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Effects of Cultivation Media on *In vitro* Callus Induction and Regeneration Capabilities of Pakaumpuel Rice (*Oryza sativa* L.), Thai Rice Landrace

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Abstract

This study determined the effects of different cultivation media and some factors on *in vitro* callus induction and shoot regeneration of Pakaumpuel rice (*Oryza sativa* L.), the Thai rice landrace. Pakaumpuel's dehusked seeds were surface sterilized by using 20 % Sodium hypoclorite for 20 min before washed and cultured on Murashige and Skoog (MS) medium in various concentrations of BAP (6-Benzylaminopurine) (0, 0.1, 0.5 and 1 mg/l) and 2,4-D (2,4-Dichlorophenoxyacetic acid) (0, 1, 2 and 3 mg/l) and cultivated in off-light or dark cycle condition for 15 days. Results showed that MS medium with 2 mg/l 2,4-D where incubated in light condition provided the highest callus induction percentage (80 %). For shoot regeneration, calli were desiccated by using silica gel for 0, 30, 90 and 180 min prior to culture on MS medium with 1 mg/l NAA (1-Naphthaleneacetic acid) and various concentrations of BAP (0, 1, 2, 3 and 4 mg/l) for 30 days. Two-way ANOVA results revealed that desiccation periods did not affect plant regeneration percentage of Pakaumpuel rice. Therefore, MS medium with 1 mg/l NAA and 3 mg/l BAP without desiccation induced the significantly highest regeneration percentage (53.33 %), number of shoot per callus (2.80 shoots), longest shoot (12.16 cm) and the highest number of leaves per shoot (2.40 leaves). This discovery can be applied for micropropagation of other Thai rice landraces.

Keywords: Pakaumpuel, Rice landrace, *in vitro* callus induction, plant regeneration, desiccation

Introduction

Rice (*Oryza sativa* L.) is important stable food in the world and the most important exported agricultural product of Thailand. Nowadays, the global rice demand tends to increase because world population is continuously increasing [1]. Therefore, rice breeding with landrace germplasms as genetic donor is an alternative choice to improve rice cultivated production.

Rice landraces have evolved from their wild progenitor and still retain high genetic diversity [2]. These landraces can be identified by use of morphological characters and named by local farmers [3]. Since genetic structure of rice landraces is heterogeneous, these landraces can show variable phenology and are able to growth in biotic and abiotic stress environments, which are the valuable characters for crop improvement production [4]. Thailand is the center of rice diversity. Therefore, Thai rice landrace is a necessary and valuable resource to use in rice breeding program and cultivation to improve productivity [5].

