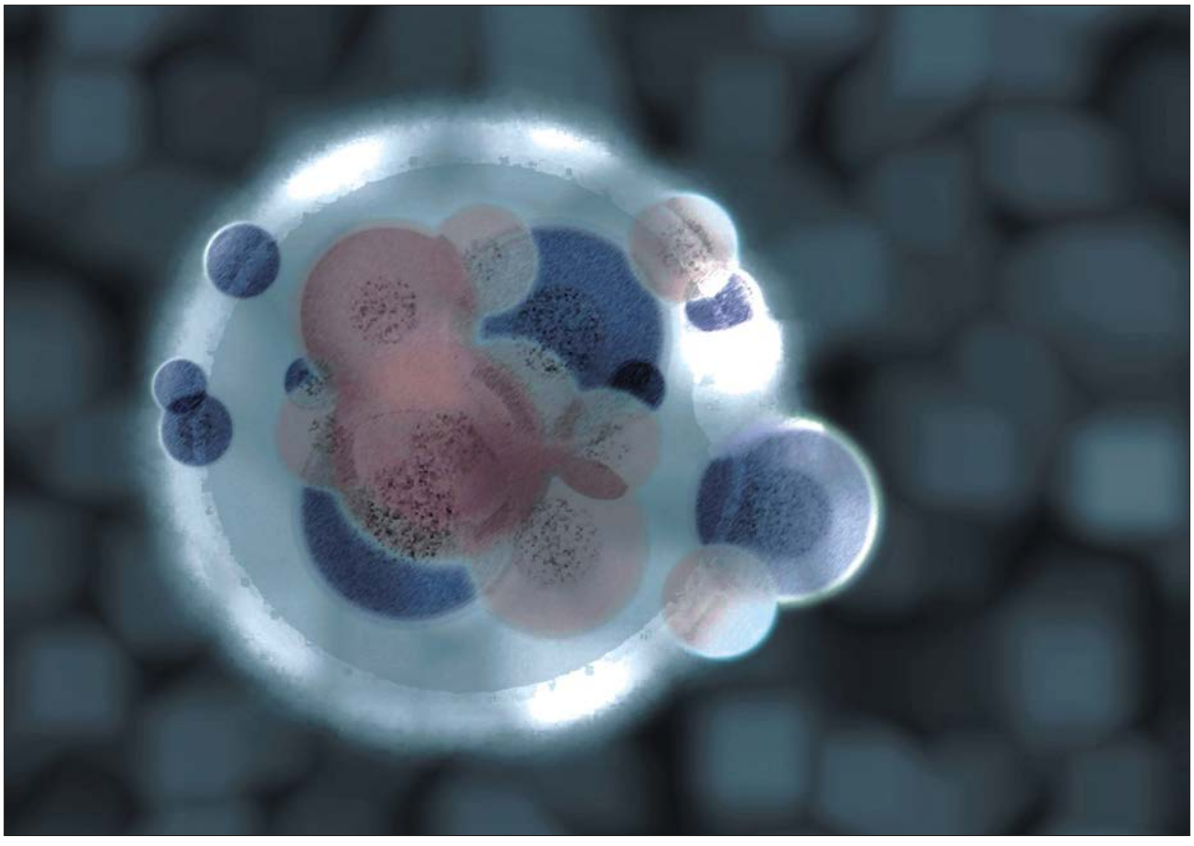

Journal of Biological Sciences

ISSN 1727-3048

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Journal of Biological Sciences 8 (3): 570-576, 2008
ISSN 1727-3048

Improvement of Plant Regeneration from Embryogemic Suspension Cell Culture of Japonica Rice

