

## EFFECT OF ILLUMINATION, CASEIN HYDROLYSATE AND PROLINE ON CALLUS INDUCTION OF *Oryza sativa* L. CV. MR219

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### ABSTRACT

In this study, factors such as illumination, casein hydrolysate and proline were shown to produce high numbers of calli from MR219 mature seeds. The effect of different concentrations (1, 2, 3, 4 and 5 mgL<sup>-1</sup>) of 2,4-dichlorophenoxy acetic acid (2,4-D) under different illuminations (24 hours light, 16/8 hours photoperiod, dark) on callus induction and growth were investigated. The results revealed that the highest percentage of callus induction (100%) and growth (90%) was observed in the Murashige and Skoog (MS) medium supplemented with 5 mgL<sup>-1</sup> 2,4-D incubated under 16/8 hours photoperiod. The effect of different concentrations of casein hydrolysate (0, 300, 400, 500, 600, 700, 80 and 1000 mgL<sup>-1</sup>) and proline (0, 500, 1000 and 1500 mgL<sup>-1</sup>) on callus induction and growth of MR219 were also studied. It was found that increasing concentrations of casein hydrolysate and proline did not give a significant effect on the percentage of callus induction. However, a significant percentage of callus growth (90%) was recorded when the callus induction medium was supplemented with 1000 mgL<sup>-1</sup> proline.

### ABSTRAK

Dalam kajian ini, faktor seperti iluminasi, kasein hidrolisat dan prolina telah dapat menghasilkan peratusan induksi kalus yang tinggi. Kesan 2,4-diklorofenoksi asetik asid (2,4-D) pada kepekatan yang berbeza (1, 2, 3, 4 dan 5 mgL<sup>-1</sup>) di bawah iluminasi yang berbeza (cahaya, gelap dan 16/8 jam fotoperiod) terhadap induksi dan pertumbuhan kalus telah dikaji. Hasil menunjukkan peratusan induksi (100%) dan frekuensi pertumbuhan kalus (90%) yang tertinggi dicatatkan pada medium Murashige dan Skoog (MS) yang mengandungi 5 mgL<sup>-1</sup> 2,4-D di bawah iluminasi 16/8 jam fotoperiod. Kesan kasein hidrolisat pada kepekatan yang berbeza (0, 300, 400, 500, 600, 700, 800 dan 1000 mgL<sup>-1</sup>) dan prolina (0, 500, 1000 dan 1500 mgL<sup>-1</sup>) pada kepekatan yang berbeza terhadap induksi dan pertumbuhan kalus juga dikaji. Hasil analisis menunjukkan bahawa peningkatan kepekatan kasein hidrolisat dan prolina tidak memberi kesan yang signifikan terhadap peratusan induksi kalus. Walau bagaimanapun, peratusan perkembangan kalus yang tertinggi (90%) telah dicatatkan dengan menggunakan medium induksi kalus yang mengandungi 1000 mgL<sup>-1</sup> prolina.

**Key words:** Rice (*Oryza sativa* L.) cv. MR219, callus induction, 2,4-dichlorophenoxy acetic acid (2,4-D), illumination, casein hydrolysate, proline

### INTRODUCTION

Rice is economically important and is a staple food for the people in Malaysia. Indica rice, MR219 used in this investigation is a commercially grown cultivar in Malaysia and has primarily been bred for high yield. The total production of MR219 is 6.5 to 10.7 tonnes per hectare. Despite all this, yield per unit area of rice in Malaysia is far below from the goal of rice self sufficiency. Hence, there is a need to improve these commercial cultivars by

biotechnology application in order to secure sufficient food supplies. Biotechnology can contribute to the agronomic improvement of rice in terms of yield and nutritional quality as a supplement to traditional breeding methods (Giri & Vijaya Laxmi, 2000).

The availability of a tissue culture system, which consists of callus production and its subsequent regeneration, is necessary for the manipulation of any plants by biotechnological means (Saharan *et al.*, 2004). Therefore, for successful application of the tissue culture technique for transformation in crop improvement,

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the production of callus and plant regeneration system of the Malaysian indica rice MR219 must be determined. It has been known that the potential for callus induction in rice tissue cultures depends on many factors, such as the genotype of the donor plant, the type and physiological status of the explants, the composition and concentration of the basal salt, and the organic components and plant growth regulators in the culture medium (Ge *et al.*, 2006). Among these factors, genotype difference is the most important. Successful callus induction and improvements have been reported in indica rice (Ge *et al.*, 2006; Lin & Zhang, 2005; Shaikat Ali *et al.*, 2004), specifically on MR219 by Syaiful *et al.* (2009). Although the tissue culture system for MR219 has been established, the search for new promotive factors could be applied in order to improve high numbers and the quality of calluses obtained. These could include illumination as well as amino acids.