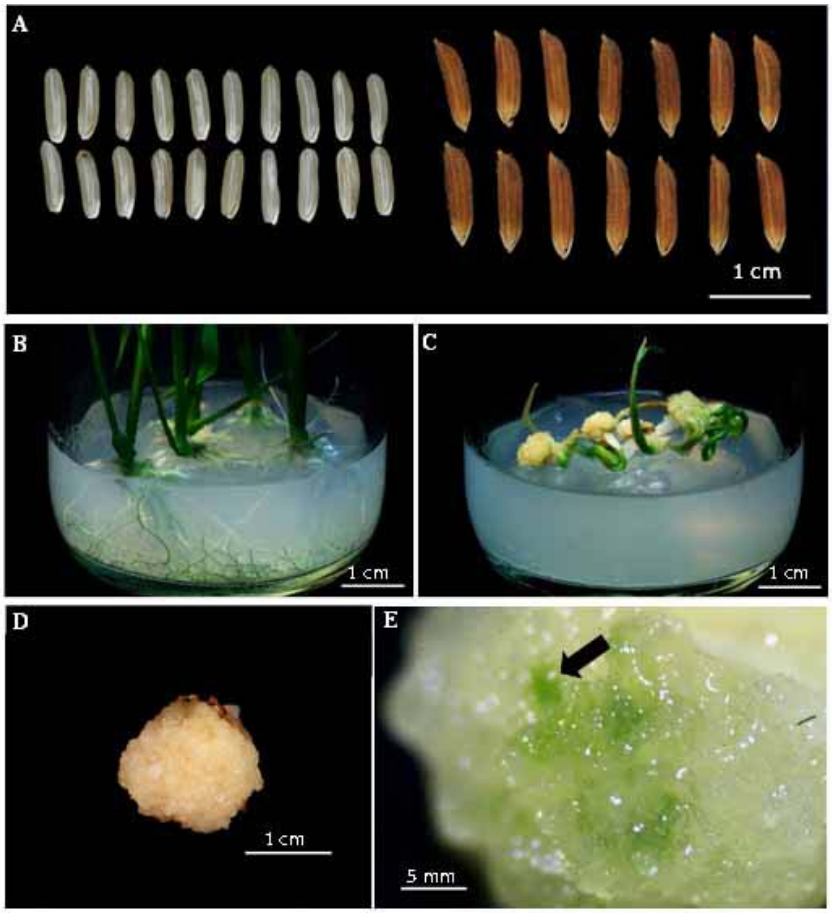

Figure 1 Pakaumpuel rice seed and different stages of *in vitro* response in different culture media, *A*-Brown rice (left) and unmilled rice (right) of Pakaumpuel rice landrace; *B*-seedling of Pakaumpuel rice in medium without PGRs under light condition; *C*-callus formation of Pakaumpuel seeds cultured on MS medium with 2 mg/l 2,4-D (C_9) after cultured for 15 days; *D*-creamy compact granular callus; *E*-surface of callus grown under light condition and green spot (*arrow*).

Pakaumpuel is a rice landrace (**Figure 1A**) grown in Surin Province, Thailand and other provinces in border between Thailand and Cambodia. Although Pakaumpuel is a non-glutinous rice possessed drought tolerance characters, its production is less than some Thai commercial varieties. However, this landrace provide greater vitamin E, lutein and iron than Khao Dok Mali (KDML) 105 about 0.8, 0.4 and 1.1-fold, respectively [6]. Callus induction and plant regeneration are the processes to produce a large number of plants rapidly, and plant growth regulators (PGRs) are always the important factor for the both processes. In callus induction, PGRs that always used for callus induction is 2,4-Dichlorophenoxyacetic acid (2,4-D) because 2,4-D can suppress organogenesis and trigger proliferation to form callus [7], while 6-Benzylaminopurine, benzyl adenine (BAP) is used for shoot regeneration because it can promote cell division and maintain shoot meristem [8,9]. In addition to PGRs, it has been found that light condition influence callus induction because light can induce the calli with green spots that show high efficiency of plant regeneration [10]. Moreover, some research reported the positive effects of callus desiccation before culture on plant regeneration medium because the desiccation can activate gene expression of late embryogenesis abundant (LEA) proteins and increases the levels of Abscisic acid (ABA) [11,12]. ABA-

induced LEA proteins can alleviate damage from abiotic stress by stabilizing of membrane structure and integrity of other proteins [13,14]. Although many reports have been established about callus induction and regeneration in some rice varieties, callus induction and regeneration of landraces rice are poorly perceived, including Pakaumpuel rice. Therefore, the present study were undertaken with aims to i) investigate the effects of 2,4-D and BAP, on-off light or dark condition on *in vitro* callus induction, ii) examine effects of BAP, desiccation period on regeneration of Pakaumpuel, Thai rice landrace. The present research has the possibility of providing source for first step of *in vitro* propagation according to rice improvement demand production.

Materials and methods

All experiments were investigated at Department of Biology, Faculty of Science, Khon Kaen University, Thailand from April - September 2017.

Plant material and sterilization

Mature seeds of the Thai rice landrace, Pakaumpuel, were obtained from Surin Rice Research Center, Thailand. The dehusked seeds were sterilized with 20 % (v/v) Sodium hypochlorite (Clorox) supplemented with 2 - 3 drops of Tween 20 for 20 min with shaking, and then rinsed 3 times with sterile distilled water.

Callus induction

Sterilized seeds were cultured on *in vitro* callus induction media, which consisted of MS medium supplemented with 3 % (w/v) sucrose and various concentrations of 2,4-D and BAP (**Table 1**). The pH of the media was adjusted at 5.70 - 5.80 with 1 M NaOH and HCl and solidified by adding 0.8 % (w/v) agar powder. After that, cultures were incubated at 25 ± 2 ºC under different light conditions: white light (16/8 h light/dark) providing 40 $\mu mol.m^{-2}.s^{-1}$ and dark (continuous dark) conditions. Callus induction percentage, length, width and fresh weight of callus were determined for 15 days after culture initiation. Dry weight was evaluated after incubation at 70 ºC for 3 days. Each treatment consisted of 5 replicates, and 5 seeds were cultured in each replicate. Total numbers of samples were 25 in each treatment and 400 of all treatments.

Table 1 *In vitro* Callus induction media supplemented with 2,4-D and BAP for growth of Thai rice landrace, Pakaumpuel (*Oryza sativa* L.).

Treatments	Plant growth regulators (mg/l)		Treatments	Plant growth regulators (mg/l)	
	BAP	2,4-D		BAP	2,4-D
C1	0	0	C9	0	2
C2	0.1	0	C10	0.1	2
C3	0.5	0	C11	0.5	2
C4	1	0	C12	1	2
C5	0	1	C13	0	3
C6	0.1	1	C14	0.1	3
C7	0.5	1	C15	0.5	3
C8	1	1	C16	1	3

Remark: C_1-C_{16} were assigned as different cultivation media treatments.

