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## DEVELOPMENT OF AN EFFICIENT *in vitro* PLANT REGENERATION PROTOCOL FOR INDICA RICE VARIETIES (*Oryza sativa* L.) IN THE MEKONG DELTA OF VIETNAM

Tran Thi Xuan Mai<sup>1</sup>, Nguyen Thi Lien<sup>1</sup>, Tran Thi Cuc Hoa<sup>2</sup>, Geert Angenon<sup>3</sup>

<sup>1</sup>Biotechnology Research and Development Institute, Can Tho University, Vietnam

<sup>2</sup>Omon Rice Institute, Vietnam

<sup>3</sup>Laboratory of plant genetics, Vrije Universiteit Brussel, Belgium

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### ABSTRACT

*In this study, the effects of medium components and environmental conditions on callus induction and regeneration potential of two indica rice varieties (IR64 and MTL250) were evaluated. Mature seeds were cultured on four different media including either (MS) Murashige and Skoog (1962) or N6 Chu (1978) for callus induction. The MS medium was found more suitable than N6 medium in terms of callus induction frequency. The highest callus induction rate with good quality was obtained on MS medium containing 2 mg/l 2,4D, 112 mg/l B5 vitamin, 500 mg/l proline, 500 mg/l glutamine, 300 mg/l casein hydrolysate, 30 g/l sucrose and 4 g/l phytigel. To investigate the best condition for callus growth, the influences of incubation temperatures and light conditions on callus induction of IR64 and MTL250 varieties were examined. The experimental results clearly showed that incubation temperatures at both 28°C and 32°C had no significant effects on callus induction in both varieties. However, periodic illumination for 16 hours/day and complete darkness proved the best effect on callus induction in MTL250 and IR64, respectively. Among 07 different media used for shoot induction, the best results were obtained when calli of IR64 and MTL250 were cultured on medium containing MS including vitamins + 2.0 mg/l BAP + 0.5 mg/l NAA + 20 g/l sucrose + 30 g/l sorbitol + 6 g/l phytigel. Shoot regeneration by using medium supplemented with kinetin, moreover, was very low or even failed in both varieties. This plant regeneration protocol is considered to be significant improved and promises to serve for breeding or genetic engineering of indica rice varieties in the Mekong Delta of Vietnam.*

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### 1 INTRODUCTION

Rice (*Oryza sativa* L.), especially indica rice, is an important food crop in tropical and some subtropical regions of Asia. Vietnam has been the third rice exporter in the world (Mottaleb *et al.*, 2016) and

the Mekong Delta is well known as the biggest rice basket in Vietnam, providing more than 90% of total rice exports and making an important contribution to the food security across the region (GSO, 2010). However, Vietnam is being one of the countries most affected by climate change such as

drought and salt-water intrusion and leading to the great losses in the crop productivities, especially rice in the Mekong Delta. In recent years, new rice varieties have been developed by applying conventional breeding to cope with climate change (Mottaleb *et al.*, 2016) but the success rate is not high. Therefore, conventional breeding methods need to be assisted by recent achievements in biotechnology to meet the increasing demand for rice production.

Plant tissue culture techniques are prerequisite for successful applications of plant biotechnology and being applied for varietal development of cereal crops including rice in various countries (Dorosieva, 1996; Islam *et al.*, 2014a). Among these techniques, the cultures of anther, leaf, root and dehusked seeds are important in rice tissue culture to exploit somaclonal variations, select *in vitro* and produce new lines from genetic transformation.

Many protocols have been developed for the *in vitro* regeneration of rice from different explants, such as immature seeds (Hiei and Komari, 2008; Islam *et al.*, 2014b), mature seeds (Sah and Kaur, 2013; Islam *et al.*, 2014a; Upadhyaya *et al.*, 2015), leaf (Karthikeyan *et al.*, 2011), shoot apex (Dey *et al.*, 2012), and root (Mandal *et al.*, 2003). Calli induced from scutellar tissue of mature seeds are the excellent source of cells for *in vitro* regeneration and for the production of transgenic rice (Wani *et al.*, 2011). The regeneration of plants is highly dependent on the quality of callus. It has been reported that many factors including genotypes, types of explant, culture medium, plant growth regulators and culture conditions can affect callus induction, quality of callus and regeneration potential (Khanra and Raina, 1998).

