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# Effects of Plant Growth Regulators on *In vitro* Regeneration of Malaysian Indica rice (*Oryza sativa* L.) cv. MR219 by Shoot Apical Meristem

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## ABSTRACT

An efficient plant regeneration protocol for indica rice (*Oryza sativa* L.) cv. MR219 was established by means of Shoot Apical Meristem (SAM) obtained from 4-day old germinating seedlings. The effect of liquid and solid Murashige and Skoog (MS) medium towards the primary establishment of meristem tissue as well as effects of Plant Growth Regulators (PGRs) on shoots and root formation was investigated. Primary shoot induction was most effectively promoted by liquid MS medium supplemented with 0.1 mg $L^{-1}$ Kinetin (KIN) with 83% of explant survival and 0.88 explant vigor. Overall, liquid MS medium produced significantly higher percentage of explant survival and vigor compared to solid MS medium. Best treatment for shoot multiplication was solid MS medium supplemented with a combination of 1.5 mg $L^{-1}$ KIN and 0.05 mg $L^{-1}$ Indole-3-Acetic Acid (IAA) with an average of 8.8 shoots, followed by solid MS medium supplemented with 1.5 mg $L^{-1}$ KIN with an average of 7.6 shoots. However, shoots produced in MS medium supplemented with a combination of 1.5 mg $L^{-1}$ KIN and 0.05 mg $L^{-1}$ IAA was in concomitant with root formation. In this combination, plantlets produced had an average of 5.4 roots. Complete plantlets that were successfully transferred to soil and grown in the greenhouse were able to grow as healthy plants. This optimized protocol regenerated whole rice plantlets without any intervening callus formation. The highly efficient protocol suggested in this study can serve as an alternative to conventional propagation methods for large-scale plant regeneration throughout the year.

**Key words:** Indicia rice MR219, shoot apical meristem, kinetin, IAA, tissue culture, shoot induction

## INTRODUCTION

Rice is the staple food of many countries throughout the world. Thus, it is very important to ensure the constant availability of this crop. However, conventional propagation methods have several disadvantages such as the unavailability of large-scale true-to-type planting materials and vulnerability to environmental changes (Siwach *et al*., 2011). *In vitro* regeneration has an advantage over conventional propagation method in producing disease free plants at high multiplication rate (Gantait *et al*., 2011).

The Malaysian indica rice MR219 is currently the most popular rice variety grown in Malaysia since its release in 2001 (FFTC, 2002). A few advantageous characteristics of this indica rice variety include short maturation period of only 105-111 days, fairly tall but strong culms and resistance to blast and bacterial leaf blight.

Regeneration studies have been established for rice using various sources of explants including mature seeds (Sandhu *et al.*, 1995; Yoshida, 1996; Bano *et al.*, 2005; Zuraida *et al.*, 2011), shoot segments (Padua *et al.*, 1998), embryos (Yoshida, 1996) and axillary shoot segments (Sandhu *et al.*, 1995). In previous studies, rice was regenerated for genetic transformation purposes using explants such as, SAM (Padua *et al.*, 2001; Sticklen and Obray, 2005), coleoptile (Kant *et al.*, 2001), mature embryo (Ahmad *et al.*, 2002) or shoot and root tips (Khanna and Raina, 1999). However, to date, there are no reports of an ideal protocol that bypasses the intermediate callus phase for the *in vitro* regeneration of indica rice.

Meristem tissue as an explant has the ability to regenerate shoots without the intermediate callus development in the ideal concentration and combination of PGRs. Callus formation requires sub culturing processes that are laborious and time consuming (Valizadeh *et al.*, 2007). Meristem tissue ensures genetic stability and minimizes somaclonal variations by avoiding adventitious organogenesis (Alam *et al.*, 2010). Also, juvenile tissue is advantageous because it contains most actively dividing cells; it is highly responsive in tissue culture medium and has high regenerative ability compared to tissue obtained from mature sources. Hence, the present study uses SAM obtained from juvenile stage (plumule of 4-day old germinating rice seedling) as an explant for plant regeneration.

Apart for the type of explant, PGRs also determine the effectiveness of an *in vitro* regeneration protocol. The type and concentration of PGRs shows significant variability in *in vitro* regeneration of various species and cultivars (Poudel *et al.*, 2005). According to Bejoy *et al.* (2006), the culture medium and PGRs determine the optimum response of an explant.