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Abstract: Improvement on plant regeneration system from suspension cells culture of different japonica rice cultivar was attempted in the present study. The present study shows that callus induction varied depend on genotype. *Nipponbare*, *Hayahishiki* and *Fujisaka 5* variety showed 100, 93 and 53% of callus induction respectively on LS medium supplemented with 2,4-D after 4 weeks in culture. Callus proliferation was observed at the scutellum and yellowish in colour. Suspension cell culture was initiated from 4 weeks old callus in N_6 liquid medium contained with 3 mg L^{-1} 2,4-D and 1 mg L^{-1} kinetin for one month with regular subculture. The embryogenic cells were characterized with 1% (w/v) Evans Blue. Primary study shows that shoot regeneration was highest on *Nipponbare* at 60%, while *Hayahishiki* and *Fujisaka* were at 30 and 20%, respectively when culture on MS basal medium contained 3 mg L^{-1} Kinetin and 0.5 mg L^{-1} NAA (SRM3). After 6 weeks, the callus covered with green shoot bud and later formed shoot with an average of 3-8 shoot per callus. Increase of shoot regeneration was observed when callus treated with partial desiccation in all variety tested. The regeneration frequency was highest at 76, 70 and 33% when *Nipponbare*, *Hayahishiki* and *Fujisaka 5*, respectively were treated with 48 h desiccation. However, shoot multiplication was low in 0 h desiccation ranging from 10-16%. After 8 weeks in optimal SRM medium, the plantlets was hardened in growth chamber for 2 weeks and later transferred into soil with 100% survival in all variety. The present study shows that rice regeneration system from suspension cells can be improved with modification of plant growth regulators and desiccation.

Key words: Cell suspension, *Oryza sativa*, 2, 4-D (2,4-dichlorophenoxyacetic acid), K (kinetin), proline, desiccation, sorbitol

INTRODUCTION

Efficient of plant regeneration for rice has been widely reported in japonica and indica (Carsono and Yoshida, 2007; Rashid *et al*., 2003; Lee *et al*., 2002; Visarada *et al*., 2002). In the literature, there many references concerning the regeneration conditions of indica and japonica varieties and the additional of amino acids such as proline (Chowdhry *et al*., 2000) and glutamine (Poné *et al*., 2000) has been demonstrated to improve shoot morphogenesis while macronutrient, growth regulator compositions strongly influence embryogenic callus and shoot formation in some varieties (Poonsapaya *et al*., 1989; Rueb *et al*., 1994). Many agronomically valuable genotypes are recalcitrant to *in vitro* manipulation because of their poor regeneration ability. These rates could be increase if appropriate improvements were made to the tissue culture medium (Zhu *et al*., 1996) and partial desiccation (Rance *et al*., 1994). Partial desiccation treatments have been reported to be beneficial for embryogenesis and rice plant regeneration such as reported by Tsukahara and Hirosawa (1992a, b) where 24 h partial desiccation increased shoot regeneration from 5 to 47% in japonica cell suspension. Partial desiccation has also been reported to promote indica rice regeneration (Saharan *et al*., 2004; Chand and Sahrawat, 2001; Diah and Bhalla, 2000).

Somatic embryogenesis is the process by which the somatic cells give rise to bipolar structure, which develop into whole plants without gamete fusion. Somatic embryogenesis and suspension cell culture provide a useful experimental model to study the involvement of plant hormones and protein in the regulation of cell proliferation. It strongly depended on plant genotype and type, physiological stage of explants, the compositions of culture medium and condition of culture. Advantages of using embryogenic cell suspension culture are chimeric cells can be avoided if used for transformation, constant availability of competent embryogenic cells and easiness for scaling up. However, the procedures are laborious,

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frequently the regeneration capacity is lost and a great number of albino or non fertile plants are obtained (Poné *et al*., 2000) or loss of totipotency by the suspension cells (Tang *et al*., 2001). Establishment of japonica suspension rice culture has been reported such as in *Nipponbare* variety (Lee *et al*., 2004), japonica Chinese variety (Tang *et al*., 2001) and japonica rice (Wenjing *et al*., 1997). However, Lee *et al*. (2004) and Tang *et al*. (2001) only focus on developing suspension cell culture while Wenjing *et al*. (1997) shows regeneration potential of suspension after 14 months culture, but non of plant regeneration from fresh callus-derived mature seeds. Alternatively, pectinase also can be use to maintained fine culture and homogenize cells hence reducing aggregate in *Nipponbare* suspension cells (Lee *et al*., 2004).

