Paper No :

MSAE2018-ARWM025

Embryogenic Callus Induction and Regeneration of Local Malaysian Rice (Oryza sativa L.) var. MR 219 and MR 253 from Mature Dehusked Seed Explants

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ABSTRACT

The present study was carried out to develop protocol for optimum callus induction and regeneration of two local Malaysian rice varieties; MR 219 and MR 253 using mature dehusked seed explants. The frequency of callus induction was determined on MS medium supplemented with different concentrations of 2,4-D. Optimum callus induction frequency of rice var. MR 219 at 90% was recorded from the explants cultured on MS medium treated with 1.0 mg/L 2,4-D giving the optimum callus fresh weight of 0.33 ± 0.05 g after 4 weeks. Meanwhile, 70% callus induction frequency was recorded from the dehusked seed explants of rice variety MR 253 cultured on MS + 1.0 mg/L 2,4-D after 4 weeks. The optimum callus fresh weight was recorded at 0.28 ± 0.04 g. Clonal roots was observed

KEYWORDS

2,4-D, Callus, Rice, Regeneration, MR 219, MR 253

**Paper presented at the 2018 MSAE Conference, Serdang, Selangor D. E., Malaysia.
7 & 8 February 2018**

INTRODUCTION

Rice (*Oryza sativa* L.) is a highly protected crop in a strategically important industry in Malaysia. Rice remains as the country's most important crop in cultivation, being the principle staple food for most of the population (Daño and Samonte, 2002). The importance of rice as staple food emphasizes its improvement undoubtedly (Sikder, 2006). MR 219 is a successful Malaysian local rice variety developed through a combination of bulk and pedigree methods of selection through a direct seeding planting system. MR 219 was officially released in January 2001 by the Malaysian Agricultural Research and Development Institute (MARDI) and has been since widely accepted and cultivated by Malaysian farmers. It has at least four improved traits from the previously released rice varieties which includes higher yields, maturation period of between 105-111 days, with large grain size and good eating quality (FFTC, 2011). However, after more than 13 years being cultivated, its resistance to diseases is weakening followed by limited yield production (Abdul-Shukor, 2008).

Thus, a new variety of rice, MR 253 has been developed by MARDI in the year of 2012; with the characteristic of being resistant to blast disease and suitable for planting on marginal soil (Buletin Teknologi MARDI, 2012). The introduction of MR 253 reduced the production cost directed pests and diseases management and minimizing the usage of pesticides and herbicides which lead to unnecessary side effects to the farmers and environment. Though MR 253 has been proven to be half resistance to most of the rice pests and diseases, it is susceptible to sheath blight disease (MARDI, 2010). With the reported limitations of both rice varieties, positive genetic manipulation through biotechnological methods can improve the existing rice varieties.

In many crop plants, callus production and its subsequent regeneration are the prime steps to be manipulated by biotechnological means (Saharan et al., 2004). Callus cells are wound tissues produced in response to injury (Smith, 1992). Callus formation is induced from plant tissues after surface sterilization and plating onto *in vitro* culture medium consisting of gelling agar and a mixture of macronutrients and micronutrients for the given cell type (Kumar and Singh, 2009). Also, in order to produce callus, culture medium usually supplemented with synthetic auxin, mainly 2,4-D (Sahoo, 2001). Callus cultures are also used to initiate cell suspensions which later used in multiple ways for plant transformation studies. The significance of callus culture studies is including that the regeneration of plants from callus is found to be an important basis of somaclonal variation essential for the establishment of novel plant cultivars with improved traits (Evans and Sharp, 1986).

The present research was thus carried out to study and develop protocol for optimum callus induction and its subsequent regeneration of rice varieties MR 219 and MR 253 using mature dehusked seed explants. The effect of different concentrations of 2,4-D supplemented to the *in vitro* culture medium for maximum callus induction frequency of the explants was also evaluated.

MATERIALS AND METHODS

Preparation of plant materials

Plant materials of rice varieties MR 219 and MR 253 were collected from MARDI Tanjung Karang, Selangor, Malaysia. Dehusked seeds of mature grain of both rice varieties were used as explants. The present research work was carried out in Plantation and Agrotechnology Laboratory, UiTM Shah Alam, Selangor, Malaysia in the year of 2010-2011.