It is especially vital to regenerate indica rice genotypes since these varieties are principal food source for most tropical regions in the world. Therefore, the present study was undertaken to regenerate whole Malaysian indica rice cv. MR219 plants from SAM, in optimized PGRs without the intermediate callus phase. Also, in this study we evaluate the effects of different PGRs towards the *in vitro* regeneration of MR219 rice from SAM.

## MATERIALS AND METHODS

This study was carried out in the Plant Physiology Laboratory, Biology Department of University Putra Malaysia from September 2010 until August 2011.

**Plant material and explant sterilization:** Indica rice (*Oryza sativa* L.) cv. MR219 seeds from Agriculture Research and Development Institute (MARDI), Tanjung Karang, Malaysia were surface sterilized in 70% (v/v) ethanol for 30 sec, followed by 5% (v/v) sodium hypochlorite solution containing two drops of Tween 20 for 20 min with agitation. The seeds were then rinsed 4 times with sterilized deionized water and germinated on a clean petri dish.

**Explant isolation and inoculation:** On the 4th day of germination, SAM measuring about 0.3-0.5 mm was excised aseptically from plumule region (Fig. 1). Prepared explants were cultured onto either liquid or solid MS basal media (Murashige and Skoog, 1962) supplemented with various combinations and concentrations of KIN and IAA (Table 1) for the primary establishment of SAM. After 4 weeks of culture, primary shoots induced on best treatment were inoculated on eleven different solid MS media compositions (Table 2), of different KIN and IAA combinations and concentrations.

Table 1: The effects of PGRs on the primary establishment of SAM on liquid and solid medium

| | Explant survival (%) | | Average explant vigor | |
|---|---|---|---|---|
| PGR (mg $L^{-1}$) | Liquid MS medium | Solid MS medium | Liquid MS medium | Solid MS medium |
| Control | 50[b] | 0[b] | 0.51[b] | 0.00[b] |
| 0.1 IAA | 15[c] | 0[b] | 0.15[c] | 0.00[b] |
| 0.15 IAA | 9[c] | 3[b] | 0.06[c] | 0.03[b] |
| 0.20 IAA | 36[b] | 0[b] | 0.53[b] | 0.00[b] |
| 0.1 KIN | 83[a] | 34[a] | 0.88[a] | 0.22[a] |
| 0.15 KIN | 80[a] | 20[a] | 0.73[a] | 0.18[a] |
| 0.20 KIN | 61[b] | 22[a] | 0.60[b] | 0.18[a] |
| 0.1 KIN+0.01 IAA | 66[b] | 5[b] | 0.62[b] | 0.03[b] |
| 0.1 KIN+0.02 IAA | 20[c] | 21[a] | 0.30[c] | 0.15[a] |
| 0.1 KIN+0.05 IAA | 32[b] | 18[a] | 0.42[b] | 0.14[a] |

Values followed by different letters within each column are significantly different according to Tukey multiple comparison test at $p<0.05$

Table 2: The effects of PGRs on the shoot number, shoot length and root number produced from SAM

| PGR treatments (mg $L^{-1}$) | Shoot No. | Shoot length (cm) | Root No. |
|---|---|---|---|
| Control | 2.3[b] | 0.9[b] | 0.6[b] |
| 1.0 IAA | 0.4[b] | 0.3[b] | 0.0[b] |
| 1.5 IAA | 0.0[b] | 0.0[b] | 0.0[b] |
| 2.0 IAA | 0.0[b] | 0.0[b] | 0.0[b] |
| 1.0 KIN | 4.2[b] | 1.6[b] | 0.0[b] |
| 1.5 KIN | 7.6[a] | 3.2[a] | 0.9[b] |
| 2.0 KIN | 3.6[b] | 1.2[b] | 0.3[b] |
| 1.5 KIN+0.05 IAA | 8.8[a] | 5.9[a] | 5.4[a] |
| 1.5 KIN+0.10 IAA | 3.9[b] | 1.3[b] | 1.2[b] |
| 3.0 KIN+0.05 IAA | 3.1[b] | 0.8[b] | 0.9[b] |
| 2.0 KIN+0.10 IAA | 3.2[b] | 0.9[b] | 0.0[b] |

