

The Effects of Temperature on Callus Induction and Regeneration in Selected Malaysian Rice Cultivar *Indica*

(Kesan Suhu ke atas Induksi Kalus dan Pertumbuhan Semula
dalam Kultivar *Indica* Padi Malaysia Terpilih)

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ABSTRACT

The development of an efficient tissue culture protocol for somatic embryo would facilitate the genetic modification in breeding program. The present study describes the reproducible protocols for three wetland Malaysian rice cultivars (MR232, MR220 and MR220-CL2) and upland rice (Bario) via somatic embryogenesis. In the present study, four pre-heat treatments (35, 40, 45 and 50°C) were applied to mature seeds with different imbibition periods (3, 5 and 7 days) prior to culture on MS media with 3 mg/L 2,4-D. The results showed that the cultivars exhibited the highest callus induction percentage from 45°C pre-heated seeds and 3 days imbibition (100%, 96%, 100% and 95% for MR232, MR220, MR220-CL2 and Bario, respectively). Callus was induced early ranging from 3 to 12 days compared to without pre-heat treatment. The regeneration efficiency for MR220 and MR220-CL2 cultivars was significantly higher compared to the control treatment. However, both 45°C and 25°C (control) treatments produced higher plantlet regeneration for MR232 and Bario. This study observed that pre-heat treated seeds prior to callus induction did promote callusing and hence regeneration. These findings can be used to establish a suitable protocol for the in vitro regeneration system for several genetic improvements in the numerous stress tolerances of Malaysian rice.

Keywords: Bario; callus; MR220-CL2; pre-heat treatment; regeneration; rice

ABSTRAK

Pembangunan protokol kultur tisu untuk penghasilan embrio somatik dapat membantu dalam pengubahsuaian genetik bagi rancangan pembiakbakaan. Kajian ini melaporkan protokol boleh ulang bagi tiga kultivar padi bendang Malaysia (MR232, MR220 dan MR220-CL2) dan padi bukit (Bario) melalui embriogenesis somatik. Dalam kajian ini, biji benih matang didedahkan kepada empat rawatan pra-pemanasan (35, 40, 45 dan 50°C) dengan masa pedapan yang berbeza (3, 5 dan 7 hari) sebelum dikultur pada medium MS yang mengandungi 3 mg/L 2,4-D. Keputusan kajian ini menunjukkan semua kultivar menghasilkan peratusan induksi kalus tertinggi daripada rawatan pra-pemanasan benih pada suhu 45°C dan 3 hari pedapan (100%, 96%, 100% dan 95% untuk MR232, MR220, MR220-CL2 dan Bario). Kalus diinduksi lebih awal pada kadar 3 ke 12 hari dibandingkan dengan tanpa rawatan pra-pemanasan. Keberkesanan penjanaan semula adalah lebih tinggi untuk kultivar MR220 dan MR220-CL2 dibandingkan dengan kawalan manakala kedua-dua rawatan pra-pemanasan 45°C dan kawalan mempunyai penjanaan semula anak pokok yang tinggi untuk MR232 dan Bario. Kajian ini menunjukkan bahawa rawatan pra-pemanasan ke atas biji benih sebelum penginduksian kalus menggalakkan pertumbuhan kalus dan juga penjanaan semula. Penemuan ini boleh digunakan untuk menghasilkan protokol yang sesuai untuk sistem penjanaan semula in vitro sebagai penambahbaikan kepada pelbagai jenis daya tahan padi Malaysia.

Kata kunci: Bario; kalus; MR220-CL2; padi; penjanaan semula; rawatan pra-pemanasan

INTRODUCTION

Oryza sativa L. is the second most widely cultivated crop in the world and a staple food in Malaysia. It belongs to the genus *Oryza* under the family Gramineae (Poaceae) and the tribe Oryzaceae. It is comprised of two subspecies, *indica* and *japonica*. Malaysian rice belongs to the *indica* subspecies. This rice species is grown both in wetland and upland areas of the country. The yield of wetland rice is comparatively higher than upland rice. However, upland rice is advantageous due to its low cost of production and

low irrigation requirements (Fageria & Baligar 2003). In the context of Malaysia, the upland rice Bario is famous for its nutritional value, long shape and exquisite taste (Wong et al. 2009). It could also be promoted as health food due to low glycemic index (Nicholas et al. 2014). Another aspect is that rice production in Malaysia is highly vulnerable to weather change such as drought and flooding. In order to ensure food security, the country needs to develop new high yield rice cultivars (FAOSTAT 2015) through available options such as new breeding.