Surface sterilization method

Seeds of mature grain to be used as explants and as plant material to obtained aseptic seedlings were manually dehusked and surface sterilized using 100% ethyl alcohol (C_2H_5OH) for 2 minutes followed by three sequential rinses with sterile distilled water. Later, the seeds were soaked in 100% sodium hypochlorite (NaOCl) added with two drops of Tween 20 for 30 minutes and rinses 3 times with sterile distilled water to remove the remaining chemical agents. The surface sterilized seeds were left to dry on sterile filter papers in a laminar air flow chamber prior to culture.

ISBN 978-967-16145

Culture medium preparation

MS (Murashige and Skoog, 1962) culture medium containing 4.41 mg/L MS salt mixture, 30.0 g/L sucrose and 8.0 g/L technical agar was prepared. 2, 4-Dichlorophenoxyacetic acid (2, 4-D) at different concentrations (0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5 and 4.0 mg/L) was added to the culture media to promote callus formation in culture. All culture media were adjusted to pH 5.8 prior to autoclaving for 20 minutes at 121^{o}C.

Callus induction and maintenance

Surface sterilized mature dehusked seed explants of rice varieties MR 219 and MR 253 were cultured on the modified MS media to obtain callus formation. All cultures were incubated under complete darkness for one week at the temperature of 25 ± 2^{o}C. Later, the cultures were transferred to the growth chamber under continuous white fluorescent light at the temperature of 25 ± 2^{o}C and 16/8 hours photoperiod. Subculture were carried out at 2-week intervals.

Statistical analysis

The callus induction experiment of rice varieties MR 219 and MR 253 was laid out using Completely Randomized Design, CRD. Each treatment was replicated 10 times with a single explant for each vial. The callus induction frequency (%) per treatment, degree of callus formation (DCF), callus fresh weight (g) and callus morphology were monitored as growth parameters (Table 1). The significant of differences among the treatments was determined by analysis of variance (ANOVA) followed by Tukey post-hoc test using Minitab Version 14 software. Each mean value was expressed as mean ± standard error (SE). Statistical significance was set up at $P < 0.01$.

RESULTS

Callus induction of mature dehusked seed explants of rice variety MR 219

Callus induction performance was investigated on mature dehusked seeds of rice variety MR 219. From the experiment, it was observed that MS media supplemented with different concentrations of 2,4-D promoted different callusing response to the explants tested. For instance, the highest callus induction frequency (100%) was recorded from the explants cultured on MS medium supplemented with 1.5 mg/L 2,4-D. The calluses were generally medium sized (DCF: +++) with an optimum callus fresh weight of 0.14 ± 0.04 g after 4 weeks. The calluses continued growing (DCF: ++++) and their fresh weight increased to 0.33 ± 0.05 g after 8 weeks of culture following two subculture cycles.

The lowest callus induction frequency at 90% was observed on MS + 4.0 mg/L 2,4 – D with the minimum callus fresh weight of 0.06 ± 0.05 g after 4 weeks. Calluses were very small in size (DCF: +). After 8 weeks, the calluses increased in size (DCF: ++) and the callus fresh weight was recorded at 0.12 ± 0.15 g. Statistical analysis showed a significant difference between treatments at $P < 0.01$ (Table 1 and Table 2). In general, calluses were compact, pale yellowish or yellowish color. Some calluses were observed to develop necrosis and deteriorated on certain treatments, while some others were observed with regenerated white roots (Figure 1a and Figure 1b).