One of the requirements for the establishment of cell culture is to count on a reliable and efficient method to estimate cell viability. The cell viability can be evaluated by staining the dead cells or living cells are coloured, because the colour is a product of cell metabolic activity (Widholm, 1972). The most used stain for dead cells are Evans blue, methylene blue and phenosafranin. The Evans blue are reduced by the living cells turning colourless while the dead cells remain blue. Therefore in the present study, Evans blue was use as a staining method because of cheap, reliable and can be examined fast under light microscope. The present study show a simple method to enhance regeneration frequency from suspension culture cell and regeneration is faster after partial desiccation which can be used for genetic transformation studies.

MATERIALS AND METHODS

Plant materials and seed sterilisation: This study was conducted from February to November 2007 at School of Bioscience and Biotechnology, Faculty of Science and Technology, UKM Bangi, Selangor, Malaysia. Mature seed of japonica rice var *Nipponbare*, *Hayahishiki* and *Fujisaka 5* were collected from MARDI, Penang (Accession Number: *Nipponbare* (10010), *Hayahishiki* (2883), *Fujisaka 5* (443). Seeds of these varieties were stored at 4°C prior to use. The seeds were dehusked and surface sterilised in 70% (v/v) ethanol for 1 min followed by 30 min shaking in 30% (v/v) of sodium hypochloride contained 2 drops of Tween 20. After rinsing 5 times with sterile distilled water, the sterilised seeds were used for callus induction.

Callus induction: For initiation of calli, dehusked sterile seeds were cultured horizontally on the surface of LS2.5 medium (Linsmaier and Skoog, 1965) contained 2.5 mg L^{-1} 2, 4-D, 500 mg L^{-1} glutamine, 30 g L^{-1} sucrose and 3 g L^{-1} gelrite (LS 2.5) and incubated at 25°C ±1 under dark condition for 1 month. After 2 weeks, the primary callus was removed from the scutellum and subculture on fresh medium and then used to establish embryogenic callus or to initiate suspension cell culture at 4 weeks culture. The plate (10 seeds/plate) was then subculture at 2 weeks interval.

Establishment of rice cell suspension cultures: Suspension cell culture was generated from actively-growing undifferentiated 4 weeks old callus. To test the function of proline, 2 g of callus were cultured in 25 mL N_6 liquid medium (Chu *et al*., 1975) containing 3 mg L^{-1} 2,4-D and 1 mg L^{-1} kinetin (designated $N_6$3K) with or without 500 mg L^{-1} proline in 150 mL conical flask. Serial subculture was carried out every 3 days by adding fresh $N_6$3K medium for one week and then subculture weekly. The cultures were shake on the rotary shaker at 140 rpm and maintained at 26±1°C for 1 month. Each week, 1 mL of cell suspension was observed under microscope to determine its morphology. Growth of cell suspension was determined by fresh weight every 2 days for 2 weeks. Each variety contained 2 replicate and repeated twice. The regeneration potential of the embryogenic suspension cell culture were tested on different shoot regeneration media and later used for desiccation treatment.

To determine the cell viability, 1 mL of cell suspension culture were immersed in 1% (w/v) Evans blue for 10 min at room temperature while control treatment in 70% (v/v) ethanol according to method of Fernandez-Da Silva and Menendez-Yuffa (2006). This procedure was repeated each month to check the cells viability.

Plant growth regulators and plant regeneration: One month old of 1-2 mm embryogenic cell culture was placed on MS medium, B_5 vitamin (Gamborg *et al*., 1968), 500 mg L^{-1} glutamine supplemented with different kinetin concentrations (1, 2, 3 mg L^{-1} kinetin) and 0.5 mg L^{-1} NAA and incubated at 16 h light and 8 h dark for one month and subculture at 2 weeks interval.