Indica rice varieties are popularly grown in the Mekong Delta region, but they are recalcitrant to *in vitro* regeneration because of poor callus induction and regeneration efficiency. Although there have been some successful reports in plant regeneration from indica rice tissue culture, the protocol is not applicable for all rice cultivars. Therefore, the objective of this study was to find a suitable medium and culture condition for callus induction and plant regeneration of IR64 and MTL250 varieties, two important genotypes for breeding. Results of this study can be applied to the *in vitro* regeneration studies of indica rice varieties in the Mekong Delta.

## 2 MATERIALS AND METHODS

### 2.1 Plant materials

Mature seeds of two indica rice (*Oryza sativa* L.) varieties with high quality and yield in the Mekong Delta, namely, IR64 and MTL250, were kindly provided by Mekong Delta Development Research Institute.

### 2.2 Disinfection of rice seeds

Mature seeds of IR64 and MTL250 were carefully dehusked manually. The dehusked seeds were washed with 70% alcohol for 1 minute and then sterilized with 50% commercial bleach, which contains 5% sodium hypochloride) plus two drops of Tween-20, by shaking the whole bottle at 120rpm for 25 minutes. After surface sterilization, the solution was removed, and the seeds were washed 5-6 times with sterile distilled water. The seeds were finally placed on the sterilized petri dish having sterile filter papers with the help of forceps to remove excess water.

### 2.3 Callus induction

The basal media used in this experiment were MS (Murashige and Skoog, 1962) and N6 (Chu, 1978). To evaluate the best medium for good quality callus induction, four different treatments were tested (Table 1). The pH of media was adjusted to 5.7-5.8 by HCl or NaOH. All media were autoclaved at 121°C and 1-atm pressure for 20 minutes. Plant growth regulators were filter-sterilized and added to the medium after autoclaving. Sterilized seeds were placed in petri dishes (90cm in diameter) prepared with 25ml medium with the endosperms soaking deeply into the medium and the embryo sides facing upward. Embryos needed to be placed on the surface of the medium to increase the contact area between the scutellum tissue and the medium. After 3 weeks being incubated in the dark at 28°C, the proliferated calli derived from the embryo scutella were observed under a stereoscope to select good quality calli (yellowish white, dry and compact with globular structures) and aseptically dissected to remove the shoots, roots and endosperm with a flamed scalpel. Good quality calli were subcultured to fresh callus induction media after 3 weeks. The frequency of callus induction was determined by the following formula:

Callus induction frequency (%) = (No. of seeds producing good quality calli/ No. of seeds cultured) x100

**Table 1: Callus induction medium (CIM)**

Components	Callus induction medium (treatment)			
	CIM1-MS	CIM2-MS	CIM1-N6	CIM2-N6
MS basal salt	4.3 g/l	4.3 g/l	-	-
N6 basal salt	-	-	4.0 g/l	4.0 g/l
B5 vitamin	112 mg/l	-	112 mg/l	-
Proline	500 mg/l	-	500 mg/l	-
Glutamine	500 mg/l	-	500 mg/l	-
Casein hydrolysate	300 mg/l	1 g/l	300 mg/l	1 g/l
Sucrose	30 g/l	30 g/l	30 g/l	30 g/l
2,4-D	2.0 mg/l	2.0 mg/l	2.0 mg/l	2.0 mg/l
Phytigel	4.0 g/l	4.0 g/l	4.0 g/l	4.0 g/l

**2.4 Environmental conditions for callus induction**

To determine the best environmental conditions for callus induction, two parameters including incubation temperature (28°C or 32°C) and light conditions were tested. The number of seeds producing good quality calli was recorded.