Table 1: Callus induction from mature dehusked seed explants of rice variety MR 219 after 4 weeks

Treatment	2,4-D (mg/L)	Callus induction frequency (%)	Degree of callus formation	Callus fresh weight (g ± SE)
1.	MS + 0.5 mg/L 2,4-D	100	+++	0.11± 0.05
2.	MS + 1.0 mg/L 2,4-D	100	+++	0.13 ± 0.04
3.	MS + 1.5 mg/L 2,4-D	100	+++	0.14 ± 0.04
4.	MS + 2.0 mg/L 2,4-D	100	+++	0.12 ± 0.03
5.	MS + 2.5 mg/L 2,4-D	100	++	0.10 ± 0.04
6.	MS + 3.0 mg/L 2,4-D	100	++	0.08 ± 0.03
7.	MS + 3.5 mg/L 2,4-D	90	+	0.10 ± 0.03
8.	MS + 4.0 mg/L 2,4-D	90	+	0.06 ± 0.05

Note: *Each callus induction frequency (%) and mean callus fresh weight (g ± SE) values are the result of ten replications from a single experiment. Degree of callus formation, DCF: (+) = very small; (++) = small; (+++) = medium; (++++) = large*

Table 2: Callus induction from mature dehusked seed explants of rice variety MR 219 after 8 weeks

Treatment	2,4-D (mg/L)	Callus induction frequency (%)	Degree of callus formation	Callus fresh weight (g ± SE)
1.	MS + 0.5 mg/L 2,4-D	100	++++	0.17± 0.08
2.	MS + 1.0 mg/L 2,4-D	100	++++	0.29 ± 0.05
3.	MS + 1.5 mg/L 2,4-D	100	++++	0.33 ± 0.05
4.	MS + 2.0 mg/L 2,4-D	100	++++	0.21 ± 0.09
5.	MS + 2.5 mg/L 2,4-D	100	+++	0.23 ± 0.04
6.	MS + 3.0 mg/L 2,4-D	100	+++	0.20 ± 0.09
7.	MS + 3.5 mg/L 2,4-D	100	++	0.16 ± 0.06
8.	MS + 4.0 mg/L 2,4-D	100	++	0.12 ± 0.15

Note: *Each callus induction frequency (%) and mean callus fresh weight (g ± SE) values are the result of ten replications from a single experiment. Degree of callus formation, DCF: (+) = very small; (++) = small; (+++) = medium; (++++) = large*

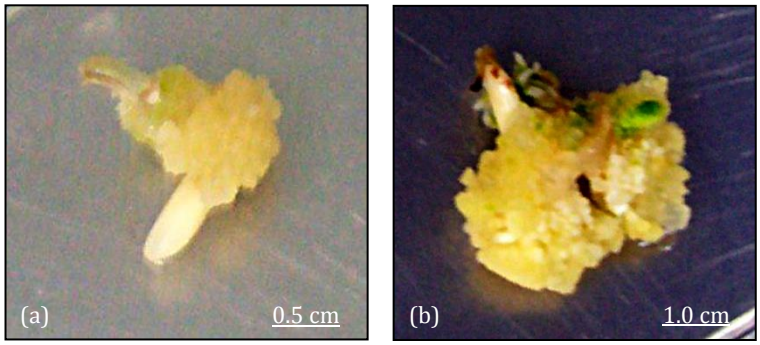


Figure 19: Callus induction from dehusked mature seed explants of rice variety MR 219 after (a) 4 weeks and (b) 8 weeks on optimum MS medium (MS + 1.5 mg/L 2,4-D)

Callus induction of mature dehusked seed explants of rice variety MR 253

The effect of different concentrations of 2,4-D was also investigated on the callus induction response of mature dehusked seeds of rice variety MR 253. It was observed the highest callus induction frequency was recorded from the explants cultured on MS medium treated with 1.0 – 1.5 mg/L 2,4-D with the rate of 90% after 4 and 8 weeks of culture. The callus fresh weight was recorded at 0.10 ± 0.01 g after 4 weeks and increased to 0.28 ± 0.04 g after 8 weeks on the optimum MS medium; MS + 1.0 mg/L 2,4-D. Statistical analysis proved that there is significant difference between treatments at $P < 0.01$. The least callus induction response of explants was observed on MS medium supplemented with high concentration of 2,4-D (4.0 mg/L) at the rate of 20% after 4-8 weeks. Depended to the MS media treatments, the developed calluses were either very small (DCF: +), small (DCF: ++), medium (+++) or large (DCF: ++++) in sizes (Table 3 and Table 4).