Desiccation and plant regeneration

Similar size calli were used as explants. Calli were transferred to sterilized glass Petri dishes containing 14 g of sterile silica gel and a dry sterile filter paper. The Petri dishes were kept in laminar air flow in light for 30, 90, and 180 min. Callus weight was measured before and after desiccation. The desiccation percentage was calculated by the following formula [15]. Calli without partial desiccation (0 min) were used as control treatment.

$$\text{Desiccation percentage} = \frac{\text{fresh weight - desiccated weight}}{\text{fresh weight}} \times 100 \quad (1)$$

Desiccated calli with different periods and control were cultured on plant regeneration media containing MS medium supplemented with 3 % (w/v) sucrose, 1 mg/l NAA and different concentrations of BAP (0, 1, 2, 3 and 4 mg/l). The pH of the media was adjusted at 5.70 - 5.80 and solidified by adding 0.8 % (w/v) agar powder. The cultures were incubated at 25 ± 2 ºC with a 16/8 h light/dark cycle condition providing 40 $\mu mol.m^{-2}.s^{-1}$. After 30 days, shoot regeneration percentage, number of shoots per callus, number of leaves per shoot and shoot length (mm) were determined. Each treatment consisted of 5 replicates, and 3 calli were cultured in each replicate. Total number of samples was 15 in each treatment and 300 of all treatments.

Statistical analysis

Both experiments were factorial in completely randomized design (CRD) with 5 replications. Data from callus induction with 2 factors, which were PGRs and light condition, were tested by the two-way Analysis of Variance (ANOVA) and difference between means was separated by the Duncan's Multiple Range test ($p < 0.05$). Data from plant regeneration were submitted to one-way ANOVA and difference between means was separated by the Duncan's Multiple Range test ($p < 0.05$). The result was expressed as the means ± standard error of mean (SE).

Results and discussion

Callus induction

In callus induction, there were only seedlings germination from dehusked seeds of Pakaumpuel culture on media without 2,4-D in both light (**Figure 1B**) and dark conditions. The two-way ANOVA results showed that there was interaction between PGRs in media and light conditions, and both factors affected callus induction of Pakaumouel rice. Although callus could be generated in all treatments containing 2,4-D with or without BAP, significantly the highest callus induction percentage (80 ± 8.94 %; **Table 2**), length (7.18 ± 0.47 mm) and width (5.06 ± 0.40 mm) was obtained from MS medium supplemented with 2 mg/l 2,4-D (C9) in light condition (**Figure 1C**, **Table 3**). In addition, the highest fresh weight (68.98 ± 22.23 and 69.66 ± 7.21 mg) and dry weight (10.64 ± 3.44 and 10.54 ± 0.96 mg) were found in treatments with 1 and 2 mg/l 2,4-D in light condition, respectively (**Table 3**). In addition to PGRs, it was found that the effects of different light conditions on callus induction showed the higher average of callus induction percentage (43.50 ± 3.55 %), length (4.28 ± 0.30 mm), width (3.19 ± 0.22 mm), fresh weight (34.06 ± 3.09 mg) and dry weight (5.76 ± 0.49 mg) were obtained from cultures under light condition. In both light and dark conditions, calli derived from Pakaumpuel seeds predominantly were shown creamy or yellow compact and granular calli shaped (**Figure 1D**). Moreover, calli with many green spots only found under light condition (**Figure 1E**), showing a high ability of regeneration.

Plant growth regulators are the most important factor to induce callus. Therefore, efficiency of callus induction depends on the ratio between auxins and cytokinins in medium cultivation. Callus can be effectively induced by culturing on media supplemented with relatively high level of both auxin and cytokinin found in some plants [16-18]. Auxin commonly used in callus induction is 2,4-D, while BAP is the most common of cytokinin used in callus induction process. Our results indicated that 2 mg/l 2,4-D without other PGR was the most effective on callus induction of Pakaumpuel rice. Therefore, 2,4-D is a necessary PGRs to induce calli from Pakaumpuel seeds, because 2,4-D is the most effective auxin to

suppress organogenesis and trigger proliferation of cells in scutellum and mesocotyl to form callus [7]. There are many researches confirmed that only 2 mg/l 2,4-D was adequate for callus induction of rice [19,20], however, some researcher reported that MS medium with 1 mg/l 2,4-D and 2 mg/l kinetin was the optimum condition for callus induction of *Barringtonia racemosa* L. [21]. Therefore, type and concentration of PGRs are required for callus induction depend on plant species.