**2.5 Shoot induction**

This experiment was designed to compare the effects of two cytokinins, Kinetin (Kn) and 6-Benzylaminopurine (BAP), in combination with auxin, 1-Naphthaleneacetic acid (NAA), on shoot

induction. After 3 weeks of subculture on callus induction medium, the selected calli were transferred onto petri dishes containing MS including vitamins medium supplemented with different concentrations of Kn (2-4 mg/l) or BAP (2-5 mg/l) and NAA (0.5-2 mg/l) for shoot regeneration (Table 2). All cultures were first incubated at 28°C in dark condition for 1 week and then in light condition (16 hours of light per day) with 3000 lux light intensity at 28°C for 3 weeks. The cultures were sub-cultured in fresh medium after every 4 weeks. Green spot formation was observed every week. The frequency of shoot induction was determined as follows:

**Table 2: Shoot induction medium (SIM)**

Components	Shoot induction medium (treatment)						
	SIM K1	SIM K2	SIM K3	SIM K4	SIM K5	SIM B1	SIM B2
MS including vitamins	4.4 g/l	4.4 g/l	4.4 g/l	4.4 g/l	4.4 g/l	4.4 g/l	4.4 g/l
Proline	-	500 mg/l	-	-	-	-	-
Casein hydrolysate	-	500 mg/l	-	-	-	-	1 g/l
Kinetin	2 mg/l	2 mg/l	4 mg/l	4 mg/l	2 mg/l	-	-
BAP	-	-	-	-	-	2 mg/l	5 mg/l
NAA	1 mg/l	0.5 mg/l	2 mg/l	1 mg/l	0.5 mg/l	0.5 mg/l	1 mg/l
Sucrose	30 g/l	30 g/l	30 g/l	30 g/l	20 g/l	20 g/l	20 g/l
Sorbitol	-	-	-	-	30 g/l	30 g/l	30 g/l
Phytigel	6.0 g/l	6.0 g/l	6.0 g/l	6.0 g/l	6.0 g/l	6.0 g/l	6.0 g/l

Shoot induction frequency (%) = (No. of shoots produced from calli/No. of calli cultured for regeneration) x100

**2.6 Root induction and acclimatization**

For root induction, elongated shoots (2-3 cm in length) were carefully plucked out and transferred to 250 ml conical flask containing 60 ml rooting medium prepared with MS including vitamins (Duchefa) supplemented with 3% sucrose, 0.8% plant agar and pH adjusted to 5.8. After 2 weeks being cultured under light condition (16/8 hours photoperiod, 3000 lux light intensity, 28°C), *in vitro* plantlets with healthy root systems were

washed under running tap water to clear off the entire residual agar medium to minimize fungal attack and transferred to pots containing a mixture of 1 vermiculite and 1 soil (v/v) and filled with Yoshida's nutrient solution (Yoshida *et al.*, 1976). Each pot was covered with a polythene bag to maintain high humidity inside for the first 3 days. Small holes were subsequently made on the polythene bags to gradually reduce the humidity to harden the plants for 3-4 days under light condition (16/8 hours photoperiod, 3000 lux light intensity, 28°C). Plantlets were then transplanted to soil in natural conditions.

### 2.7 Statistical analysis

Each treatment was replicated 3 times with 50 seeds per replication for the induction of callus and 20 calli per replication for the induction of shoot. All collected data were assessed by analysis of variance (ANOVA) for factorial complete randomized design (CRD) using computer software Statgraphics Centurion XV.

## 3 RESULTS

### 3.1 Effect of media on callus induction

Many reports demonstrated that good quality callus (dry and compact in structure, yellowish white in color and globular in shape) is one of the most essential factors for efficient plant regeneration. Therefore, in this study, only good quality calli were selected to evaluate the efficiency of callus induction. Calli started protruding from the scutellum region and were clearly visible after 4 to 5 days of culturing. However, good quality calli were observed under a stereoscope after 21 days (Fig. 1). On MS medium, both IR64 and MTL250 obtained optimum frequency of callus formation on MS-CIM1 medium, being 46% in IR64 and 44.7% in MTL250, followed by MS-CIM2 medium with 32.7% in IR64 and 40.3% in MTL250 (Fig. 2).

