ge *et al.*, 2006). The composition and strength of mineral salts in the media is the main reason which caused the differences of callus induction and plantlet regeneration. Ge *et al.* (2006) optimized the medium S for callus subculture through increasing the $MnSO_4 \cdot 4H_2O$ and other trace elements, base on MS maximum constituent, which improved the regeneration of embryogenic callus. In this present study, the concentration of $MnSO_4 \cdot 4H_2O$ in ARDA medium is higher than that in MS and $N_6$. Therefore, it might be one of the results that improve plantlet regeneration in landrace *Hom Kra Dang Ngah* rice like the report of Ge *et al.* (2006). It is well known that *indica* rice has strong genotype unique, so, it is essential to optimize culture media separately for each genotype before carrying out any transformation experiments (Zaidi *et al.*, 2006; Abolade *et al.*, 2008).

Gelling agent also play role in callus induction subsequent to plantlet regeneration. Both type and strength have significant important in those responses. Agar is thought to contain agropectins with its sulphate side groups and some other organic impurities that might have inhibitory effects on the explant growth and callus proliferation (Bhojani *et al*., 1996). Phytagel is said to be free of such impurities as have been found in agar. In this present study, the different results between phytagel and agar were investigated. The increase in concentration of phytagel resulted in the increment of plantlet regeneration frequency. Available of water under low concentration of gelling agent and high humidity of *in vitro* culture conditions were reported to decrease the plantlet regeneration frequency from calli (Bhojani *et al*., 1996). So, in this study, 0.3% phytagel was the most suitable for plantlet regeneration in *indica* rice landrace *Hom Kra Dang Ngah* in comparison with the normal concentration of 0.17-0.25%. The contribution of these gelling agents might adjust the humidity, available of water and mineral salts uptake by the callus under *in vitro* culture conditions like those reported by Zaidi *et al.* (2006).

In most tissue culture experiments, a high auxin to cytokinin ratio is used for initiation of embryogenic callus whereas a low ratio of those PGRs is used for plantlet regeneration (Ge *et al*., 2006). Usually, cytokinins are negative regulators of root growth and development (Werner *et al*., 2003). Auxins are also thought to exert control over the cell cycle by regulating key genes (Blilou *et al*., 2002; Del Pozo *et al*., 2002). The cell cycle is therefore under the influence of both PGRs. Hence, auxin to cytokinin ratio is important for the control of many developmental processes, including organ regeneration from differentiated tissue. The type of interaction may be synergistic, antagonistic or additive (Coenen and Lomax, 1997) depending upon the tissue, its developmental stage and culture conditions (Jaillais and Chory, 2010). Together, these mechanisms coherently coordinate developmental decisions (Busch and Benfey, 2010) culminating into callogenesis, organogenesis or embryogenesis. The present study has also demonstrated that the auxin to cytokinin ratio is decisive to *in vitro* response of plant tissues.

The higher ratio of cytokinin to auxin enhanced the plantlet regeneration. In addition, the ratio of (BA+Kn) to NAA ranging from 3:1 to 7:1 promoted plantlet regeneration in landrace *Hom Kra Ngah* rice. However, the ratio at 6:1 showed highest efficiency. Lu *et al*. (2006) also reported that the ration of (BA+Kn) to NAA at 7.7:1 was suitable for regeneration in *indica* rice cultivar boB. In case of *indica* rice cultivar Super Basmati a low ration of Kn to NAA at 3:1 was reported to be the most suitable for plantlet regeneration (Faiz *et al*., 2012). The different responses of the cultivar might be due to strong genotype-independent and recalcitrant in *indica* rice. So, it is necessary to regulate the ratio of cytokinin to auxin according to various genotypes in order to promote regeneration frequency.

The response of landrace *Hom Kra Dang Ngah* to tissue culture was affected by the type and concentrations of nitrogen sources and some osmoregulatory substance. Our results showed that sorbitol was more effective than L-proline, glutamine and casein hydrolysate with respect to regeneration response. It was reported that casein hydrolysate stimulated callus induction and regeneration frequencies and it provides a source of amino acids. These findings are in conformity with those by Zaidi *et al.* (2006) and Afolabi *et al.* (2008), in which the positive effect of casein hydrolysate had been shown.

Proline is sometime act as an osmotic regulatory and can have a positive impact on regeneration ability. Our findings showed only a slight effect of L-proline and glutamine on *in vitro* regeneration response of landrace *Hom Kra Dang Ngah* rice. However, 500-600 mg/L L-proline has been recommended for rice tissue culture experiments (Ge *et al.*, 2006; Afolabi *et al.*, 2008). The impact of incorporating appropriate sorbitol on the plantlet regeneration was

also examined. Adding 82mM sorbitol to medium combined with 1g /L casein hydrolysate created a positive impact on regeneration frequency. This finding is similar to the previous research of Shahsavari *et al*. (2010). In fact, they reported that addition of appropriate amounts of sorbitol in the culture media increased regeneration rate drastically. It seems using sorbitol in tissue culture acts as primary carbon source to enhance regeneration frequency of embryogenic calli (Geng *et al.*, 2008).

## Conclusion

In this study, successful optimizations on the regeneration system of *indica* rice landrace *Hom Kra Dang Ngah* were performed on ARDA medium supplemented with (BA+KN) and NAA at ratio of 6:1. The culture medium solidified with phytagel at concentration of 0.3% plus 82mM sorbitol in combination with 1g/L casein hydrolysate gave the highest frequency of plantlet regeneration at 75%. This high regeneration frequency will lead to improve it by genetic transformation technology.

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