Plant tissue culture consists of a wide range of applications which can be divided into three categories: Basic research, environmental issues and commercial applications. Understanding the concept of physiology and the molecular pathways in plant cells is part of basic research, whereas conservation strategies fall under the environmental application category. Research in plant tissue culture has focused on commercial applications such as crop improvement, secondary metabolite production and various strategies for inducing genetic interference (Chawla 2002). Somatic embryogenesis is an important plant propagation method used as a tool for crop improvement. The success of somatic embryogenesis depends on various factors including genotype, tissue, explant, cultural conditions and developmental stages of the mother plant (Rahman et al. 2010). Callus induction and regeneration are the main steps used for crop plants in biotechnological approaches. A lack of efficient tissue culture methods for generating new plantlets is one of the main barriers when it comes to developing high yield and well-developed transgenic plant species. The recalcitrance of *Oryza sativa* cv. *indica* has been attributed to low callusing and regenerating abilities (Zuraida et al. 2011), as well as to the frequent occurrence of albino plant regeneration (Silva 2010).

Different types of heat and cold treatments of seeds have been used for tissue culturing methods on various plant species such as wheat, millet and oat (Haque et al. 2017; Kiviharju & Pehu 1998; Touraev et al. 1996). Seeds treated using these methods have shown increase in callus formation over non-treated seeds (Chaar et al. 2012). The positive effects of pre-heat treatment have been studied in many plant species and it has been shown to encourage an androgenetic response during the *in vitro* culture (Kiviharju & Pehu 1998) of which five plants (haploids). It was also found that pre-heat treatment of 32°C for 5 days enhances embryo induction in oats. Therefore, pre-heat treatment will help to increase potential callus formations involved in the potential regeneration of the *indica* rice species (Silva 2010).

Although several researchers (Abdollahi et al. 2015; Hirano et al. 1999; Kiviharju & Pehu 1998; Kumar et al. 2015; Wang et al. 2014) have examined pre-heat treatment for callus induction and regeneration of different cereal crops, no protocols have been established for a pre-heat treatment for the callus induction of Malaysian *indica* rice. The production of embryogenic competent cells using pre-heat treatment could be an alternative solution for inducing callus and furthering regeneration protocols. In this research, the seeds underwent pre-heat treatment for the development of potential callus induction protocols in different upland and wetland Malaysian *indica* rice varieties. The study specifically focused on discovering the effect of pre-heat treatment on callus induction and regeneration.

MATERIALS AND METHODS

PLANT MATERIALS

Three wetland rice cultivars i.e. MR220, MR232 and MR220-CL2 (hybrid MR220 cultivar) and an upland rice cultivar Bario were used in this study. The wetland rice cultivars were obtained from MARDI, Seberang Prai, Penang, Malaysia and the upland cultivar was obtained from Sarawak, Malaysia.

SEED SURFACE STERILIZATION

Manually dehusked mature rice seeds were sterilised using 100% ethanol for 1 min. Afterwards, the ethanol was discarded and a mixture of 100% Clorox (Sodium hypochlorite 5.99%) with 2-3 drops of Tween-20 (Sigma –Aldrich, USA) was added to the seeds. The seeds were then shaken on an orbital shaker at 120 rpm for 30 min. Then, the solution was removed and the sterilised seeds were rinsed with sterile double distilled water and finally blotted onto sterilised filter paper (Binte Mostafiz & Wagiran 2018).

CALLUS INDUCTION EXPERIMENT AND PRE-HEAT TREATMENT

Rice seeds from four cultivars were incubated for 3, 5 and 7 days at 25, 35, 40, 45 and 50°C separately in the incubator prior seed sterilization, with 25°C incubation representing the control. Callus induction media (CIM) was prepared based on Murashige and Skoog (1962) medium, 3 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D) and 2.5 g/L phytigel were used as a solidifying agent. The pH of the medium was adjusted to 5.7 before autoclaving. Sterilised seeds from pre-heat treatment were cultured horizontally onto the CIM and then incubated for 8 weeks at 25±2°C under dark condition. The callus were sub-cultured onto the same media at an interval of 3 weeks. The callus thus induced were then examined under the microscope for morphological characteristics such as colour and structure. The callus induction percentage was recorded at eight weeks of culturing. The following formula was used for determining the callus induction percentage:

$$\text{Callus induction percentage} = (\text{number of induced callus}) / (\text{number of incubated seeds}) \times 100\%$$

MEASUREMENT OF CALLUS FRESH WEIGHT AND DRY WEIGHT

Approximately 8-weeks callus were collected and the fresh weights (FW) and dry weights (DW) were recorded in order to determine the biomass. Readings were obtained from three replicates in each treatment. The callus were directly transferred onto an empty petri dish with filter paper to weigh the FW. For the DW, the callus were incubated at 60°C for 3 days. The DW was recorded by weighing the dried callus.