During the first 4 weeks of development, most of the calluses were friable, translucent white in color and with or without white roots regeneration. Some calluses even developed necrosis and deteriorated (Fig. 2a). By 8 weeks, the calluses grew in sizes and were generally compact, yellowish or beige in color (Fig. 2b)

Table 3: Callus induction from mature dehusked seed explants of rice variety MR 253 after 4 weeks

Treatment	2,4-D (mg/L)	Callus induction frequency (%)	Degree of callus formation	Callus fresh weight (g ± SE)
1.	MS + 0.5 mg/L 2,4-D	70	+	0.03 ± 0.01
2.	MS + 1.0 mg/L 2,4-D	90	+++	0.10 ± 0.01
3.	MS + 1.5 mg/L 2,4-D	90	+++	0.12 ± 0.03
4.	MS + 2.0 mg/L 2,4-D	60	+	0.02 ± 0.02
5.	MS + 2.5 mg/L 2,4-D	50	+	0.02 ± 0.00
6.	MS + 3.0 mg/L 2,4-D	50	+	0.02 ± 0.01
7.	MS + 3.5 mg/L 2,4-D	20	+	0.01 ± 0.01
8.	MS + 4.0 mg/L 2,4-D	20	+	0.01 ± 0.01

Note: *Each callus induction frequency (%) and mean callus fresh weight (g ± SE) values are the result of ten replications from a single experiment. Degree of callus formation: (+) = very small; (++) = small; (+++) = medium; (++++) = large*

ISBN 978-967-16145

Table 4: Callus induction from mature dehusked seed explants of rice variety MR 253 after 8 weeks

Treatment	2,4-D (mg/L)	Callus induction frequency (%)	Degree of callus formation	Callus fresh weight (g ± SE)
1.	MS + 0.5 mg/L 2,4-D	70	++	0.06 ± 0.01
2.	MS + 1.0 mg/L 2,4-D	90	++++	0.28 ± 0.04
3.	MS + 1.5 mg/L 2,4-D	90	++++	0.20 ± 0.08
4.	MS + 2.0 mg/L 2,4-D	60	++	0.08 ± 0.02
5.	MS + 2.5 mg/L 2,4-D	50	++	0.05 ± 0.01
6.	MS + 3.0 mg/L 2,4-D	50	++	0.08 ± 0.04
7.	MS + 3.5 mg/L 2,4-D	20	++	0.06 ± 0.02
8.	MS + 4.0 mg/L 2,4-D	20	+	0.03 ± 0.02

Note: *Each callus induction frequency (%) and mean callus fresh weight (g ± SE) values are the result of ten replications from a single experiment. Degree of callus formation: (+) = very small; (++) = small; (+++) = medium; (++++) = large*

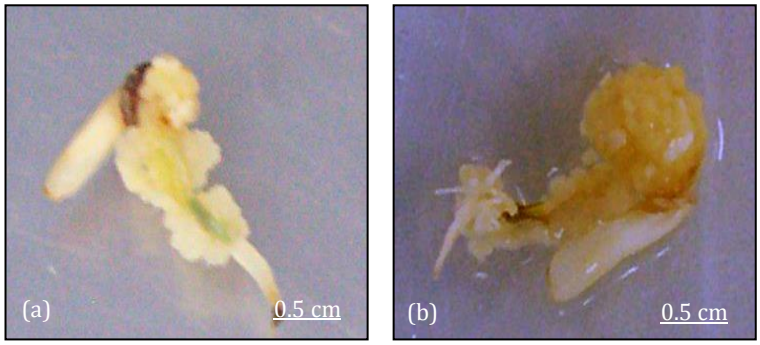
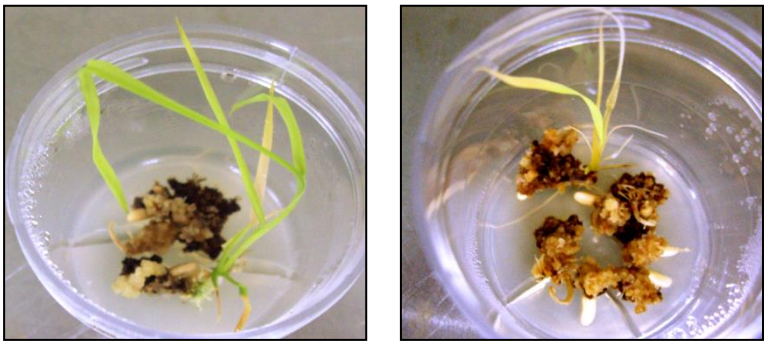


