Journal of Phytology 2010, 2(3): 30–38
An Open Access Journal
ISSN: 2075-6240
Available Online: www.journal-phytology.com

REGULAR ARTICLE

REGENERATION OF MALAYSIAN INDICA RICE (*ORYZA SATIVA*) VARIETY MR232 VIA OPTIMISED SOMATIC EMBRYOGENESIS SYSTEM

Zuraida Abdul Rahman[1]*, Suri Roowi[2], Wan Zaliha W.S[3] and Sreeramanan Subramaniam[4]

[1]*Biotechnology research centre, Persiaran MARDI-UPM, MARDI Headquarter, Serdang 43400 Selangor, Malaysia*
[2]*Food Technology Research Centre, Persiaran MARDI-UPM, MARDI Headquarter, Serdang 43400 Selangor*
[3]*Department of Agrotechnology, Faculty of Agrotechnology and Food Science, Universiti Malaysia Trengganu, 21030 Kuala Terengganu, Terengganu, Malaysia*
[4]*School of Biological Sciences, Universiti Sains Malaysia, Georgetown, 11800, Penang, Malaysia*

SUMMARY

In vitro studies of indica rice variety MR232 via somatic embryogenesis system was established by using mature seeds. By manipulating various plant growth regulators, the optimum medium was obtained by using MS medium containing 5 mg/L NAA (α-naphthaleneacetic acid) and 1 mg/L 2, 4-D (2,4-dichlorophenoxyacetic acid) without browning effect. This treatment showed higher percentage of callus induction and frequency of embryogenesis at the range of 91-97%, which was further, confirmed with the histology studies. The highest whitish somatic embryos frequency (87%) was initiated by incubating embryogenic calli on media containing 10 mg/L ABA and 9 mg/L gelrite agar for 4 weeks. However, the numbers of regenerated plant on medium containing NAA and that was previously pre-treated with 10 mg/L ABA, 9 mg/L gelrite agar and incubation at 8 weeks was the best treatment for shoots induction with 10 plantlets per 3 gm of somatic embryos.

Key words: Indica rice MR232, Callus induction, Embryogenic callus, Regeneration

Zuraida Abdul Rahman et al. Regeneration of Malaysian Indica Rice (*Oryza sativa*) Variety Mr232 via Optimised Somatic Embryogenesis System . J Phytol 2/3 (2010) 30-38

*Corresponding Author, Email: azuraida@mardi.gov.my

1. Introduction

Rice is the main staple food for more than half of the world population and is a model monocot system for genetic and functional genomics studies. Considerable efforts were being directed towards improvement of important agronomic traits of rice through biotechnological techniques (Xiaojia et al. 2006). Asian cultivated rice (*Oryza sativa*) consists of two major groups, the indica (*Oryza sativa* ssp. indica) and *jopanica* (*Oryza sativa* ssp. japonica). The indica subspecies is the most widely cultivated form of rice in the world especially in South and South-East Asia countries (Zhang et al. 2005).

Genetic transformation has become an important tool for breeding improvement and gene function studies in rice. However, transformation system in indica rice remains difficult (Zhang et al. 2005). It appears that indica rice is more specific than the japonica rice to various tissue culture conditions and generally was less responsive to callus induction (Mikami and Kinoshita, 1988). Therefore, establishment of an efficient *in vitro* protocol is an essential prerequisite in harnessing the advantage of cell and tissue culture for genetic improvement. Callus induction and regeneration in rice tissue culture depended on a number of factors, such as the genotype of the donor plants, the

type and physiological status of the explant, the composition and concentration of the basal salts, organic components and plant growth regulator in the tissue culture medium (Khaleda and Al-Forkan, 2006). Among these factors, genotypic different in rice is the most important and significant. Khanna and Raina (1998) reported that indica rice was less responsive to callus indica rice compared with japonica rice. Even within indica subspecies, significant variation of *in vitro* culture response still exist in different genotypes (Seraj et al., 1997, Asit et al., 2003).