VISUAL OBSERVATION BY LIGHT MICROSCOPE

Visual observation was performed using a light microscope (Nikon Eclipse E200, Japan) to determine the colour and surface structure of the callus. In this study, the callus were divided into four categories depending on colour characteristics according to Visarada et al. (2002): (i) white and cream compact callus that usually show excellent regeneration; (ii) yellow organised callus; (iii) brown unorganised callus that has turned brown; and (iv) rhizogenic callus that only produce roots. Types (i) and (ii) have high embryogenic potential, while types (iii) and (iv) are non-embryogenic.

PLANT REGENERATION AND ACCLIMATIZATION

Embryogenic callus were selected and placed on MS basal media according to Mohd Din et al. (2016) protocols. Callus were taken from four different rice cultivar (MR220, MR220-CL2, MR232 and Bario) and placed on regeneration media (designed as RM media composed of MS supplemented with a combination of BAP (3 mg/L), Kinetin (2 mg/L) and NAA (0.5 mg/L) and 30 g/L maltose). The media were solidified with 4 g/L gelrite. All culture plates were then incubated under a 16/8 h (light/dark) photoperiod at 27°C for 8 weeks. The *in vitro* regeneration responses based on number of callus initiation with green spot were observed every 2 weeks. The regenerated plantlets were transferred to culture tubes containing the same MS basal medium for shoot elongation after 4 weeks. To facilitate root proliferation, the regenerated *in vitro* plantlets were transferred to a half-strength MS basal medium without plant growth regulators for 7 days. For acclimatization, any residue entrapped on the root system was gently washed with sterile water before transplanted into soil. Then, plants with healthy roots were transferred to pots filled with 4:1 v/v mixture of soil and organic manure and placed in glasshouse. The regeneration percentage = (number of green spot producing callus)/(number of inoculated callus piece) × 100% (Binte Mostafiz & Wagiran 2018).

STATISTICAL ANALYSIS

The experiments used a Randomised Complete Block Design (RCBD) with four blocks, where three replicates for each block. The callus induction frequency and callus initiation days were recorded for three replicates from each treatment. Plantlet regeneration rates and the number of plantlets after transferring into soil were recorded for three replicates for each treatment. The data was analysed using a one-way analysis of variance (ANOVA) and the mean differences were evaluated using Tukey's test of SPSS software version 22.0 (SPSS, Chicago, IL, USA). The differences were considered statistically significant when $p \leq 0.05$. Different means were marked with different letters (a, b, c and d) in order to represent the significant value of the differences.

RESULTS AND DISCUSSION

PRE-HEAT TREATMENT AND CALLUS INDUCTION

In this study, the wetland and upland rice cultivars began producing callus from the scutellum region between 2.4 and 20 days after culturing on callus induction media (Table 1). The study showed that early callus were seen on different days in all the wetland and upland rice cultivars at 45°C compared to those without heat treatment. The earliest was 2.4 days for MR232, 3.9 days for MR220, 3 days for MR220-CL2 and 12 days for Bario cultivar. The early callus initiation was significantly affected by pre-heat treatment in the present study. Even though there was not much information on the effect of pre-heated seed prior to rice callus induction (Reddy et al. 1985), there have been several reports on other monocot seeds, e.g. maize (Afele et al. 1992) and wheat (Li et al. 1988), showing optimal duration of temperature was more or less similar in cultivars. Previous literature showed that the callus initiation of Basmati rice took 7–10 days on MS media supplemented with 2 mg/L 2,4-D (Haq et al. 2009), presumably from untreated heat seed. However, in this study the callus initiation from rice seeds was found to

TABLE 1. The effects of various pre-heat treatments on callus initiation of MR220, MR220-CL2, MR232 and Bario rice

Heat treatment (°C)	Cultivar name			
	Callus initiation after seed culture (days)			
	MR220	MR220-CL2	MR232	Bario
25*	9 ^b	5.8 ^c	6.8 ^c	21.0 ^b
35	7.1 ^b	5.2 ^c	5.4 ^c	18.8 ^b
40	5.2 ^b	4.7 ^b	4.2 ^b	15 ^a
45	3.9 ^a	3.0 ^a	2.4 ^a	12 ^a
50	8 ^b	4.2 ^b	5.5 ^c	20 ^b
P	0.001	0.045	0.043	0.047

Means followed by different letters were significantly different within column at $p \leq 0.05$ in Tukey's test ($n = 12$). *25°C used as control

be earlier, i.e. after 3-4 days at 45°C for the wetland rice cultivars as compared to 12 days in case of Bario upland rice cultivar. The present study also showed the optimal temperature was different between upland rice and wetland rice in this study. Pre-heat treatment at 45°C was found contributing to early callus initiations in Malaysian rice, as shown in our findings. In rice anther cultures, previous reports have indicated that both pre- and post-cultures had positive effects on callus initiation (Silva 2010). Pre-heat treatment could be beneficial not only for rice anther culture, but also for other rice explants.