Desiccation treatment and plant regeneration: Desiccation treatment was performed by transferring 1 month old suspension cell culture to sterile Petri dishes containing one sterile Whatmann filter paper (Saharan *et al*., 2004). The petri dish were sealed with Parafilm and incubated at 25°C ±1 in dark for 24 and 48 h to obtain desiccated calli. After desiccation treatment, the desiccated calli were transferred into optimal shoot regeneration media (designated SRM3) contained MS (Murashige and Skoog, 1962), Gamborg B_5 vitamin, 3 mg L^{-1} kinetin, 0.5 mg L^{-1} NAA and 3 g L^{-1} gelrite. To

check the regeneration potential, the optimal shoot regeneration was compared to same MS media but contained 1 mg L^{-1} BAP and 10 g L^{-1} sorbitol (designated SRM4). Ten to fifteen centimeters plantlets with good rooting were hardening at 85% humidity and 25°C ±1 in the growth chamber for 2 weeks. Later the plantlets were transferred under normal glasshouse conditions.

Culture conditions: All chemicals used in the present study were from Sigma Aldrich otherwise stated. All the media used in this study was adjusted to pH 5.8 before autoclaving at 121°C ±1 for 20 min. The callus induction (LS2.5) and shoot regeneration media (SRM3 and SRM4) plates (~25 mL/plate) were sealed using Parafilm (Chicago, USA) and were incubated at 28°C ±1 at dark condition or 16 h day and 8 h night length respectively. Each treatment has 3 replicate and repeated twice. Observation on callus induction were made 4 weeks after incubation and the number of shoot regenerated per callus were counted after 4 weeks on shoot regeneration media.

RESULTS

Callus induction: Callus formation varied among the rice varieties tested. In general, the callus was developed from the scutellar region of the seeds and was visible after 14 days (Fig. 4). The primary callus 1-2 mm was later proliferated into yellowish to white callus after 3 weeks. The callus frequency was 100, 93 and 53% for variety *Nipponbare* followed by *Hayahishiki* and *Fujisaka 5* respectively after 4 weeks in culture (Table 1).

Inclusion of proline show increase of fresh weight in all variety tested compared to without proline (Fig. 1). All variety shows increased in fresh weight gradually after 2 days incubation in media contained proline while slow response was observed in medium without proline (Fig. 1). *Fujisaka 5* showed the best response to proline compared to *Nipponbare* and *Hayahishiki*. The present study shows that inclusion of proline in the medium culture increase the cell mass of suspension culture.

The suspension cells proliferate rapidly and well dispersed which consisted of heterogenous cells where embryogenic cells were smaller, spherical in shape and had yellow dense cytoplasm with very few vacuoles while elongated cells with obvious vacuoles were non embryogenic (Fig. 3). One month old of embryogenic suspension cell was then cultured on MS supplemented with different concentration of kinetin and NAA to induce shoot for obtained *in vitro* plant. After 4-6 weeks the calli were covered with green area approximately 3-10 shoots regenerated per callus. From the Table 2, it shows that

Table 1: Percentage of callus formation from different rice variety on LS media supplemented with 2.5 mg L^{-1} 2, 4-D after 4 weeks in culture

Rice variety	Callus formation (%)
Nipponbare	100
Hayahishiki	93
Fujisaka 5	53

Table 2: Shoot regeneration of embryogenic cell suspension from different variety of rice on different MS media after 4 weeks in culture

	Plant growth regulator		
Rice variety	Kinetin	NAA	Shooting (%)
Nipponbare	1	0.5	20
	2	0.5	40
	3	0.5	65
Hayahishiki	1	0.5	10
	2	0.5	18
	3	0.5	30
Fujisaka 5	1	0.5	3
	2	0.5	10
	3	0.5	20