Therefore, the primary aim of this study was to develop an efficient procedure using the factors mentioned for callus induction employing mature seeds of MR219 for future genetic transformation studies. The successful development of callus induction will be used for the MR219 transformation procedure and it will pave the way to introducing agronomically useful traits into MR219, providing the best opportunity for maximising yields.

## MATERIALS AND METHODS

### Seed material and explant preparation

Mature and healthy seeds of MR219 were selected by physical appearance and then dehusked manually. The dehulled mature seeds of MR219 were then surface sterilised by immersion in 100% ethanol for two minutes and followed by 50% of Clorox solution supplemented with four drops of Tween 20. Seeds were continuously shaken in Clorox for 30 minutes. Then, the seeds were rinsed 5 to 10 times with sterile distilled water and dry blotted. After rinsing with sterile distilled water, seeds were cultured onto callus induction medium.

### Culture media and treatments

The basal medium MS (Murashige and Skoog, 1962) with different concentrations of 2,4-dichlorophenoxy acetic (1, 2, 3, 4 and 5 mgL<sup>-1</sup>) supplemented with 30 gL<sup>-1</sup> sucrose and 3.5 g L<sup>-1</sup> gelrite, pH 5.8 were used as the treatment for callus induction. The cultures then were incubated under different illuminations (24 hours light, 16/8 hours photoperiod, 24 hours dark) at 27±2°C for 6 weeks without subculturing. Separate experiments were conducted to study the effect of casein hydrolysate

and proline, the basal medium MS (Murashige and Skoog, 1962) containing (a) different concentrations of casein hydrolysate (0, 300, 400, 500, 600, 700, 800, 1000 mgL<sup>-1</sup>) and (b) media supplemented with different concentrations of proline (0, 500, 1000 and 1500 mgL<sup>-1</sup>). Every medium was supplemented with 3 mgL<sup>-1</sup> 2,4-D, 30 gL<sup>-1</sup> sucrose and 3.5 g L<sup>-1</sup> gelrite, pH 5.8. Cultures were incubated under 24 hours light condition, 27±2°C for 6 weeks without subculturing.

### Parameters

The data that were recorded were percentage of callus induction (%) and percentage of callus growth (%) for different concentrations of 2,4-D, illuminations, casein hydrolysate and proline. For the casein hydrolysate treatment, the average weight (g) of callus was recorded as well. The data for percentage of callus induction were collected after one week of culture but the percentage of callus growth frequency (%) was recorded after three weeks of culture. On the other hand, the average of callus weight (g) was recorded after 6 weeks of culture.

### Experimental design and statistical analysis

The experiments were arranged in a Completely Randomized Design (CRD). All the data were analysed using the Statistical Analysis System (SAS). Analysis of variance (ANOVA) was done and the DUNCAN New Multiple Range Test (DNMRT), at  $\alpha=5\%$  was employed.

## RESULTS

### Effect of 2,4-D and illuminations

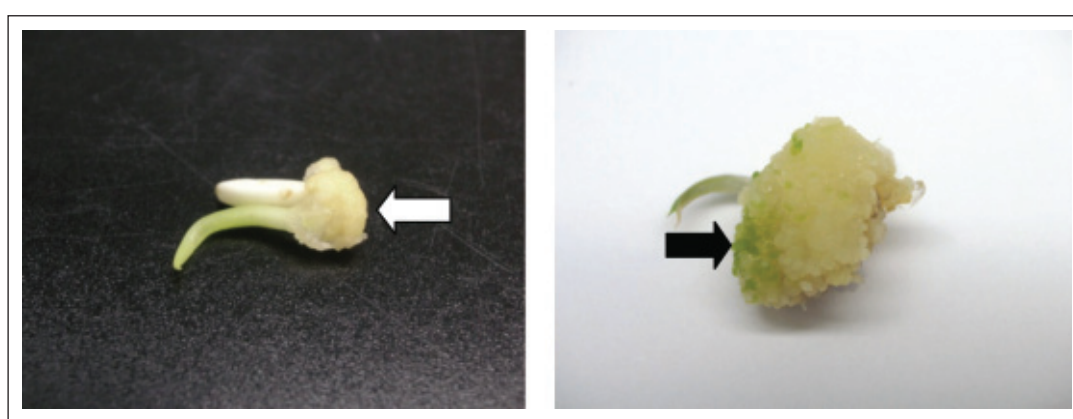
The result in Table 1 showed that increasing concentrations of 2,4-D increased the percentage of callus induction from 65% up to 100% and 52.5% up to 100% when the explants were cultured under 24 hours light and 16/8 hours photoperiod respectively. However, under dark condition, callus induction percentage were highest (95%) at 4 mgL<sup>-1</sup> 2,4-D but when the 2,4-D concentration was increased more than 4 mgL<sup>-1</sup> 2,4-D, the percentage of callus induction decreased to 85%. Regarding the percentage of callus growth (%), 5 mgL<sup>-1</sup> 2,4-D at illumination 16/8 hours photoperiod, gave the highest percentage (90%) among the treatments carried-out. The second highest percentage of callus growth is 4 mgL<sup>-1</sup> 2,4-D under 24 hours light. This treatment resulted in 85% of callus growth. On the other hand, in the dark condition, the highest percentage of callus growth is 70% when treated with 2 mgL<sup>-1</sup> 2,4-D.