In N6 media, callus induction frequency ranged from 9.7% to 13.3% in both two varieties, and the highest callus induction rate at 12.7% was observed in IR64 with N6-CIM1 medium and 13.3% in MTL250 with N6-CIM2 medium. These results indicated that callus induction ability of MS medium was significantly higher than that of N6. In addition, the average diameter of callus on MS media was 6 to 7 mm while on N6 media it was only 2 to 3 mm (data not shown). Therefore, MS medium was found to be more suitable for callus induction of both IR64 and MTL250 varieties. From this result, CIM1-MS medium was selected as the culture medium for callus induction in subsequent experiments.

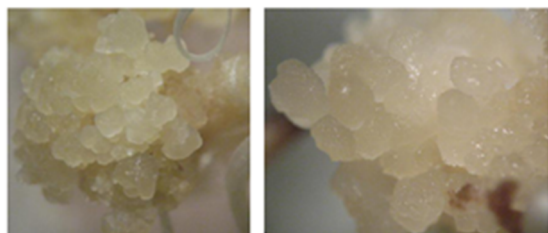


Fig. 1: Embryogenic callus from IR64 (A) and MTL250 at 3 weeks

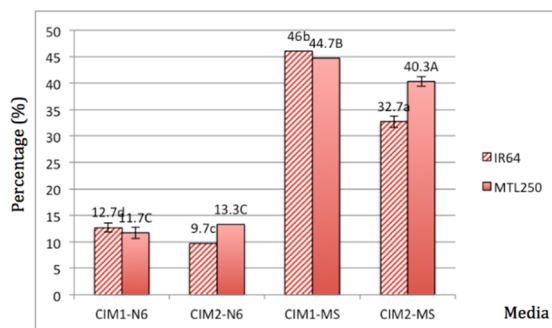


Fig. 2: Effect of medium on callus induction in IR64 and MTL250

### 3.2 Effect of incubation temperature on callus induction

The effect of incubation temperature in callus induction was illustrated in Figure 3. No significant difference in callus induction frequency was observed between 28°C and 32°C in both varieties, being 39.5% and 41.5% in IR64, 37% and 40.5% in MTL 250, respectively.

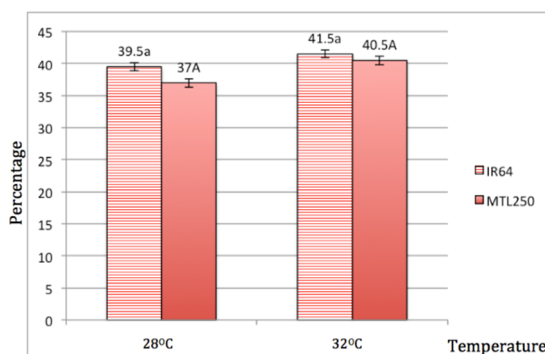
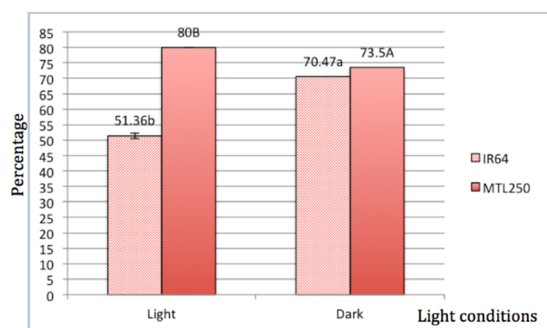


Fig. 3: Effect of incubation temperature on callus formation

### 3.3 Effect of light condition on callus induction

The results from Figure 4 showed that callus induction frequency of rice seeds cultured in the dark was significantly higher (70.47%) than under the light condition (51.36%) in IR64, being contrary to the situation of MTL250 with a significantly higher percentage of seeds (80%) forming good callus under lighting than 73.5% in the dark condition. Such a difference might be due to the differences in genotypic efficiency of rice varieties for callus induction.