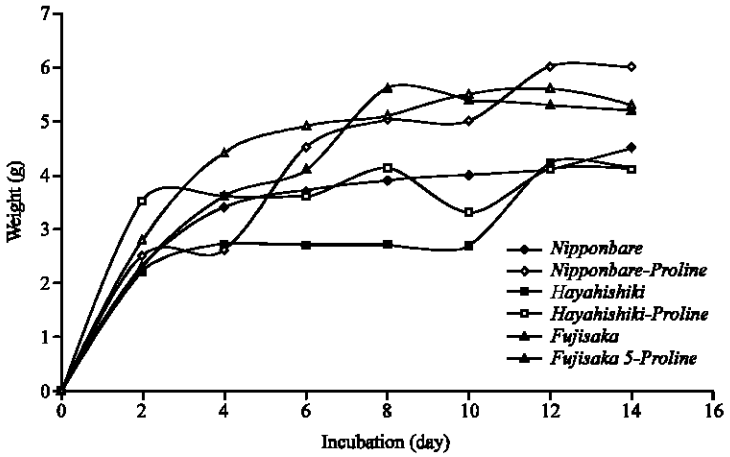

Fig. 1: Growth of rice suspension cell culture in $N_6$3K liquid media contained with or without proline after 2 weeks in different rice variety

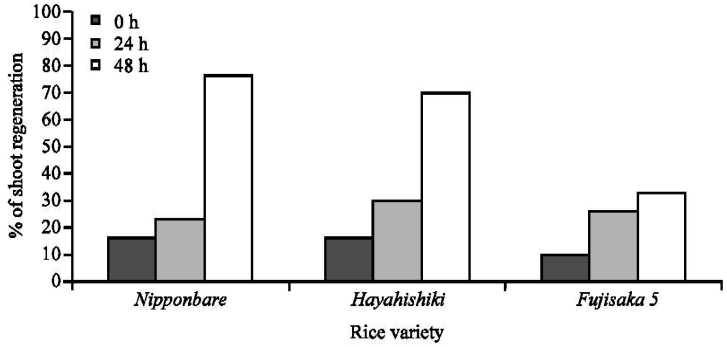

Fig. 2: Effect of partial desiccation (0, 24 and 48 h) on shoot regeneration of embryogenic cell suspension of different rice varieties when culture on MS medium contained 3 mg L^{-1} kinetin, 1 mg L^{-1} BAP and 0.5 mg L^{-1} NAA after 2 month in culture

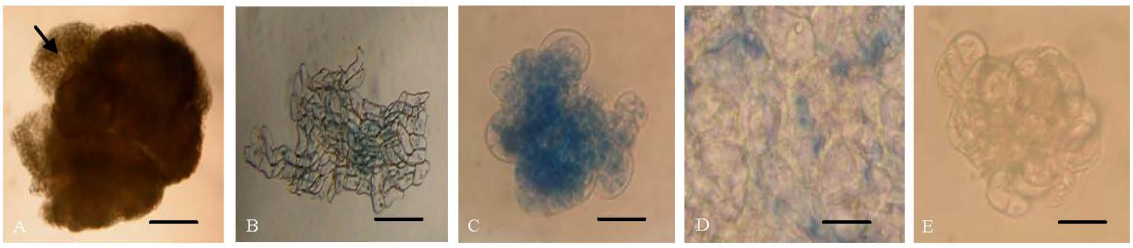

Fig. 3: (A) Cluster of embryogenic cell suspension (show with arrow). (B) Non embryogenic cells suspension (C) Non viable cell suspension stained with 1% (w/v) Evans blue. (D) Viable cells in 1% (w/v) Evans blue staining (E) Non-stained cells in 70% (v/v) ethanol. — 10 μm

increment of kinetin concentration increase shoot regeneration. Maximum shooting percentage observed in var *Nipponbare* at 60% followed by *Hayahishiki* and *Fujisaka 5* at 30 and 20%, respectively on MS media contained 3 mg L^{-1} kinetin and 0.5 mg L^{-1} NAA. However, *Fujisaka 5* variety showed poor response on shoot regeneration media. The optimal shoot regeneration media (MS supplemented with 3 mg L^{-1} kinetin and 0.5 mg L^{-1} NAA:SRM3) were then used for shoot regeneration of desiccated calli.