The culture that was incubated under 24 hours light and 16/8 hours photoperiod started to grow calluses within three days incubation (Fig. 1a). The

**Table 1.** Effect of different concentrations of 2,4-D and illuminations (24 hours light, 16/8 hours photoperiod and dark) on the percentage of MR219 callus induction (%) and growth (%)

Illumination 2,4-D concentration (mgL <sup>-1</sup> )	Light		16/8 hours Photoperiod		Dark	
	Callus induction (%)	Callus growth (%)	Callus induction (%)	Callus growth (%)	Callus induction (%)	Callus growth (%)
1.0	65 <sup>c</sup>	40 <sup>c</sup>	52.5 <sup>b</sup>	30 <sup>b</sup>	75 <sup>ab</sup>	67.5 <sup>a</sup>
2.0	85 <sup>b</sup>	55 <sup>b</sup>	80 <sup>a</sup>	67.5 <sup>a</sup>	77.5 <sup>ab</sup>	70 <sup>a</sup>
3.0	97.5 <sup>ab</sup>	72.5 <sup>ab</sup>	92.5 <sup>a</sup>	82.5 <sup>a</sup>	67.5 <sup>b</sup>	60 <sup>a</sup>
4.0	100 <sup>a</sup>	85 <sup>a</sup>	100 <sup>a</sup>	80 <sup>a</sup>	95 <sup>a</sup>	60 <sup>a</sup>
5.0	100 <sup>a</sup>	80 <sup>a</sup>	100 <sup>a</sup>	90 <sup>a</sup>	85 <sup>ab</sup>	65 <sup>a</sup>

Means followed by the same letter(s) in the same column are not significantly different using the Duncan New Multiple Range Test (DNMRT) at  $p=0.05$ .



**Fig. 1.** a) Callus formed at the scutellum of a mature seed after 3 days of culture (white arrow) when incubated in 24 hours light or 16/8 hours photoperiod. b) Green spots formed on the surface of callus (black arrow) that were incubated in 24 hours light or 16/8 hours photoperiod.

green spots were also observed on callus (Fig. 1b). On the other hand, dark incubated cultures showed that very few calluses formed response which takes about one week after culture. In addition, there are some explants that formed shoots and roots rather than calluses when the culture was incubated under 24 hours light and 16/8 hours photoperiod using 1 mgL<sup>-1</sup> 2,4-D.

#### Effect of casein hydrolysate

Increasing concentrations of casein hydrolysate has no significant effect on the percentage of callus induction and callus growth (Table 2). The highest callus induction recorded is 97.5% using 0.5 gL<sup>-1</sup> and 1.0 gL<sup>-1</sup> casein hydrolysate. Whereas, 80% is the highest percentage that was recorded for callus growth using 0.6, 0.7 and 0.8 gL<sup>-1</sup> casein hydrolysate.

#### Effect of different concentrations of proline on callus induction and growth

Data summarised in Table 3 indicated that different concentrations of proline did not give a significant effect on the percentage of MR219 callus induction but it gave a significant result on

percentage of callus growth. The addition of proline in the media at 1.0 gL<sup>-1</sup> showed the optimum percentage of callus growth at 90% and followed by 0.5 gL<sup>-1</sup> at 82.5%. We can also note that increasing concentrations of proline up to 1.0 gL<sup>-1</sup> may inhibit the callus growth.

#### DISCUSSION

The critical step for this experiment is to obtain high numbers of callus. Calluses are masses of undifferentiated cells that are a good starting material for *in vitro* manipulation (Wani *et al.*, 2011). Keeping this in view, an attempt was made to improve the numbers of callus by employing different illuminations, concentrations of proline and casein hydrolysate using matured seeds of MR219 as explants. The availability of matured seeds (Jiang *et al.*, 2000) make them suitable as explants used in this study.