**Fig. 4: Effect of light conditions on callus induction**

**3.4 Effect of media on regeneration potential**

Combinations of cytokinins and auxins have stimulated *in vitro* regeneration in several plant species including rice. As shown in Table 3 and Figure 5, both IR64 and MTL250 varieties formed green spots after 4- 5 weeks in SIM-B1 medium and after

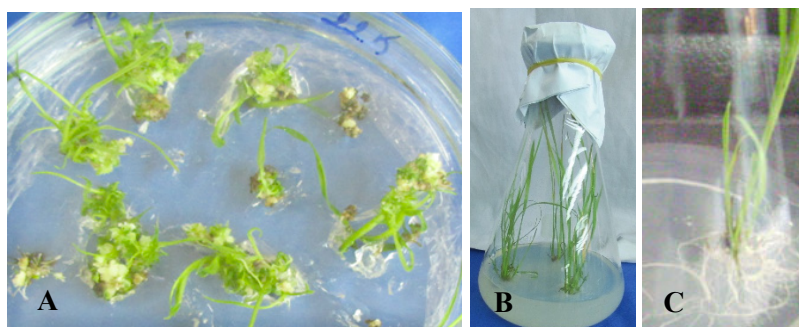
6-7 weeks in SIM-K2 and SIM-B2. No green spot was observed in SIM-K1, SIM-K3, SIM-K4 and SIM-K5 while SIM-K2 supplemented with 2.0 mg/l Kn and 0.5 mg/l NAA had the shoot induction frequency of 9.3% in IR64 and 11.1% in MTL250. In case of the treatments using different combinations of BAP and NAA, the highest shoot induction frequency was recorded in SIM-B1 medium with 2.0 mg/l BAP and 0.5 mg/l NAA, being 46.3% in IR64 and 37.0% in MTL250. However, shoot induction frequency was found at only 3.7% in both two varieties when calli were cultured in SIM-B2 medium using higher concentrations of BAP and NAA (5.0 mg/l BA and 1.0 mg/l NAA). Moreover, as seen in Table 2, the SIM-B1 medium had a similar composition to SIM-K2 except for kinetin but it resulted in significant difference in shoot induction as mentioned above. Those results indicated that cytokinin BAP at 2 mg/l concentration was more effective to induce shoot formation compared to cytokinin Kn in IR64 and MTL250 varieties.

**Table 3: Shoot regeneration after 8-week culturing of IR64 and MTL250**

Medium	Time required for green spot formation (week)		Percentage of shoot regeneration	
	IR64	MTL250	IR64	MTL250
SIM-K1	X	X	0	0
SIM-K2	7	6	9,3 <sup>b</sup>	11,1 <sup>b</sup>
SIM-K3	X	X	0	0
SIM-K4	X	X	0	0
SIM-K5	X	X	0	0
SIM-B1	4	5	46,3 <sup>a</sup>	37,0 <sup>a</sup>
SIM-B2	6	6	3,7 <sup>bc</sup>	3,7 <sup>bc</sup>

X: no shoot observed

a, b, c: in the same column, means followed by the same letters revealed no significant difference.



**Fig. 5: (A) Shoot regeneration from callus, (B and C) Shoot on root induction medium**

**4 DISCUSSION**

MS and N6 are the two well-known media used for callus induction (Rashid *et al.*, 1996; Toki, 1997). Hussain *et al.* (2010) reported that MS medium was better for callus induction of GNY-53, Basmati-370 and JP-5 varieties. Similar findings of other researchers (Khatun *et al.*, 2003; Azria and Bhalla, 2000) also demonstrated that MS medium was the

most effective for callus induction. However, these results are contradictory to the findings of Rashid *et al.* (2004) in which N6 medium was suggested as a relatively better basal medium than others for callus induction. Having the same conclusion, Tariq *et al.* (2008) also reported the better callusing effect of N6 medium in comparison to MS in four varieties of rice - Super Basmati, Fakhre Malaknd,

Basmati-370 and Basmati-371. In another report by Summart *et al.* (2008), N6 medium resulted in the highest rate of callus formation while most of the high quality calli were obtained in MS medium.