Calli regenerated from desiccated embryogenic cell culture were covered with green bud on SRM3 after 2 weeks in culture. During subculture, the shoot buds further elongated and multiple vigorously. The shoot initials appeared as green areas and average of 3-8 shoots per callus were observed. Shoot regeneration was 10-16% in all variety when treated with 0 h desiccation while maximum shoot regeneration frequency was observed when 48 h desiccation was used as treatment in all variety. The shoot regeneration was highest at 76 and 70% for var. *Nipponbare* and *Hayahishiki* while 33% for *Fujisaka 5* (Fig. 2). Although the plantlet could regenerated on SRM3 medium without BAP and retained the ability to regenerate plant but addition of 1 mg L^{-1} BAP increased the growth rate, number of regenerated plantlet and portion of taller plantlet increased (Fig. 4). After 8 weeks, the plantlet was hardened for 2 weeks in growth chamber and later transferred to the pot for further growth. All varieties showed 100% survival when transfer into normal glasshouse conditions.

DISCUSSION

Rapidly growing, fine texture plant cell cultures can provide a homogenous cell population. The present study shows that embryogenic cells having high proliferation potential, small size, isodiametric, dense cytoplasm content with prominent enlarged nuclei and small vacuole as reported by Tang *et al*. (2001) while non embryogenic cells shows elongated cell with large nuclei (Fehér, 2005). The yellow pigmentation of cytoplasm indicated the embryogenic characteristic of the suspension cells. The same results were also reported by Ganapathi *et al*. (2001) and Jalil *et al*. (2003) in banana. The embryogenic characteristic were confirmed by Evans blue staining (Fig. 2). The suspension cells in liquid media contained proline shows increment of fresh weight compare to without proline treatment (Fig. 1). Therefore, proline may

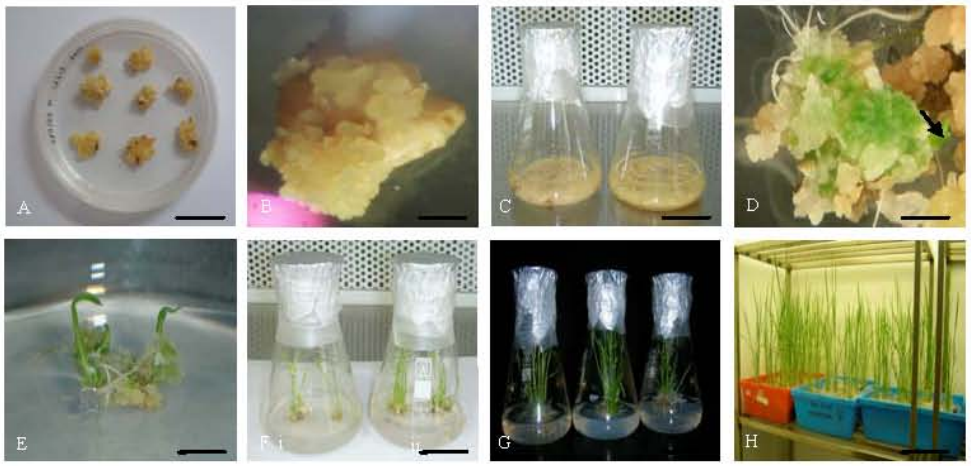

Fig. 4: Plant regeneration of japonica rice; (A-B): callus induction on LS2.5 medium after 4 weeks in culture. — 0.5 mm, (C): embryogenic culture at 1 month old in N_6 liquid media contained 3 mg L^{-1} 2, 4-D and 1 mg L^{-1} kinetin, (D): Shoot bud on SRM3 after 14 days in culture (show with arrow) — 0.5 mm, (E): shoot regenerated after 4 weeks in culture — 0.5 cm, (F): 6 weeks of plantlet (i) without BAP (ii) with BAP, (G): plantlets at 8 weeks culture, (H): plantlets in the soil at 2 weeks hardening period

