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Effect of 2,4-D on Embryogenic Callus Induction of Malaysian *indica* Rice (*Oryza sativa* L.) Cultivars MR123 and MR127

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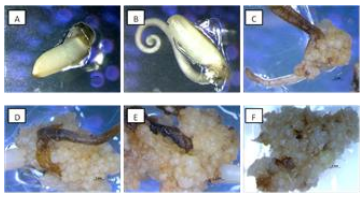
Graphical abstract



Abstract

The aim of this study is to study the effect of various concentrations of 2,4-D on embryogenic callus induction of *indica* rice MR123 cultivar and MR127 cultivar using mature seeds. Optimal media for induction of callus of both cultivars was MS basal media supplemented with 30g/L sucrose, 500mg/L glutamine and 500mg/L proline supplemented with 2.5 mg/L 2,4-D. The highest percentage of callus induction was 70% and 76% for MR123 and MR127 respectively. Both cultivars produced an embryogenic callus from scutellum after a week in culture with white-yellowish in color. The viability tested using Evans blue show callus obtained was embryogenic. This simple protocol using staining method could be used for screening embryogenic callus before further experiments can be conducted.

Keywords: Malaysian rice; *indica*; Callus induction; embryogenic; 2,4- Dicholophenoxyacetic-acid; viability

Abstrak

Kajian ini bertujuan untuk melihat kesan pelbagai kepekatan 2,4-D terhadap penginduksian kalus padi yang embriogeni menggunakan bijibenih matang. Sebanyak 70% dan 76% kalus daripada kultivar MR123 dan MR127 masing-masing berjaya bertukar menjadi kalus embriogenik apabila dikultur di atas medium MS yang mengandungi 30g/L sukrosa, 500mg/L glutamin, 500mg/L prolin dan 2.5 mg/L 2,4-D (media optimum). Kalus embriogeni ini dilihat tumbuh dari skutelum selepas seminggu dikulturkan dengan warna putih kekuningan. Kalus yang diperolehi kemudiannya ditentukan kebolehhidupannya menggunakan pewarna Evans biru yang menunjukkan ciri-ciri embriogeni. Kajian ini menunjukkan protokol mudah ini mampu menyaring kalus embriogeni padi sebelum eksperimen lanjutan dilakukan.

Kata kunci: Padi Malaysia; *indika*; induksi kalus; embriogeni; 2,4-diklorofenoksiasetik asid; kebolehhidupan

1.0 INTRODUCTION

Oryza sativa (rice) is one of the most important crops in the world and Asia is the largest rice consumption. It account for calorie consumptions in Cambodia at 70%, Sri Lanka at 40% and 37% for Pakistan (FAOSTAT, 2012). Three countries with highest rice production in 2012 in Asia are China, India and Vietnam (FAOSTAT, 2012; Datta, 2004). In Malaysia, rice is a staple food for all range of aged. The increasing of population growth and rice demand, BERNAS totals import increase from 590,000 metric tons in 1996 to 1,130000 metric tons in 2009 (Tey and Radam, 2011). Therefore, the need for alternative resources and exploration of new rice variety to meet demand that focused on local rice cultivars compare to imported rice. There are two major subspecies of rice (*indica* and *japonica*) which long grained *indica* rice comprised 80% of cultivated rice (Ramesh *et al*., 2009).

Malaysian rice belongs to subspeciess *indica* are in general are less responsive to callus induction as well as regeneration compared to subspecies japonica (Abe and Futsuhara, 1986). Despite a large number of reports on rice tissue culture, there is significantly genotype-dependence, and *in vitro* regeneration of *indica* rice is still a challenging task (Kumria *et al*., 2000). This problem is due to some factors which include genotype of the donor plants, the type and physiological status of the explants, the composition and concentration of the basal salts, organic components and plant growth regulator in the tissue culture medium (Zuraida *et al*., 2010). Among the different factors