Values followed by different letters within each column are significantly different according to Tukey multiple comparison test at $p<0.05$

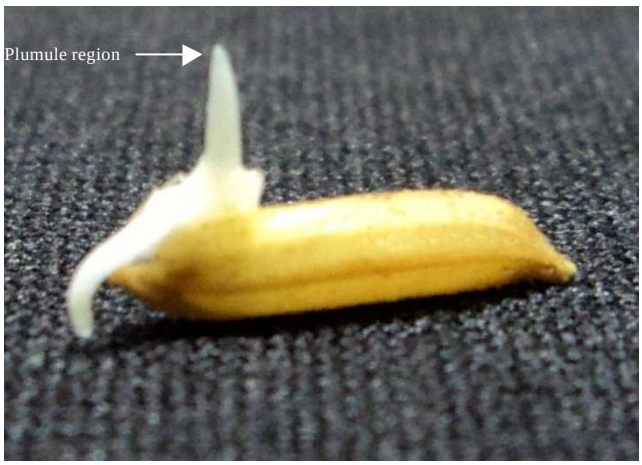


Fig. 1: Four-day-old germinating MR219 rice seedling

Control treatment was MS growth regulator-free medium. In all treatments, medium was supplemented with 20 g $L^{-1}$ sucrose and 10 g $L^{-1}$ glucose. The pH of medium was adjusted to 5.8 prior to addition of gelling agent for solid medium. The medium was autoclaved at 15 psi for 15 min at 120°C. All cultures were maintained in 16/8 h (light/dark) cycled fluorescent light cooled incubators with temperature regulated at 20°C.

**Data collection and analysis:** For each treatment, a total of 16 replicates were used and experiment was repeated thrice. The experiment was conducted in a completely randomized design. For the primary establishment of SAM, data was collected after a period of 4 weeks while the number of shoots and roots and shoot length were collected 8 weeks after culture. All data were analyzed using SPSS version 17.0. A one-way ANOVA and Tukey multiple comparison test was performed to access the significant difference between treatments at $p<0.05$.

## RESULTS

Primary shoot induction was observed in liquid MS medium supplemented with either 0.1 mg $L^{-1}$ KIN (Fig. 2a) or 0.15 mg $L^{-1}$ KIN as early as 15-20 days after culture. Liquid MS medium supplemented with 0.1 KIN $L^{-1}$ produced the best results with 83% explant survival and 0.88 explant vigor. Solid MS medium did not effectively promote primary establishment of SAM (Table 1). Among the 10 solid MS medium treatments tested, the highest percentage of explant survival was only 34% in solid MS medium supplemented with 0.1 mg $L^{-1}$ KIN. None of the explants survived in solid PGR-free MS medium, solid MS medium supplemented with 0.1 mg $L^{-1}$ IAA and 0.15 mg $L^{-1}$ IAA. Generally, the results indicate that exogenous cytokinin, (KIN) did have an effect towards shoot induction from SAM. Thus, the best treatment for the primary establishment of SAM is liquid MS medium supplemented with 0.1 mg $L^{-1}$ KIN.

Following the primary establishment of SAM, induced shoots from the best treatment mentioned above was cultured in solid MS medium supplemented with different PGRs. Solid MS medium supplemented with a combination of 1.5 mg $L^{-1}$ KIN and 0.05 mg $L^{-1}$ IAA and solid MS medium supplemented with 1.5 mg $L^{-1}$ KIN produced significantly the highest number of shoots and shoot length compared to all other treatments (Table 2). The average number of shoots produced in these two treatments was 8.8 and 7.6 with average shoot length of 5.9 and 3.2 cm, respectively. Healthy shoot development (Fig. 2b). and shoot multiplication (Fig. 2c) were observed on solid MS medium supplemented with a combination of 1.5 mg $L^{-1}$ KIN and 0.05 mg $L^{-1}$ IAA as early as 2nd and 4th week of culture, respectively Shoot formation was in concomitant with root formation in MS medium supplemented with a combination of 1.5 mg $L^{-1}$ KIN and 0.05 mg $L^{-1}$ IAA (Fig. 2d). However, Fig. 2e shows that although shoot multiplication was effectively promoted on solid MS medium supplemented with 1.5 mg $L^{-1}$ KIN, root formation was not observed on this treatment on the 8th week of culture. Solid MS medium supplemented with a combination of 1.5 mg $L^{-1}$ KIN and 0.05 mg $L^{-1}$ IAA was the only treatment that produced significantly the highest number of roots (an average of 5.4 roots) compared to all other treatments. Also, in this treatment, shoots and roots were produced without any intervening callus formation. Solid MS medium supplemented with either 1.5 mg $L^{-1}$ IAA or 2.0 mg $L^{-1}$ IAA did not promote shoot multiplication. In fact, explants inoculated in these treatments turned brown and died within 3 weeks. Figure 2f shows that plantlets regenerated from MS medium supplemented with a combination of 1.5 mg $L^{-1}$ KIN and 0.05 mg $L^{-1}$ IAA were able to grow normally as healthy plants once transferred to soil and grown in the greenhouse, making this the best treatment for the regeneration of MR219 rice from SAM.