have promotive effect on somatic embryogenesis in the present study. The present of proline in the culture medium seems to produce a required stress condition, decreasing water potential, increased the accumulation of nutritional elements in the cells and finally enhance embryogenesis (Moghaddam *et al*., 2000). Callus formation in the present study was found to be genotype dependent. The results are in agreement with Rashid *et al.* (2003) who reported that rice varieties differed in the degree of callusing. Callus induction was a critical stage where the regeneration of plants is highly dependent on the quality of callus. The embryogenic callus of Graminea species has a relatively dry, compact and nodular appearance (Quiroz-Figueroa *et al*., 2006; Jiminez and Bangerth, 2001; Nabors *et al*., 1983) while in embryogenic rice, coloration has been used as criterion to select embryogenic callus (Oinam and Kothari, 1995) similar observation in the present study. Combination of auxin and cytokinin is known to affect the regeneration of rice plants from embryogenic calli (Rueb *et al*., 1994). Regeneration frequency in the present study depend on concentrations of plant growth regulator used and it similar with previous report on other japonica rice varieties which reported highest regeneration frequency from 67-77% (Lee *et al*., 2002). In general, *Nipponbare* variety show good response to Kinetin treatment compared to *Fujisaka 5* in the present study. This probably because of high proliferation of callus induction in *Nipponbare* compared to *Fujisaka 5*.

Callus dehydration enhanced plantlet regeneration in all variety tested (Fig. 2). The present study shows that 48 h partial desiccation resulted in an up to sevenfold increase in the shoot regeneration frequency in var *Nipponbare* and *Hayahishiki*. Forty eight hour desiccation treatment proved optimal as this treatment shows up to 76 and 70% plant regeneration of var *Nipponbare* and *Hayahishiki* respectively. Forty eight hour desiccation treatment was also reported to enhance shoot regeneration from 63 to 82% but in indica rice cv HKR-46 and HKR-125 (Saharan *et al*., 2004). Shoot regeneration was 10-16% in all variety without desiccation treatment. The present studies, shows that partial desiccation alone is not enough for the promotion of regeneration frequency. The present study was contradict on previous report where 24 h desiccation treatment on cell suspension of japonica rice var *Sasanishiki* increase shoot regeneration from 5 to 47% (Tsukahara and Hirosawa, 1992a). Jain *et al.* (1996) also reported that 24 h partial desiccation increase three time of shoot regeneration in indica rice. Somatic embryoids arise from single cells and obviate the problem of possible genetic chimerism arising from developmental mode (Chowdhry *et al*., 2000). No albino plant was reported in the previous study, where partial desiccation has no deleterious effect on Australian regenerated shoots rice (Diah and Bhalla, 2000), similar observation in the present study. This improvement in regeneration potential has been suggested to be due to decreased water containing the dehydrated callus leading to an increase in endogenous ABA levels (Higuchi and Maeda, 1990; Yang *et al*., 1999) and improvement of oxygen supply to the callus (Jain *et al*., 1996). Acceleration of shoot

regeneration frequency in the present study after 48 h desiccation may be because of inclusion of sorbitol and BAP increase embryogenesis and restoring the regeneration ability. The present study was agreement with previous study who reported that addition of sorbitol enhance *in vitro* culture growth and morphogenesis in certain rice genotypes (Geng *et al*., 2008; Huang and Huang, 1999; Al-Khayri *et al*., 1996; Yoshida *et al*., 1994). The low regeneration frequency of *Fujisaka 5* variety may be because of poor callus induction even though after desiccation treatment.

In addition on its positive effect on the number of plantlet regenerated, partial desiccation also accelerated the organogenesis and development and thus shortened the time in tissue culture in the present study where first leaflet were obtained 10-14 days after 48 h desiccation while control treatment after 21 days on shoot regeneration media. This result was accordance to Rance *et al*. (1994) who reported that mature embryo-derived calli indica rice regeneration was enhanced by partial desiccation. No difference of plant regeneration duration was observed with or without partial desiccation in the present study. However, the percentage of regeneration frequency was enhanced in partial desiccation treatment.

CONCLUSION

On the basis of the results and discussion, an efficient protocol can be concluded for improvement regeneration of embryogenic cell culture of rice from mature seed derived callus. This system could be employed to produce large scale of plant from small quantity of mature embryo seeds as starting source.

ACKNOWLEDGMENTS

We thank Malaysian Agriculture of Research and Development Institute (MARDI) for providing mature seeds of japonica rice. This research is funded by research grant from the Ministry of Science, Technology and Innovation of Malaysia awarded to Ismanizan Ismail.

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