CALLUS INDUCTION PERCENTAGE

In general, all the treatments were found to produce callus when cultured on CIM from heat treated seed and at different imbibition time (Figure 1) ranging at 33-100%. Callus induction rates from pre-heat treatment seeds were higher than without pre-heat treatment (control). The exposure of seeds for 3 days at different temperatures showed a high frequency of callus induction compared to 5 and 7 days in all the cultivars. The results also showed that incrementing the temperature and duration of pre-heat treatment caused a reduction in callus induction percentage. The results further demonstrated that high temperatures (i.e. 50°C) caused a reduction in callus induction percentages for both the upland and wetland rice cultivars.

The percentages of callus induction percentages for MR220, MR220-CL2, MR232 and Bario were 96, 100, 100 and 95.7%, respectively, when exposed to 45°C for 3 days (Figure 1). The percentage of callus recorded in the current study was much higher compared to those reported by previous researches which ranging from 60 to 87% (Abiri et al. 2017; Kadhim et al. 2016; Libin et al. 2012), we describe the reproducible protocol of Malaysian rice (*Oryza sativa* L.). Our findings showed that the pre-heat treatment in seed may facilitate earlier callusing as well as increase the percentages. Pre-heat treatment was also reported to enhance callus induction in cereals such as oats (Kiviharju & Pehu 1998) and rice (Reddy et al. 1985).

Callus morphologies from the four rice cultivars under different pre-heat treatments are presented in Figure 2. The seeds incubated at 45°C for 3 days produced callus that were compact, nodular, dry, iso-diametric and had a relatively whitish to light yellowish colour with a smooth surface (Figure 2; M-P). In contrast, non-embryogenic callus which had loosely held cells, were watery and yellowish to brownish in colour. Our finding was in accordance with previous works (Abiri et al. 2017; Visarada et al. 2002). Based on colour and structural morphology, the callus obtained from the present experiment were mostly embryogenic at 45°C. It was illustrated that pre-treatment at 10°C for 7-8 days of incubation for an elite *indica* rice hybrid (BS6444G) was effective for callusing and plant regeneration (Naik et al. 2017).

FRESH WEIGHT AND DRY WEIGHT OF CALLUS

The present study showed that increments in temperature decreased FW and DW (Figure 3). The highest callus formation was found at 45°C and as a consequence, all the cultivars exhibited the highest amount of FW and DW. For FW, no significant differences were found among the cultivars. However, significant differences were found when pre-heat treatment was used, and the highest FW values were at 45°C (381, 378.3, 394.3 and 390.3 mg for MR220, MR220-CL2, MR232 and Bario, respectively).