Table 2 Callus induction percentage of Thai rice landrace, Pakaumpuel (*Oryza sativa* L.) after cultured on callus induction media and incubated under different light conditions.

Treatments	Callus induction percentage (%) mean±SE		
	light	**dark**	**average**
C1	0 ± 0^{g}	0 ± 0^{g}	0 ± 0^{c}
C2	0 ± 0^{g}	0 ± 0^{g}	0 ± 0^{c}
C3	0 ± 0^{g}	0 ± 0^{g}	0 ± 0^{c}
C4	0 ± 0^{g}	0 ± 0^{g}	0 ± 0^{c}
C5	$56.00\pm7.48^{a-f}$	36.00 ± 4.00^{ef}	46.00 ± 5.20^{b}
C6	$40.00\pm6.32^{d-f}$	$48.00\pm10.20^{c-f}$	44.00 ± 5.81^{b}
C7	$52.00\pm17.44^{b-f}$	$48.00\pm10.20^{c-f}$	50.00 ± 9.54^{ab}
C8	$68.00\pm10.20^{a-c}$	32.00 ± 8.00^{f}	50.00 ± 8.56^{ab}
C9	80.00 ± 8.94^{a}	$52.00\pm4.90^{b-f}$	66.00 ± 6.70^{a}
C10	76.00 ± 9.80^{ab}	$52.00\pm4.90^{b-f}$	64.00 ± 4.00^{a}
C11	$64.00\pm7.48^{a-d}$	$64.00\pm4.00^{a-d}$	64.00 ± 4.27^{a}
C12	$48.00\pm4.90^{c-f}$	$60.00\pm6.32^{a-e}$	54.00 ± 4.27^{ab}
C13	$56.00\pm7.48^{a-f}$	32.00 ± 10.20^{f}	44.00 ± 7.18^{b}
C14	$52.00\pm10.20^{b-f}$	$52.00\pm8.00^{b-f}$	52.00 ± 6.11^{ab}
C15	$48.00\pm10.80^{c-f}$	$52.00\pm4.90^{b-f}$	50.00 ± 5.37^{ab}
C16	$56.00\pm7.48^{a-f}$	$52.00\pm10.20^{b-f}$	54.00 ± 6.00^{ab}
average	43.50 ± 3.55^{a}	36.25 ± 2.94^{b}	39.88 ± 2.31
Treatments		$p < 0.001$	
Light condition		$p < 0.05$	
Interaction		$p < 0.05$	

Mean ± SE followed by the different letter are significantly different according to ANOVA and Duncan's Multiple Range Test ($p < 0.05$).

Considering the effects of different light conditions on callus induction, we found that the optimum condition to induce callus from Pakaumpuel seeds was at light condition. In light condition, the large callus was found along with a tiny green shoot, whereas a long albino shoot with or without callus was always found in dark condition because continuous dark elevated the endogenous auxin levels. The endogenous auxin or IAA increased due to dark condition promotes cell expansion in stem [22]. Therefore, dark condition promotes shoot elongation and etiolation instead of callus induction. Besides, our study confirmed the vital roles of light on callus induction by appearance of green spots on callus under only light condition, because chlorophyll synthetic enzymes, NADPH-protochlorophyllide oxidoreductase, and green spot formation can be induced under light condition [7,23,24]. There are research reported the positive effects of light condition on callus induction of TDK1 rice [7], *Brassica napus* [25], and tobacco [10]. On the contrary, many studies reported that the optimum condition for rice callus induction was completely dark condition [26,27], which the major cause of this difference could be the difference of rice genotype [28]. Thereafter, calli obtained from culturing of Pakaumpuel seeds in MS medium with 2 mg/l 2,4-D under light condition, were used as explants in desiccation and regeneration showed contradictory result to the report mentioned above due to different type of rice.

Table 3 Size, fresh weight and dry weight of calli derived from Pakaumpuel seeds after cultured on callus induction media and incubated under different light conditions.