influencing callus induction and regeneration, genotype and nutrient media composition are the two major factors that decide the fate of *in vitro* culture of rice (Khanna and Raina, 1999). However, modification of factors affecting tissue culture system has been proved to improve regeneration system in rice such as reported by Sahoo *et al*., (2011). Recent publication on Malaysian rice also show the optimization of factors affecting tissue culture provides valuable information on regeneration (Zuraida *et al*., 2010; 2011). Establishment of highly efficient and widely use d tissue culture system will accelerate the genetic improvement technology or improve of cultivars especially against to abiotic stress. Coloration methods or specific enzyme tests has been used for estimation of cell death in plant cells. The most commonly dye used are Evans blue (Smith *et al*., 1982), trypan blue (Hou and Lin 1996), neutral red (Swain and De 1994), methylene blue (Huang *et al*., 1986) and phenosafranine (Widholm, 1972). This simple protocol will differentiate the non embryogenic callus faster, simple and easy. Therefore, the present study was conducted to establish the callus induction as well as the simple and easy methods for primary screening embryogenic callus through staining.

■2.0 MATERIALS AND METHODS

2.1 Plant Materials

Mature seeds of MR 123 and MR 127 *indica* rice were obtained from MARDI, Seberang Prai, Penang, Malaysia.

2.2 Surface Sterilization

Seeds were dehusked and rinsed thrice using sterile double distilled water. Seeds were then sterilized by immersed in 100% ethanol for 3-5 minutes. Then, the ethanol was discarded and 100% Clorox (Sodium hypochlorite 5.25%) with a few drops of Tween was added and stirred for 30 minutes. The sterilized seed was then rinse five times with sterilized distilled water and finally blot dried on sterilized filter paper. The sterilized seeds were then ready for callus induction experiment.

2.3 Media Preparation and Growth Condition

Seeds were cultured onto solidified MS basal medium (Murashige and Skoog, 1962) containing 30g/L sucrose, 500mg/Lglutamine, 500mg/L proline, 2g/L phytagel and different concentration of 2, 4-dichlorophenoxyacetic acid (2.5 mg/L, 3.5 mg/L and 5.0 mg/L) for callus induction. The pH of medium adjusted to 5.7 prior to autoclaving. Each 25mL plate contained 10 seeds and the experiments have three replicates with 3 weeks interval of subculture. The cultured plate was incubated in dark conditions at 26-27°C. At the end of experiment at 8 weeks, percentage of callus induction and the morphology of embryogenic and non-embryogenic callus were recorded.

2.4 Viability Test for Callus Cell

Evans blue (EB) dye was used to test the viability of callus either embryogenic or non embryogenic according to Fernandez and Menéndez (2006). Approximately 1 mg of callus culture was immersed into 1% w/v of Evans blue solution. The callus was then incubated for 10 minutes at room temperature. To remove the remaining dye, the callus was rinsed with distilled water. The viability of callus was observed under the Nikon T*I* eclipse microscope.

2.5 Statistical Analysis

The statistical analysis was conducted using SPSS. The data were analyzed using independent t-test with three replications and each replication per treatment contained ten explants.

■3.0 RESULTS AND DISCUSSION

3.1 Callus Induction and Morphology

The present study shows calli were induced from both cultivars was different at various concentrations of 2, 4-D (Figure 1). No callus induction observed in free hormone MS medium. However, callus induction percentage for cultivar MR127 response well to all the 2,4-D concentration up to 70%. The maximum percentage of callus induction for cultivar MR127 (76%) was observed at 2.5 mg/L 2,4-D treatment. From all concentration of 2,4-D tested, the callus induction percentage in MR127 cultivar is higher than MR123 cultivar but not significant differences. Increase of 2,4-D concentration did decrease the callus induction percentage for cultivar MR123. MR123 shows the maximum percentage of callus induction (70%) when the seed was cultured on MS media supplemented with 2.5 mg/L of 2, 4-D. While, 5.0 mg/L of 2, 4-D shows the lowest percentage of callus induction. It shows that, callus induction for MR123 work best at 2.5 mg/L of 2, 4-D. Therefore, 2.5 mg/L of 2, 4-D was the optimum concentration for callus induction in both cultivars.