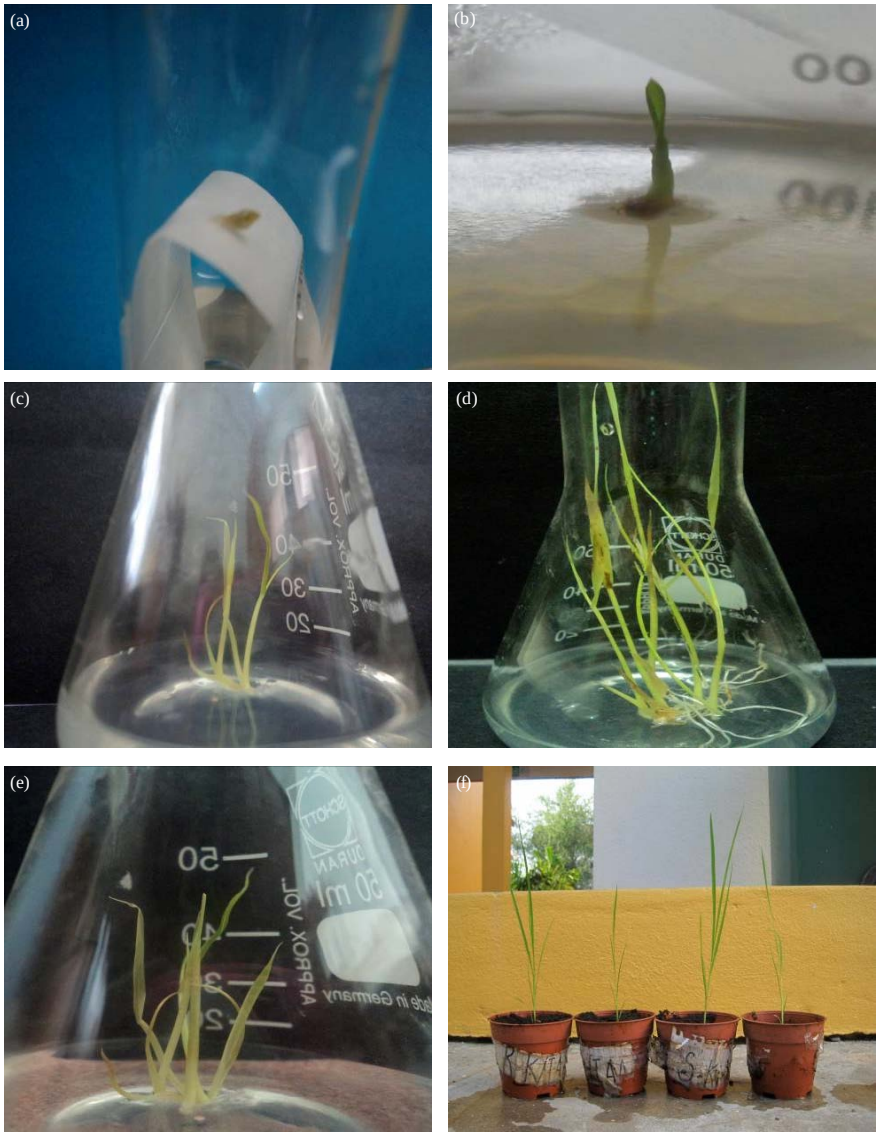


Fig. 2(a-f): Representative pictures of *in vitro* propagation of *Oryza sativa* L. cv. MR219, (a) Shoot induction from SAM on liquid MS medium supplemented with 0.1 mg $L^{-1}$ KIN, (b) Primary shoot development on solid MS medium supplemented with a combination of 1.5 mg $L^{-1}$ KIN+0.05 mg $L^{-1}$ IAA on 2nd week of culture, (c) Shoot development and multiplication on solid MS medium supplemented with a combination of 1.5 mg $L^{-1}$ KIN+0.05 mg $L^{-1}$ IAA on 4th week of culture, (d) Shoot multiplication in concomitant with roots formation on solid MS medium supplemented with a combination of 1.5 mg $L^{-1}$ KIN+0.05 mg $L^{-1}$ IAA on 8th week of culture, (e) Shoot multiplication and development without root formation on solid MS medium supplemented with 1.5 mg $L^{-1}$ KIN and (f) Complete plantlets that were successfully regenerated from MS medium supplemented with a combination of 1.5 mg $L^{-1}$ KIN+0.05 mg $L^{-1}$ IAA that were transferred to soil and grown in the greenhouse were able to grow as healthy plants

## DISCUSSION

The *in vitro* regeneration of indica rice MR219 from SAM was successfully achieved without intermediate callus phase. It seems that exogenous supply of PGRs is important to increase the mitotic activity and differentiation, hence, promoting organogenesis within SAM. In this study, primary establishment of SAM is a crucial prerequisite step for the *in vitro* regeneration of indica rice MR219. This is because SAM is a cluster of tiny, undifferentiated and free-conducting tissue. Hence, the optimum concentration of PGRs can: first, prevent formation of a mass of undifferentiated cells (callus); second, promote organogenesis of SAM, thus, inducing primary shoot formation.

Liquid medium was significantly beneficial as most SAM failed to survive or grow in solid medium (Table 1). This is because liquid medium allows good plant-medium contact, provides good aeration and increases the ability for dissolved nutrient uptake by entire surface of explant. Similar results were reported for the primary establishment of SAM of tomato plant (Alam *et al*., 2004) and sweet potato (Elliott, 1969; Alam *et al*., 2004).