Treatments	Length (mm) mean ± SE			Width (mm) mean ± SE		
	light	dark	average	light	dark	average
C1	0 ± 0^{h}	0 ± 0^{h}	0 ± 0^{e}	0 ± 0^{h}	0 ± 0^{h}	0 ± 0^{c}
C2	0 ± 0^{h}	0 ± 0^{h}	0 ± 0^{e}	0 ± 0^{h}	0 ± 0^{h}	0 ± 0^{c}
C3	0 ± 0^{h}	0 ± 0^{h}	0 ± 0^{e}	0 ± 0^{h}	0 ± 0^{h}	0 ± 0^{c}
C4	0 ± 0^{h}	0 ± 0^{h}	0 ± 0^{e}	0 ± 0^{h}	0 ± 0^{h}	0 ± 0^{c}
C5	6.54 ± 0.85^{ab}	$5.02 \pm 0.64^{c-g}$	5.78 ± 0.56^{ab}	4.62 ± 0.38^{ab}	$3.84\pm0.62^{b-f}$	4.23 ± 0.37^{a}
C6	$6.00 \pm 0.32^{b-d}$	$5.78 \pm 0.37^{b-e}$	5.89 ± 0.23^{ab}	$4.38\pm0.23^{a-d}$	$3.96\pm0.43^{b-f}$	4.17 ± 0.24^{a}
C7	$5.50 \pm 0.27^{b-f}$	$5.52 \pm 0.28^{b-f}$	5.51 ± 0.18^{bc}	$4.38\pm0.25^{a-d}$	$3.66\pm0.27^{c-g}$	4.02 ± 0.21^{a}
C8	$5.62 \pm 0.28^{b-f}$	$4.78 \pm 0.38^{d-g}$	$5.20 \pm 0.26^{b-c}$	$4.56\pm0.21^{a-c}$	$3.68\pm0.24^{c-g}$	4.12 ± 0.21^{a}
C9	7.18 ± 0.47^{a}	$5.84 \pm 0.33^{b-e}$	6.51 ± 0.35^{a}	5.06 ± 0.40^{a}	$3.70\pm0.23^{c-g}$	4.38 ± 0.31^{a}
C10	$5.90 \pm 0.75^{b-d}$	$4.62 \pm 0.28^{e-g}$	$5.26 \pm 0.43^{b-d}$	$4.24\pm0.37^{a-e}$	$3.68\pm0.09^{c-g}$	3.96 ± 0.20^{a}
C11	$5.40 \pm 0.23^{b-g}$	4.42 ± 0.40^{fg}	4.91 ± 0.27^{cd}	$4.44\pm0.29^{a-d}$	$3.42\pm0.24^{e-g}$	3.93 ± 0.25^{a}
C12	4.40 ± 0.20^{fg}	$4.98 \pm 0.13^{c-g}$	4.69 ± 0.15^{cd}	$3.56\pm0.36^{d-g}$	$4.28\pm0.30^{a-e}$	3.92 ± 0.25^{a}
C13	$5.18 \pm 0.58^{c-g}$	4.24 ± 0.54^{g}	4.71 ± 0.40^{cd}	$3.66\pm0.25^{c-g}$	2.96 ± 0.31^{g}	3.31 ± 0.22^{b}
C14	$6.22 \pm 0.20^{a-c}$	$5.62 \pm 0.47^{b-f}$	5.92 ± 0.26^{ab}	$4.36\pm0.22^{a-d}$	$4.26\pm0.28^{a-e}$	4.31 ± 0.17^{a}
C15	$5.80 \pm 0.39^{b-e}$	$5.16 \pm 0.22^{c-g}$	5.48 ± 0.24^{bc}	$4.42\pm0.23^{a-d}$	$3.86\pm0.23^{b-f}$	4.14 ± 0.18^{a}
C16	$4.76 \pm 0.47^{d-g}$	4.24 ± 0.34^{g}	4.50 ± 0.29^{d}	3.34 ± 0.19^{fg}	$3.40\pm0.21^{e-g}$	3.37 ± 0.13^{b}
average	4.28 ± 0.30^{a}	3.76 ± 0.26^{b}	4.02 ± 0.20	3.19 ± 0.22^{a}	2.79 ± 0.19^{b}	2.99 ± 0.15
Treatments		$p < 0.001$			$p < 0.001$	
Light condition		$p < 0.001$			$p < 0.001$	
Interaction		$p < 0.05$			$p < 0.05$	