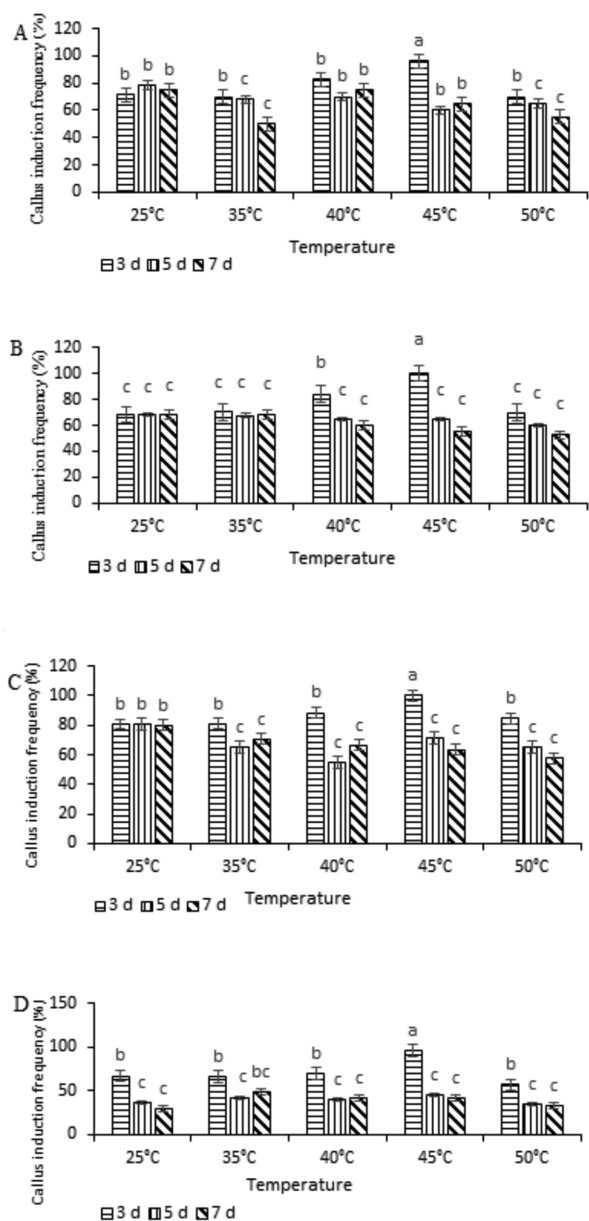


FIGURE 1. Callus induction frequency under different pre-heat treatments and different durations of (A) MR220, (B) MR220-CL2, (C) MR232 and (D) Bario cultivars. Vertical bars present \pm SEM ($n = 12$). Different letters (a-c) represent significant differences amongst treatment group where 25°C used as control

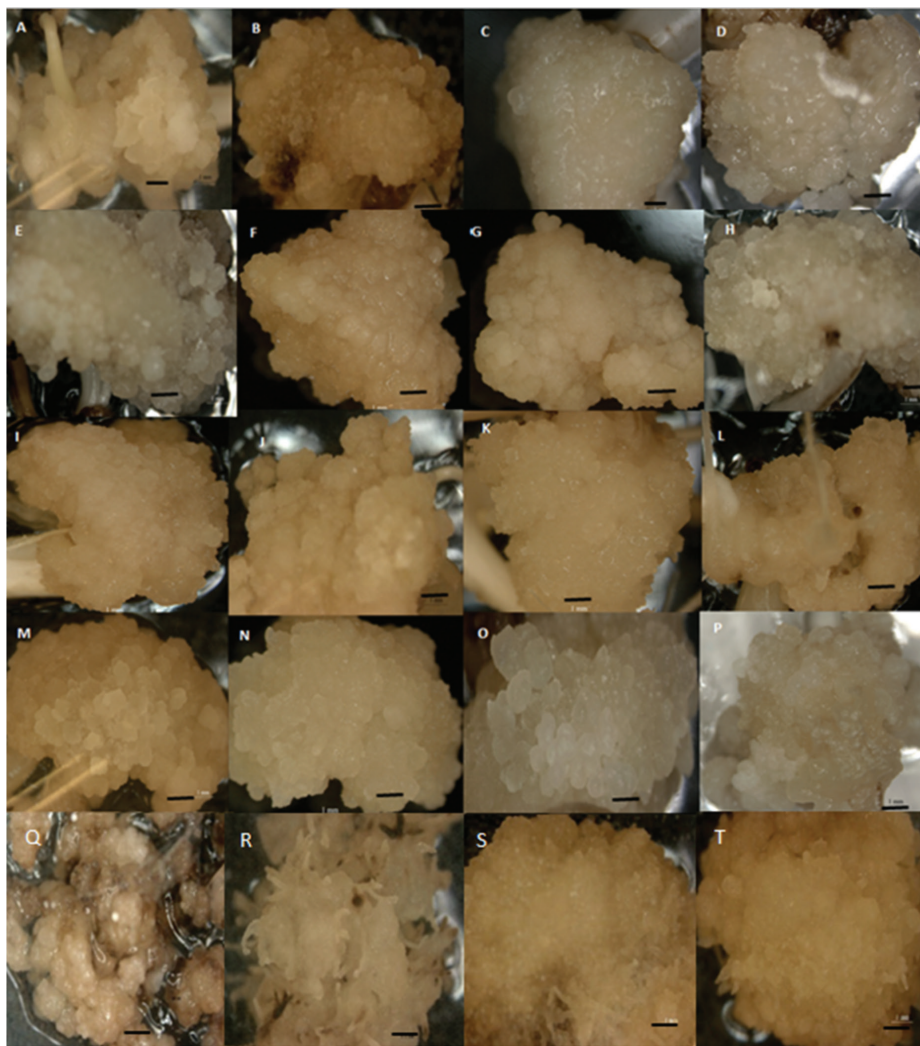


FIGURE 2. Callus inductions of four Malaysian rice cultivars after 8 weeks culture at different temperatures with 3 days of pre-heat treatment, (A–D): MR220, MR220-CL2, MR232 and Bario at 25°C; (E–H): MR220, MR220-CL2, MR232 and Bario at 35°C; (I–L): MR220, MR220-CL2, MR232 and Bario at 40°C; (M–P): MR220, MR220-CL2, MR232 and Bario at 45°C; (Q–T): MR220, MR220-CL2, MR232 and Bario, Embryogenic and non-embryogenic callus found in different cultivars at 50°C; (M–P) Globular, white and compact callus found at 45°C in all rice cultivars; (Q–T) yellow to brown and unorganised callus found at 50°C in all rice cultivars (C, G, R) Rhizogenic callus that produce only roots. 25°C used as control. Bar = 1 mm

The DW values of rice callus were the highest from 45°C treatment (167, 159, 160 and 170 mg for MR220, MR220-CL2, MR232 and Bario, respectively), while the lowest values were found at other temperatures in all the rice cultivars.