Given the ideal stimuli, the high degree of plasticity and totipotency of SAM allows the initiation of organs. Results obtained from this experiment proved that the type of organogenesis that occurs in plant tissue culture is highly dependent on the ratio and concentration of auxin and cytokinin employed. Similarly, Skoog and Miller (1965) reported that the differentiation of cultured tissues into either shoots or roots depends on the ratio of auxin to cytokinin in the culture medium. A combination of 1.5 mg $L^{-1}$ KIN and 0.05 mg $L^{-1}$ IAA promoted both shoot multiplication and root formation from SAM. Similar results were reported by Novak and Maskova (1979) in the regeneration of tomato from SAM as explants. Also, this study proved that IAA used solely in culture medium did not promote organogenesis of SAM. In all measured traits, treatments employing KIN either solely or in a combination was superior (Table 2).

The protocol suggested in this study is beneficial in comparison to other regeneration methods. Not only plantlets produced are clean, healthy and viable for acclimatization but also it is less time and cost consuming. SAM isolated from 4 day old germinating rice seedling is an excellent explant because it is more sensitive and efficient in responding to PGRs compared to mature tissues. Also, this protocol successfully regenerated MR219 indica rice plantlets without the long intermediate callus phase which requires sub-culturing and could also induce somaclonal variation.

In this study, the exogenous application of auxin and cytokinin had significant effect towards the shoot and root formation from SAM. The results show that SAM isolated from 4-day old germinating seedling cultured in liquid MS medium supplemented with 0.1 mg $L^{-1}$ KIN promoted organogenesis and the induction of primary shoots. Primary shoots that were then cultured in solid MS medium supplemented with a combination of 1.5 mg $L^{-1}$ KIN and 0.05 mg $L^{-1}$ IAA produced the best results for shoot multiplication and root formation.

## CONCLUSION

As a conclusion, the present study reported the successful *in vitro* regeneration protocol of Malaysian indica rice MR219 from SAM without the intervening callus phase. This method is time and cost saving, as well as efficient and economic. Thus, the highly ideal protocol suggested in this study can be applied as an alternative to conventional propagation method for the large-scale production of MR219 rice throughout the year.

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