Figure 2: Callus induction from dehusked mature seed explants of rice variety MR 253 after (a) 4 weeks and (b) 8 weeks on optimum MS medium (MS + 1.0 mg/L 2,4-D)

Callus regeneration and clonal shoot regeneration of rice varieties MR 219 and MR 253

Transfer of optimum calluses obtained from the mature dehusked seed explants of rice varieties MR 219 and MR 253 on basal MS medium (MS0) had resulted in the formation of green pigments indicating the formation of chlorophyll and the existence of somatic cells of the culture (Figure 3a and Figure 3b). Complete plant regeneration of both rice varieties was observed to regenerate from the callus after 4-6 weeks of transfer following subculture. MR 219 produced green plants with white roots (Figure 3c), while MR 253 produced slightly yellow plants (Figure 3d).

ISBN 978-967-16145



(c) 1.0 cm (d) 1.0 cm

Figure 3: Green spots on calluses surfaces derived of mature dehusked rice explants of rice varieties (a) MR 219 and (b) MR 253 after 4 weeks of transfer on MS0. Complete plant formation from regenerated calluses of rice varieties (c)MR 219 and (d) MR 253 after 6 weeks on MS0

DISCUSSIONS

In the present study, a scientific attempt has been carried out to investigate the effect of different concentrations of synthetic auxin, 2,4-D on callus induction response of mature dehusked seed explants of rice varieties MR 219 and MR 253. From the results analysis, it was observed that MS medium supplemented with 1.0 - 1.5 mg/L 2,4-D gave response to optimum callus formation of both rice varieties, while higher concentrations of 2,4-D promoted the least callus induction frequencies. On the optimum MS media treatments, the formation of calluses started at the caryopses within the first week following inoculation of explants. After 2-4 weeks, the initiated calluses increased in sizes with better texture and eventually covering the entire surface of explants.

This finding is supported by Joyia and Khan (2012) who reported optimum callus induction frequency of rice variety Basmathi on MS medium containing 1.0 mg/L 2,4-D, while 5.0 mg/L 2,4-D was the maximum concentration. However, our result is contradicted to Panjaitan et al. (2009) whom previously reported optimum embryogenic callus induction frequency (80%) of rice variety MR 219 on MS medium supplemented with 4.0 mg/L 2,4-D.

2,4-D is the most frequently used auxin as it happened to be exceptionally efficient for plant and cell development. Many previous studies had revealed that the manipulation of 2,4-D alone to the culture medium was efficient to induce callus in many plant cultivars (Jubair et al., 2008; Afrasiab and Jafar, 2011; Tahir et al., 2012). Prior to the callus regeneration, green spots were observed to develop on the calluses surface. According to Illahi et al. (2005), the green spots were probably the somatic embryos. Afolabi et al. (2008) reported as callus with the similar green projections were transferred to regeneration medium, the possibility of it to develop into plantlets was very high.

CONCLUSIONS

In conclusion, optimum callus induction frequency of rice varieties MR 219 (100%) and MR 253 (90%) was obtained on MS + 1.5 mg/L 2,4-D and MS + 1.0 mg/L 2,4-D respectively. Complete plant formation through callus regeneration of both rice varieties was obtained after 4-6 weeks on MS0.

ACKNOWLEDGEMENT

The authors acknowledge the support of this research by Excellence Fund under Research Management Institute (RMI) [600-RMI/ST/DANA/5/3/Dst (70/2011)] and Fellowship Scholarship, Universiti Teknologi MARA (UiTM), Shah Alam, Selangor, Malaysia.

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ISBN 978-967-16145