The use of amino acid proline and glutamine in the medium has been reported to have positive effects on frequency of callusing and regeneration in rice (Ge *et al.*, 2006; Shahsavari, 2011). Many reports demonstrated that the use of casein hydrolysate with proline is beneficial for the generation of embryogenic calli in japonica (Hiei *et al.*, 1994; Toki, 1997) as well as in indica rice (Zhang *et al.*, 1996; Hoa and Dung, 2006). According to Pawar *et al.* (2015), addition of proline or glutamine either alone or in combination resulted in a significant increase in callus formation and fresh weight of callus. The enhancement of callus growth by amino acid supplementation can be explained on the basis that amino acids provide a readily available source of nitrogen for the growing calli. In addition, proline and glutamine are relatively non-toxic which will enable the cells to maintain a high growth rate for a longer period.

The combination of 300 mg/l casein hydrolysate with 1,000 mg/l proline was appropriate to promote callus induction in Supanburi 1, using proline at 500 mg/l can increase the callusing rate in Chi Nat 1 and Patum Thani 1 rice varieties (Rattana *et al.*, 2012). Three japonica rice varieties including Nipponbare, Hayahishiki and Fujisaka 5 were observed with increasing fresh weight of cell mass after 2 days of being cultured in media supplemented with 500 mg/l proline (Wagiran *et al.*, 2008). Saharan *et al.* (2004) reported that addition of 300 mg/l casein hydrolysate in combination with 500 mg/l proline could influence the callus induction of rice. In this study, the addition of casein hydrolysate at 300 mg/l in combination of proline at 500 mg/l and glutamine at 500 mg/l to CIM1-MS medium gave the best callusing response in both IR64 and MTL250 varieties.

Summart *et al.* (2008) reported that there was no significant difference in the growth of callus but morphology of callus was remarkably different when rice seeds were cultured at  $25\pm 2^\circ\text{C}$  or  $30\pm 2^\circ\text{C}$ .

Many reports demonstrated that incubation temperature at  $32^\circ\text{C}$  could induce the rapid growth of cultured rice cells during callus formation and stimulate the removal of *Agrobacterium* from rice cells after the co-cultivation stage in transformation studies since at this temperature, plants became more resistant to the infection of *Agrobacterium tumefaciens* (Fullner *et al.*, 1996; Toki, 1997). The

present results were also in agreement with the report of Hervé and Kayano (2006), and Abayawickrama and Anai (2006), in which an increase in temperature to  $32^\circ\text{C}$  instead of  $28^\circ\text{C}$  led to a significant increase in callus formation.

Light is a very important physical factor for callus induction, cell growth, and production of plant secondary metabolites (Vom Endt *et al.*, 2002). The level of responsiveness to light depends on cell types, plant species, and cultivars. Wanthanalert and Ketudat-Cairns (2011) found that light was not an important factor for Koshihikari secondary callus induction and in the dark calli had a less browning rate than in the light condition.

However, in several studies, light seemed to have stimulative effects on callus proliferation from embryos of rice (Thadavong *et al.*, 2002; Maneewan *et al.*, 2005). Similarly, light influenced the rate of callus induction, speeded up the callus growth and also increased the browning rate in rice variety Pei'ai64s (Qian *et al.*, 2004). Afrasiab and Jafar (2011) reported that light conditions proved to be better for the callogenesis and regeneration from mature seed explants of Super Basmati and IRRI-6 varieties.