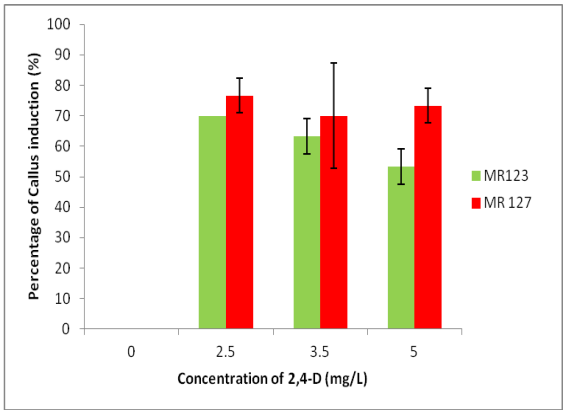


Figure 1 Percentage of callus induction in MR123 and MR127 cultivars with different concentration of 2,4-D used in MS basal medium. Data represent means of percentage and error bars represent standard deviation

From Figure 2, no callus formation was observed on hormone-free MS medium (Figure 2A). After 7 days, growth of callus was started followed by root (Figure 2B). From the observation, the callus formation was derived from scutellum of the culture mature seeds. Both cultivar shows embryogenic callus with dry, compact, light yellowish and numerous globular structures (Figure 2C and Figure 2D). The embryogenic calli of MR127 cultivar after 2 month culture was shown in Figure 2E while non-embryogenic calli on Figure 2F. This finding was supported from previous study of indica rice by Sahoo et al., (2011) which non-embryogenic callus shows a white, rough and pointed appearance.

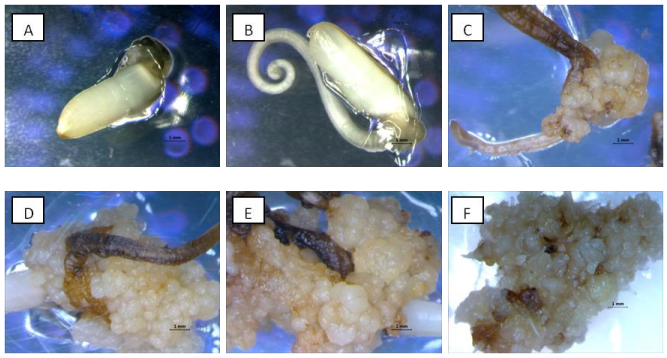


Figure 2 Callus derived from in vitro culture of MR123 and MR127 rice cultivar on MS media. (A) Dehusked mature rice seeds cultured on MS media supplemented with 2, 4-D (B) One-week old embryogenic calli (C) Embryogenic callus derived from seed after 2 weeks in culture. (D) Embryogenic calli of MR123 cultivar after 2 month of culture (E) Embryogenic calli of MR127 cultivar after 2 month culture (F) Non-embryogenic calli after 2 months in culture

Many literatures shows that variety of rice explants type can be used for starting materials for embryogenic callus induction such as mature and immature seeds, young inflorescences, rachilla, anthers and microspores; roots and coleoptiles (Mandal *et al*., 2003). Majority of literatures shows that inclusion of auxin, 2,4-D or in combination with others plant growth resulted in callus formation (Lee *et al*., 2002). The present study concurs with previous reports that show 2.5mg/L 2,4-D gave optimal results on callus induction of *indica* rice (Kartikayan *et al*., 2009). Syaiful *et al*., (2010) was also reported of optimal callus induction at 80% in MS media supplemented with higher 2,4-D at 4 mg/L in other Malaysian rice cultivars, MR219. Highest percentage of callus induction was also observed in cultivar HA-8 when 2,4-D was combined with other auxin or cytokinin such as BAP (Khaleda and Al-Forkan, 2006) and NAA (Zuraida *et al*., 2010). Combination of 2,4-D and NAA gave optimal callus induction in Malaysian rice, MR 232 cultivars up to 95%. The data presented here shows that the response of different Malaysian rice cultivars to 2,4-D tested was varied.