Previous research has shown significant differences in terms of DW and FW observed in different rice cultivars (Htwe et al. 2011), which is similar to our study. In another study, the FW of callus was measured at 160–320 mg of Malaysian *indica* rice MR269 (Kadhimi et al. 2016), whereas FW of callus was higher in the present study. According to Azizi et al. (2017), maximum fresh and dry weights of callus were recorded in different types of Malaysian *indica* rice cultivars including MR50, MR 74, MR219 and MR276. Ranging at 0.40-0.45 g and 0.01-0.02 g, respectively.

REGENERATION AND ACCLIMATIZATION

Embryogenic callus from 45°C pre-heated seeds were transferred to RM in order to investigate their potential for regeneration. The percentages of plantlets regenerated and number of plantlets are shown in Table 2. Pre-heat treatment callus at 45°C showed the highest percentages of shooting and significant differences compared to the control in only two cultivars MR220 (61%) and MR220-CL2 (82%). However, the same pre-heat treatment did not show significant difference in the other two cultivars MR232 and Bario. The highest regeneration was recorded for MR220-CL2 (82%). The pre-heat treatment was not significant difference in terms of the number of plantlets in all the cultivars except MR220. After the callus were transferred onto RM, the green spots emerged early at day 7 for pre-heated treatment (45°C) while it was in a range

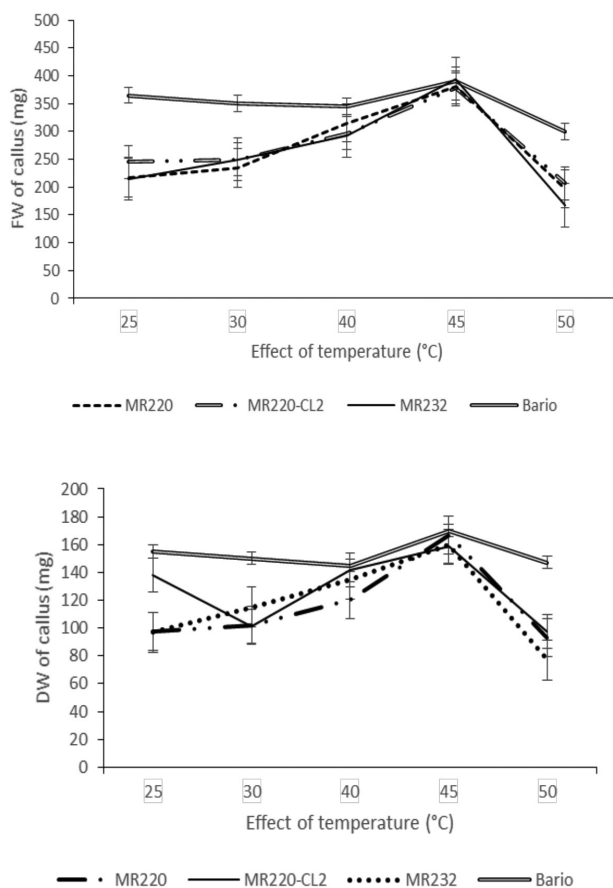


FIGURE 3. Fresh weights (FW) and dry weights (DW) of the callus of MR220, MR220-CL2, MR232 and Bario rice cultivars after 8 weeks of culturing where 25°C used as control. Vertical bars present \pm SEM ($n=12$)

of 9-12 days for control treatment in all the cultivars. Then, the green spots underwent further development into multiple shoots and *in vitro* plantlets. The healthy plants were then transferred into soil. All the cultivars started to flower within 45 days except for the Bario. The plantlet regeneration is shown in Figure 4 and fertile regenerated plants on soil are presented in Figure 5.