The types and concentrations of cytokinins combined with NAA have been considered to be important factors that might affect the shoot regeneration of both Indica (Xue and Earle, 1995) and Japonica rice (Lee *et al.*, 2002). Pons *et al.* (2000) reported that BAP yielded more shoots than Kn in all varieties while in case of using auxin NAA and IAA, it depended on the varieties. High concentration of BAP could promote the regeneration of calli (Jiang *et al.*, 2000). MS medium supplemented with 1 mg/l NAA and 5 mg/l BAP gave the highest frequency of shoot induction from calli in Basmati 385 rice (Noor *et al.*, 2005). These findings were in accordance with the earlier reports of Rashid *et al.* (2001, 2004). Afrasiab and Jafar (2011) revealed that MS medium supplemented with 1.0 mg/l NAA and 3.0 mg/l BAP performed better than all other treatments in Super Basmati while IRRI-6 had the greatest regeneration frequency with a higher concentration of BAP (5.0 mg/l). However, Hussain *et al.* (2010) obtained higher regeneration rates in two rice cultivars (JP-5 and GNY-53) by using a combination of 1 mg/l NAA and 2 mg/l BAP.

The findings in this study are in contradiction with the results obtained by Raina (1989), in which Kn was found to enhance the shoot regeneration of rice callus. Lee *et al.* (2002) also reported the highest regeneration frequency on MS medium containing NAA at 2.0 mg/l and Kn at a range of 1.0–

4.0 mg/l. Cho *et al.* (2004) concluded that using of Kn seemed to be more appropriate for regeneration of calli than BAP.

It is undeniable that the composition and concentration of the basal salt and growth regulators are important factors that may strongly affect the process of regeneration from callus. The addition of organic components such as proline and casein hydrolysate, however, may also have positive effects. Khaleda and Al-Forkan (2006) demonstrated that plant regeneration media supplemented with L-proline stimulated plant regeneration in rice. Thadavong *et al.* (2002) reported that addition of 800 mg/l casein hydrolysate can enhance the rates of callus forming green spots and shoot regeneration of rice cv. TDK1.

In this study, as comparing all treatments using cytokinin Kn, it was found that the addition of only SIM-K2 medium with proline (500 mg/l) and CEH (500 mg/l) may be the reason for the different results obtained in this treatment. According to Saharan *et al.* (2004), maximum shoot induction frequency was found when calli were cultured on SIM-K2 medium. However, this medium gave the lowest response in shoot induction of IR64 and MTL 250 varieties in our study.

In two treatments using cytokinin BAP in this study, SIM-B2 medium was also added with 2 g/l CEH but it resulted in the lowest shoot induction frequency. This was in agreement with the previous report of Khaleda and Al-Forkan (2006), in which they reported that casein hydrolysate tremendously reduced the regeneration process in rice. Sorbitol, in addition, has been used for restoring and enhancing the plant regeneration ability of rice callus (Kishor and Reddy, 1986). Cho *et al.* (2004) reported that the growth of callus was stimulated and multiple shoots of regenerated plants could be obtained in higher frequency by supplementation of sorbitol in combination with sucrose or maltose and 5 mg/l of kinetin. The addition of 10-20 g/l sorbitol also enhanced rice regeneration (Wagiran *et al.*, 2008; Shahsavari, 2010). In this study, maximum shoot induction frequency was obtained in SIM-B1 supplemented with 2.0 mg/l BAP, 0.5 mg/l NAA and 30 g/l sorbitol. Our results showed similarities to the earlier report by Hoa and Dung (2006), which indicated that SIM-B1 was the best medium for regeneration of indica rice varieties. Our findings also showed that addition of proline and casein hydrolysate to shoot induction medium seemed to be unnecessary.

## 5 CONCLUSIONS

In this study, a protocol for high frequency regeneration from mature seeds through embryogenic callus formation of two indica rice varieties, IR64 and MTL250, has been successfully established. This protocol has provided a fundamental platform to investigate regeneration potential of indica rice varieties in the Mekong Delta region. This protocol, in addition, would be very useful to further develop the genetic transformation techniques for these important rice varieties.

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