Induction of embryogenic callus in terms of number, colour, size and morphology were varied between rice genotype and depend on composition of media used, type of explants and interaction between this factors (Lee *et al*.,2002). Four type of embryogenic callus has been characterized by Visarada *et al*., (2002). Type I and II are embryogenic type with high regeneration frequency. Type I callus are white or cream in color with compact cells, type II composed same morphology with type I but white callus, type III was yellow or brown callus with cells highly uncompact while type IV with white, yellow and brown and very highly uncompact cells. Morphological characterization of rice callus has been reported such as Sahoo *et al*., (2011). Characterization of callus either embryogenic or non-embryogenic can be assessed by visual observation under light microscope or in some extent by histology's studies and scanning electron microscopic observation (Narciso and Hattori; 2010; Vega *et al*., 2009). The present study concurrent with report from Sahoo *et al*., (2011) where embryogenic *indica* rice characterization as pale yellow-white in color with a loose friable texture in appearance While, non-embryogenic callus shows a white, rough and pointed. The appearance of embryogenic callus of Malaysian MR219 were also characterized by Zuraida *et al*., (2011) with dry, compact, light yellowish and nodular.

3.2 Determination of Embryogenic Callus

Eight week-old callus for both cultivar were tested with 1% (w/v) EB solutions to test either the callus are embryogenic or non-embryogenic cells. The present study show that embryogenic callus stained were exhibited light blue colour (Figure 3B) compare to control (Figure 3A). The non embryogenic callus show intense blue colour (Figure 3C) in both cultivars tested.

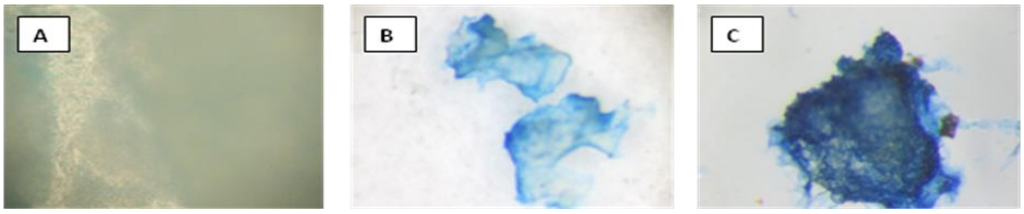


Figure 3 Staining of callus using EB solutions (A) Immersion of callus in 70 (v/v) % alcohols (control). (B) Light blue colour on embryogenic callus. (c) Non-embryogenic callus with dark blue colour

It is possible to differentiate embryogenic cells using EB (Silva *et al*., 2012). The non embryogenic callus are permeable to EB thus give a cell with blue color, while embryogenic callus will shows a lack of coloration from this dye (Silve *et al*., 2012). According to Fernandez and Mernendez (2006), embryogenic cells will turn the cells into light blue or colorless. While, non embryogenic cells will turning the cell into dark blue after stained with EB. From the Figure 3, both varieties MR123 and MR127 callus turned into light blue color after stained with 1% (w/v) EB solution. It shows both varieties are potential of embryogenic callus with light blue color after stained with 1% (w/v) EB dye. And the non-embryogenic cells turned into intense blue color after stained with 1% (w/v) EB solution for 10 minutes. Although visual observation may not provide enough information of good quality of callus, the present study shows potential of using simple staining methods for early screening of embryogenic callus.

■4.0 CONCLUSIONS

The present study shows establishment on embryogenic callus induction using 2,4-D for Malaysian rice of MR123 and MR127 cultivars. The embryogenic characteristics were differentiating using viability assay which Evans Blue methods is simple and easy for confirming its embryogenicity. This study could be

used for genetic improvement and established the suitable protocol for in vitro regeneration system of Malaysian rice.

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