Treatments	Fresh weight (mg) mean ± SE			Dry weight (mg) mean ± SE		
	light	dark	average	light	dark	average
C1	0 ± 0^{i}	0 ± 0^{i}	0 ± 0^{f}	0 ± 0^{h}	0 ± 0^{h}	0 ± 0^{e}
C2	0 ± 0^{i}	0 ± 0^{i}	0 ± 0^{f}	0 ± 0^{h}	0 ± 0^{h}	0 ± 0^{e}
C3	0 ± 0^{i}	0 ± 0^{i}	0 ± 0^{f}	0 ± 0^{h}	0 ± 0^{h}	0 ± 0^{e}
C4	0 ± 0^{i}	0 ± 0^{i}	0 ± 0^{f}	0 ± 0^{h}	0 ± 0^{h}	0 ± 0^{e}
C5	68.98 ± 22.23^{a}	$38.28\pm9.32^{b-g}$	53.63 ± 12.46^{ab}	10.64 ± 3.44^{a}	$6.36\pm1.48^{b-g}$	8.50 ± 1.91^{a}
C6	53.22 ± 9.90^{ab}	$47.54\pm5.25^{b-d}$	$50.38\pm5.37^{a-c}$	$8.24\pm1.33^{a-c}$	$7.20\pm0.88^{b-e}$	7.72 ± 0.77^{ab}
C7	$34.06\pm5.33^{b-h}$	$35.06\pm3.19^{b-h}$	34.56 ± 2.93^{de}	$6.56\pm0.81^{b-g}$	$5.86\pm0.43^{c-g}$	$6.21\pm0.45^{b-d}$
C8	50.64 ± 4.62^{bc}	$26.22\pm6.32^{e-h}$	38.43 ± 5.49^{cd}	$7.20\pm0.89^{b-e}$	$4.82\pm0.79^{d-g}$	$6.01\pm0.69^{b-d}$
C9	69.66 ± 7.21^{a}	$41.00\pm4.65^{b-f}$	55.33 ± 6.26^{a}	10.54 ± 0.96^{a}	$6.86\pm0.56^{b-f}$	8.70 ± 0.81^{a}
C10	$42.48\pm8.39^{b-f}$	$27.14\pm1.22^{d-h}$	34.81 ± 4.74^{de}	9.34 ± 1.67^{ab}	$4.56\pm0.04^{d-g}$	$6.95\pm1.12^{a-c}$
C11	$41.18\pm3.97^{b-f}$	$24.12\pm2.31^{e-h}$	32.65 ± 3.57^{de}	$7.46\pm0.78^{b-d}$	$4.40\pm0.34^{d-g}$	$5.93\pm0.65^{b-d}$
C12	$23.22\pm3.27^{f-h}$	$36.00\pm4.80^{b-h}$	29.61 ± 3.47^{de}	$4.12\pm0.33^{e-g}$	$5.66\pm0.71^{c-g}$	4.89 ± 0.45^{cd}
C13	$38.10\pm6.62^{b-g}$	$19.14\pm3.07^{g-i}$	28.62 ± 4.67^{de}	$6.42\pm0.96^{b-g}$	3.72 ± 0.55^{fg}	5.07 ± 0.69^{cd}
C14	$47.98\pm3.49^{b-d}$	$36.04\pm7.31^{b-h}$	$42.01\pm4.30^{b-d}$	$8.54\pm0.64^{a-c}$	$6.00\pm0.86^{c-g}$	7.72 ± 0.66^{ab}
C15	$45.04\pm7.24^{b-e}$	$28.32\pm1.85^{d-h}$	36.68 ± 4.49^{de}	$7.44\pm0.73^{b-d}$	$4.76\pm0.41^{d-g}$	$6.10\pm0.60^{b-d}$
C16	$30.36\pm6.33^{c-h}$	15.66 ± 1.90^{hi}	23.01 ± 3.96^{e}	$5.70\pm1.12^{c-g}$	3.46 ± 0.38^{g}	4.58 ± 0.67^{d}
average	34.06 ± 3.09^{a}	23.41 ± 1.99^{b}	28.73 ± 1.88	5.76 ± 0.49^{a}	3.98 ± 0.31^{b}	4.87 ± 0.30
Treatments		$p < 0.001$			$p < 0.001$	
Light condition		$p < 0.001$			$p < 0.001$	
Interaction		$p < 0.05$			$p < 0.05$	

Mean ± SE followed by the different letter are significantly different according to ANOVA and Duncan's Multiple Range Test ($p < 0.05$).

Desiccation and plant regeneration

In desiccation, calli obtained from the previous experiment were desiccated for 30, 90 and 180 min and non-desiccated calli (0 min) were used as control. Fresh weight and desiccated weight were used for determination of desiccation percentage. The average desiccation percentages of calli, which were desiccated for 30, 90 and 180 min, were 33.24 ± 0.75, 62.77 ± 3.72 and 81.94 ± 0.94 %, respectively

(**Table 4**). After desiccation, desiccated calli were cultured on MS medium with 1 mg/l NAA and 0, 1, 2, 3 and 4 mg/l BAP, and the results were shown in **Figure 3**. The results of two-way ANOVA demonstrated that there was no interaction between desiccation periods and BAP concentrations ($p > 0.05$), and the desiccation did not affect the efficiency of plant regeneration ($p > 0.05$). Therefore, the data from 30, 90, and 180 min were excluded and one-way ANOVA was used instead of two-way ANOVA. After cultured for 30 days, the highest average of regeneration percentage (53.33 ± 8.16 %; **Figure 2A**), number of shoots per callus (2.80 ± 0.37 shoots; **Figure 2B**), shoot length (12.16 ± 1.19 cm; **Figure 2C**) and number of leaves per shoot (2.40 ± 0.24 leaves; **Figure 2D**) were obtained from medium containing 1 mg/l NAA and 3 mg/l BAP without desiccation, whereas there were no shoot emerging from callus that cultured on either MS medium with only 1 mg/l NAA or 1 mg/l NAA combined with 1 mg/l BAP (**Figure 3**).