In the present study, it was found that plantlet regeneration was genotype-specific in relation to pre-heat treatment. Plantlet regeneration percentage was found to be increased from pre-heat treatment (45°C) with up to 17 and 15% in MR220 and MR220-CL2,

respectively. However, pre-heat treatment showed no positive significant effect on plantlet regeneration in the other two cultivars. This was also concurred with the earlier recorded of non-embryogenic callus morphology at 50°C pre-heat treatment as well as decreased of FW and DW. In our study, the adoption of higher temperature (45°C) on seeds has been shown to enhance regeneration in Malaysian rice cultivars compared to other Indian *indica* cultivars (Biswas & Mandal 2007). The Bario callus was the least affected by pre-heat treatment in terms of regeneration ability since this genotype is normally planted in upland environment. According to Zuraida et

TABLE 2. The effect of regeneration from various heat pre-treated callus of MR220, MR220-CL2, MR232 and Bario rice

Treatment (°C)	Cultivar							
	Plantlet regeneration (%)				No. of plantlet			
	MR220	MR220-CL2	MR232	Bario	MR220	MR220-CL2	MR232	Bario
25*	44 ^b	67 ^b	74	42	4 ^b	7.2	7.2	4
45	61 ^a	82 ^a	71	40	7 ^a	9.1	6.1	5
P	0.041	0.001	N.S.	N.S.	0.001	N.S.	N.S.	N.S.

Means followed by different letters were significantly different within column at $p \leq 0.05$ in Tukey's test ($n = 12$). *25°C used as control