The 2,4-D alone was normally used in rice callus induction and most reports recommended 1.5 to 2.0 mgL<sup>-1</sup> 2,4-D but there are reports by

**Table 2.** Effect of different concentrations of casein hydrolysate on the percentage of MR219 callus induction (%) and growth (%)

Casein hydrolysate concentration (gL <sup>-1</sup> )	Callus induction (%)	Callus growth (%)
0	90 <sup>a</sup>	70 <sup>a</sup>
0.3	72.5 <sup>a</sup>	57.5 <sup>a</sup>
0.4	85 <sup>a</sup>	77.5 <sup>a</sup>
0.5	87.5 <sup>a</sup>	70 <sup>a</sup>
0.6	97.5 <sup>a</sup>	80 <sup>a</sup>
0.7	90 <sup>a</sup>	80 <sup>a</sup>
0.8	90 <sup>a</sup>	80 <sup>a</sup>
1.0	97.5 <sup>a</sup>	70 <sup>a</sup>

Means followed by the same letter(s) in the same column are not significantly different using the Duncan New Multiple Range Test (DNMRT) at p=0.05.

**Table 3.** Effect of different concentrations of proline on the percentage of MR219 callus induction (%) and growth (%)

Proline concentration (gL <sup>-1</sup> )	Callus induction (%)	Callus growth (%)
0	97.5 <sup>a</sup>	70 <sup>b</sup>
0.5	97.5 <sup>a</sup>	82.5 <sup>ab</sup>
1.0	100 <sup>a</sup>	90 <sup>a</sup>
1.5	100 <sup>a</sup>	70 <sup>b</sup>

Means followed by the same letter(s) in the same column are not significantly different using the Duncan New Multiple Range Test (DNMRT) at p=0.05.

Panjaitan *et al.* (2009) that recommend the increase of 2,4-D concentration up to 6 mgL<sup>-1</sup>. The lowest concentration (1 mgL<sup>-1</sup>) of 2,4-D is not strong enough and therefore not suitable to induce callus of MR219. Our observation is in agreement with those previous reports that showed that at a low level of 2,4-D which is 1 mgL<sup>-1</sup>, the explants grow roots and shoots rather than calluses at a relatively high frequency (Lee *et al.*, 2002). The highest percentage (100%) of callus induction was recorded using 5 mg/L 2,4-D under 24 hours light and 16/8 hours photoperiod. Meanwhile, the highest percentage (95%) of callus induction under dark condition was recorded using 4 mg/L 2,4-D. This indicates that 4 mgL<sup>-1</sup> 2,4-D was the most suitable concentration to stimulate the callus induction under dark conditions. It was noted in Table 1 that the percentage of callus growth frequency is below the percentage of callus induction. This showed that there are a number of calluses that are non-prolific and hence did not grow. Hence, different concentrations of 2,4-D induced a fair amount of calluses but also promoted their growth.

Swollen scutellum developed into callus within 3 days under 24 hours light and 16/8 hours

photoperiod (Fig. 1a). Meanwhile, dark incubated cultures take about one week to form calluses. Light is a very important physical factor for callus induction, cell growth and production of secondary metabolites (Summart *et al.*, 2008). However, the degree of responsiveness to light is dependent on cell type and plant species. Some of the calluses that were incubated under light formed green spots on the surface (Fig. 1b). These phenomena cannot be seen with the calluses that were incubated under dark conditions. The callus with green areas indicate that the photosynthetic processes had begun when the calluses were exposed to a light stimulus (Maneses *et al.*, 2005). In *Poaceae*, the presence of green spots in cultures has been considered as predictors of potential shoot formation (Nabors *et al.*, 1982).

Nutritional supplements such as casein hydrolysate, proline and glutamine have been earlier reported to enhance callusing response (Lin & Zhang, 2005). However, Tyagi *et al.* (2007) stated that although casein hydrolysate led to significant improvements in the quality of the calluses, there was no improvement in the callusing frequency which remains approximately 50%. Their result is similar with our result. There are no significant results observed on the percentage of callus induction and growth using different concentrations of casein hydrolysate. However, different concentrations of proline gave significant results on the percentage of callus growth. The promotive effect of proline on the frequency of callusing and regeneration have been reported by Chowdry *et al.* (1992). Moghaddam *et al.* (2000) also stated that the presence of proline in the culture medium seems to produce a required stress condition, decreasing water potential, increasing the accumulation of nutritional elements in cells and finally enhance embryogenesis. So as to enhance green-plant regeneration, supplements such as proline have been used because the use of proline in the medium has been reported to be effective for the initiation and maintenance of embryogenic calluses (Datta, 1992).

## CONCLUSION

Optimal media compositions were chosen on the basis that give a high percentage of callus induction and growth. In our experiment, the highest percentage of callus induction (100%) and growth (90%) could be achieved by culturing the mature seeds of MR219 on MS media supplemented with 5 mgL<sup>-1</sup> 2,4-D under 16/8 hours photoperiod. Although it is not significant, it gave the highest percentage as compared to the other treatments for MR219 callus induction and growth. In addition, different concentrations of casein hydrolysate did

not give a significant result on the percentage of callus induction and growth. However, proline plays a role in the growth of calli. Therefore, supplement of proline in the media can enhance the callusing response of MR219.

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