Table 4 Desiccation percentage of calli derived from Pakaumpuel seeds.

Desiccation periods (min)	Desiccation percentage (%) mean ± SE
0	0±0
30	33.24±0.75
90	62.77±3.72
180	81.94±0.94

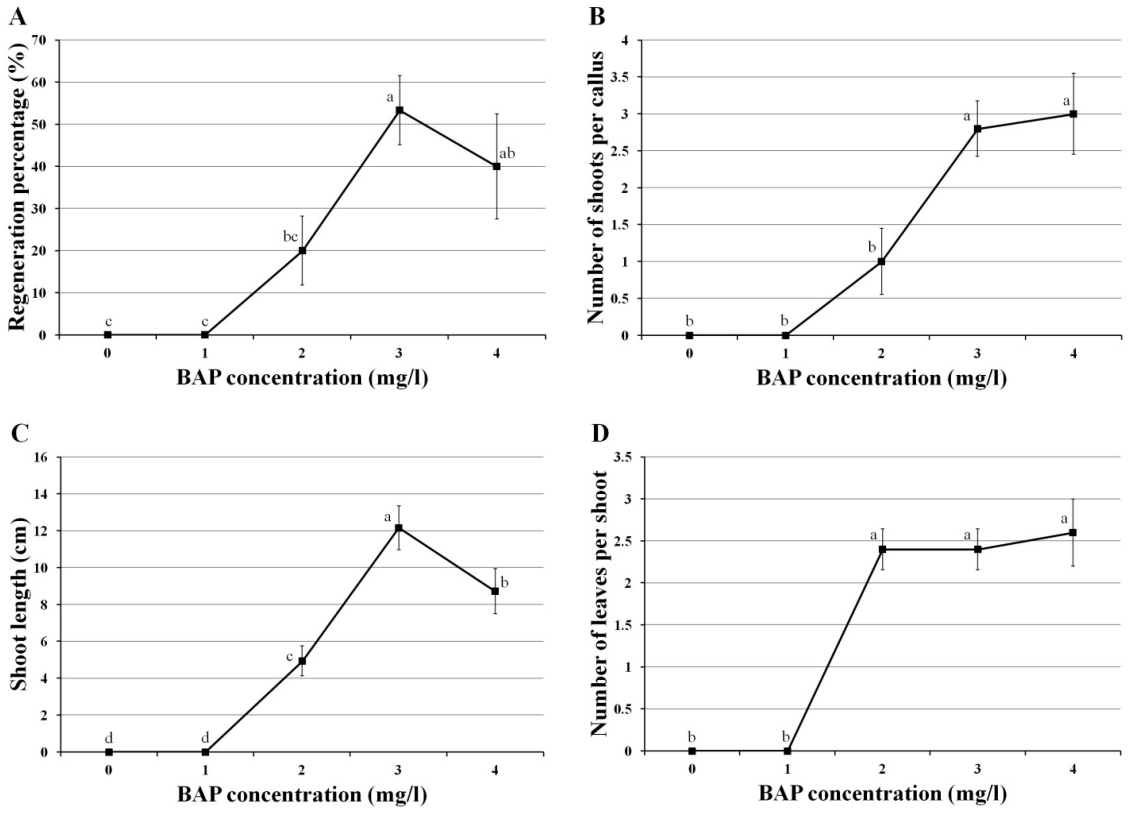

Figure 2 *A*-Regeneration percentage; *B*-number of shoots per callus; *C*-shoot length; *D*-number of leaves per shoot of Pakaumpuel rice after desiccation and cultured on regeneration media for 30 days.

The key factor of plant regeneration is PGRs, especially cytokinin. In this study, it was found that medium containing 1 mg/l NAA and 3 mg/l BAP was the optimum medium to regenerate plants from callus. The results were consistent with many studies on plant regeneration of rice. In plant regeneration of glutinous rice cultivar TDK1, the combination of 1 mg/l IAA and 4 mg/l BAP provided the highest regeneration percentage and number of shoot per callus [7]. The highest efficiency of plant regeneration of KDML105 and RD6 rice were obtained from the condition of 0.1 mg/l NAA and 3 mg/l BA in MS medium [20]. In addition, the positive effects of high auxin:cytokinin ratio were demonstrated in many plants, such as sugarcane [29] and *Chlorophytum borivilianum* [30]. These results confirmed that the suitable type and concentration of cytokinin can promote shoot regeneration from callus, because cytokinin plays important role in promotion of cell division, maintenance of shoot meristems and elevation of antioxidant activity [8,9].