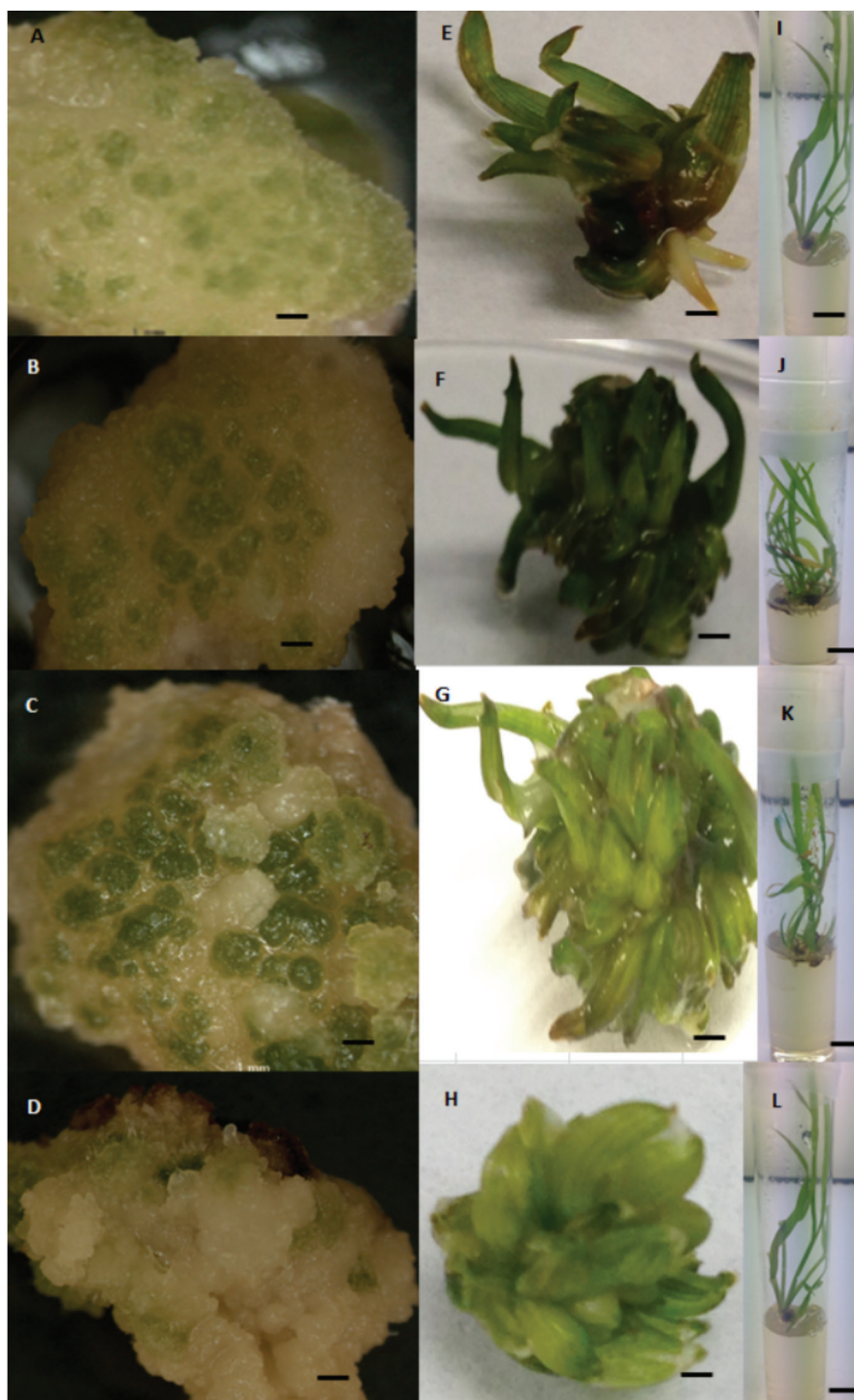


FIGURE 4. Plant regeneration through somatic embryogenesis of four Malaysian *indica* rice cultivars which were derived from pre-heat treated seeds at 45°C for 3 days, (A–D): Morphological features of green spot initiation on regeneration media under light microscope after 21 days of regeneration of MR220, MR220-CL2, MR232 and Bario, respectively (bar =1mm), (E–H): regenerated shoot (bar =1cm) and (I–L): plantlet regeneration on rooting media of MR220, MR220-CL2, MR232 and Bario, respectively. bar = 1 cm

al. (2011), pre-treatment may stimulate initial cell division and play an important role in physiological characteristics of callus. Additionally, adoption of pre-heat treatment was reported to be beneficial in androgenesis of *japonica* rice but perform poorer responses in *indica* rice (Dewi et al.

2009). Our study showed that pre-heat treatment at 45°C for initial seeds contributed to high plantlet regeneration in only two Malaysian rice cultivars. This treatment is considered beneficial for regeneration purpose in our case.

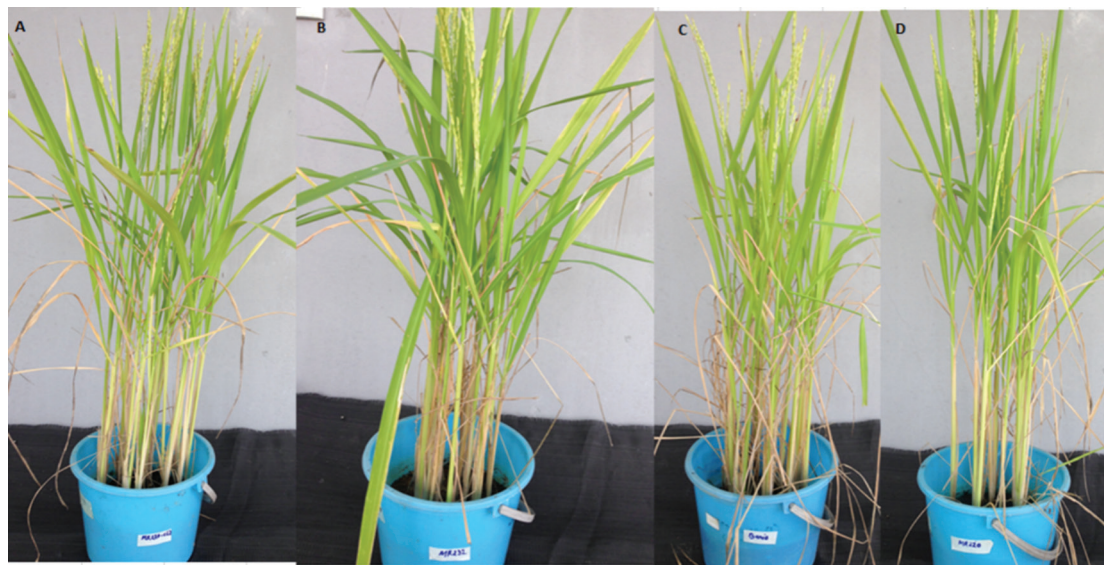


FIGURE 5. A fertile regenerated rice plants on soil after transferring from rooting media of (A): MR220, (B): MR220-CL2, (C): MR232 and (D): Bario. The pictures (A, B, C) were taken at 54-days and the picture (D) was taken at 65 days after transplanting soil

CONCLUSION

This study demonstrates the establishment of embryogenic callus induction for Malaysian rice cultivars MR232, MR220, MR220-CL2 and Bario from mature seeds through embryogenic callus formation. It was found that pre-heat treated seeds at 45°C and 3-day imbibition showed the highest callus induction percentages among all the cultivars. Moreover, early callus induction was also observed at the same treatment. Pre-heat treated seeds at 45°C and 3-day imbibition contributed to the highest regeneration percentage compared to control treatment (25°C) in the rice cultivars. This study showed that the PGR combination of 3 mg/L of BAP + 2 mg/L of Kinetin + 0.5 mg/L of NAA on MS media has the potential to promote regeneration of selected *indica* rice cultivars. Pre-heat treatment on seeds might be helpful in callus formation and regeneration of *in vitro* plantlets in Malaysian rice. This protocol could be used for other recalcitrant *indica* rice genotypes and to transfer desirable genes into Malaysian *indica* rice cultivars for crop improvement.

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