There are many reports about effects of callus desiccation on plant regeneration. Some researcher reported that the plant regeneration from callus of sugarcane could be enhanced by callus desiccation in Petri dish containing sterile filter papers for 48 h [15], whereas a lower regeneration percentage was obtained from 72 h desiccation treatment. Likewise, other researcher showed that the most effective regeneration frequency of indica rice callus cultivar HKR-46 and HKR-126 could be received by desiccation for 48 h [31]. Osmotic stresses due to high concentration of NaCl and air desiccation can improve the regeneration percentage of 3 rice varieties that were BR10, BRRI dhan32, and BRRI dhan47 [32]. There were evidences demonstrated that desiccation can enhance the efficiency of plant regeneration because the desiccated calli can rapidly absorb water and nutrients from regeneration medium and promote shoot development. Moreover, LEA proteins that involve with the membrane stabilizing can be activated by air desiccation [11,12]. However, our results were different from the previous studies because two-way ANOVA results showed that desiccation period did not affect plant regeneration of Pakaumpuel rice. Therefore although callus desiccation is effective in various plant species, including rice, it cannot promote regeneration efficiency of Pakaumpuel rice.

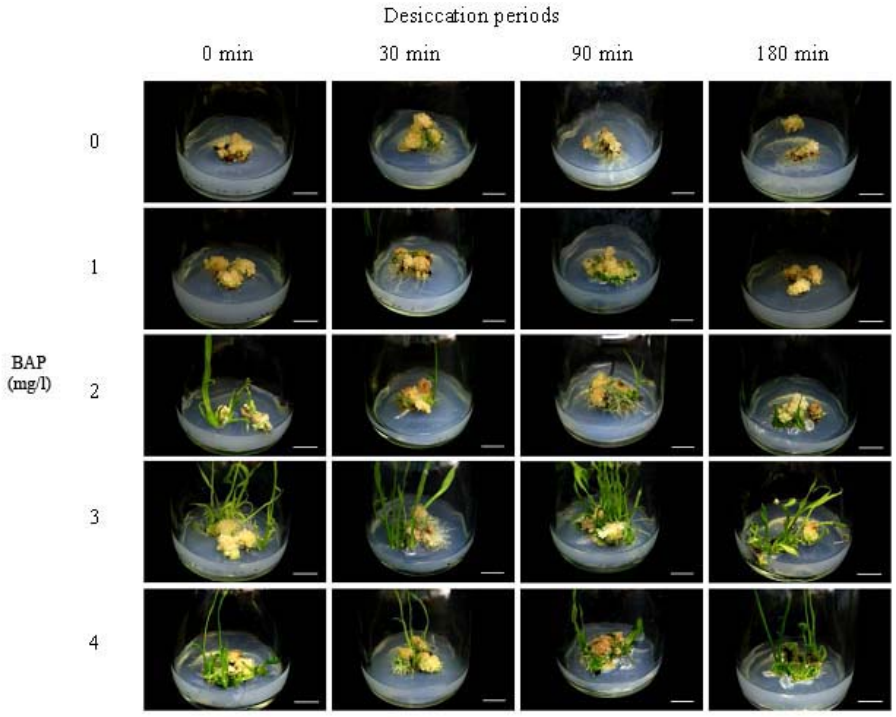

Figure 3 Plantlets of Pakaumpuel rice obtained from desiccated calli and cultured on regeneration media (bar = 1 cm.)

Conclusions

This current study reports a successful and high frequency regeneration protocol of Pakaumpuel rice from mature seed embryo through embryogenic callus formation. The study demonstrated that 2 mg/l 2,4-D without BAP under light condition provided the most effective callus induction of Pakaumpuel rice. In plant regeneration, we found that BAP was the vital factors to regenerate new plants from callus and callus desiccation did not affect regeneration percentage and number of shoots per callus, but over desiccation (180 min desiccation period) had negative effects on shoot length and number of leaves per shoot. The novel discovery found in this study is the fact that light can positively affect callus induction of Pakaumpuel rice, but callus desiccation prior to culture on regeneration medium cause negative effects on plant regeneration of Pakaumpuel rice. This discovery can be applied for micropropagation of other Thai rice landraces. In addition, the finding of this study will be useful for the first step of rice improvement or rapid *in vitro* propagation through any innovative biotechnological approaches which provide a simple and reliable protocol for *in vitro* generating high frequency callus and further cultivar genetic transformation experiment toward high yielding improved through plant tissue culture and genetic transformation techniques.

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