Somatic embryogenesis is an efficient plant generation system, is a potentially useful for the production somaclonal variants and the development of transgenic plants, and is valuable tools in the understanding of basic plant biology. Highly efficient and reproducible *in vitro* regeneration system is prerequisite for clonal propogation and for production of transgenic plants (Tang and Newton, 2003). Recently, somatic embryogenesis from scutellar embryo of *Oryza sativa* L. variety MR 219 was published (Syaiful et al., 2009). They have reported that the highest percentage of embryogenic callus formation (80%) was obtained on the modified MS medium containing 4 mg/L of 2, 4-D. Therefore, the objective of this study was to develop a regeneration system for Malaysian indica rice variety MR232 through somatic embryogenesis system.

2. Materials and Methods

Callus induction

Mature seeds of the Malaysian indica rice variety MR 232 were dehusked, cleaned and sterilized by immersion in 100% ethanol for 1-2 minutes and followed by immerse in 100% Clorox (Sodium hypochlorite 5.25%) supplemented with few drops of Tween-20 for 30 minutes. After being rinsed three times in the sterile distilled water, seeds were cultured on solidified medium containing MS basal medium (Murashige and Skoog, 1962), 3% sucrose, 0.3% gelrite agar and different concentrations of NAA (0-50 mg/L) or 2,4-D (0-50 mg/L) and combination of both. The pH of the medium was adjusted to 5.7-5.8 prior to autoclaving. Cultured seeds were maintained in the dark room at 25-27^{o}C under 16 hours photoperiod of 1250 lux provided by fluorescent tubes. Calli were produced after 3 to 4 weeks culture and scored as percentage of callus induction. The fresh calli were then transferred (sub-cultured) onto the same fresh medium, and then the percentage of browning response and frequency of embryogenic callus were determined.

Somatic embryogenesis

Selected high quality embryogenic callus were cultured on pre-regeneration MS basal medium supplemented with different concentration of gelrite agar (3, 6, 9, 12 and 15 mg/L) that were supplemented with 1, 5 or 10 mg/L of ABA. The cultures were then incubated at 2, 4, 6 or 8 weeks for pre-regeneration process. Data was recorded as the percentage of forming whitish embryos. For histology observation, whitish embryos were fixed in a fixation solution for 12-24 hours, dehydrated through an ethanol-xylol series and embedded in paraffin wax. Tissue were sectioned at 3-4µm and stained with 0.5% (w/v) fast green and 0.25% (w/v) safranin and examined under a light microscope.

Plant regeneration

Three grams of the whitish embryos that pre-treated with 6, 9, 12 and 15 mg/L solidified agar, containing 5 or 10 mg/l of ABA and incubated at 4, 6 or 8 weeks were cultured on MS basal medium with or without NAA at 1 mg/L for further regeneration. The numbers of regenerated plants were recorded within 4 to 6 weeks.

Statistical analysis

The statistical analyses were conducted using SPSS. The experiments were arranged in a Completely Randomized Design (CRD) as a single factor experiment with four replications and each replication per treatment contained ten explants. All experiments were repeated thrice.

3. Results and Discussion

Callus induction

Experiments were conducted to determine the optimum concentration of the growth regulators 2, 4-D and NAA or their combinations on the induction and quality of the embryogenic calli produced. Callus formation from plates also varied significantly depending on plant growth regulators and their combinations (Table 1). Results showed that all seeds plated on media containing 2, 4-D alone developed a pale yellow callus after 2-4 weeks (Fig.1 [A]). Addition of 2, 4-D alone resulted callus formation varying between 43-96%. The highest callus induction was obtained on medium with 10 mg/L 2, 4-D at 97%. Media supplemented with NAA or control (no hormone) showed no callus development. Moreover, the seeds were developed and germinated rapidly into plantlets.

A similar phenomena was reported by Thengane et al. (2006) with the application of NAA (5.3-10.7 µM) which gave rise to germinating embryos with well-developed shoots. In garlic, low level of 2, 4-D increased the percentage of explants producing callus (Barandiaran, 1999) and subsequently tend to give rise to fine compact callus formation. In this study, combination effects of both 2, 4-D (1-10 mg/L) and NAA (1-50 mg/L) were tested. The entire combinations hormone tested produced callus ranging from 7 to 95%. The callus cultured in medium combination of 2, 4-D and NAA grew at higher rate than those cultured in medium with 2, 4-D alone. In the combination treatment, the highest percentage of callus (95%) was obtained on the media containing 1 mg/L 2, 4-D and 10 mg/L NAA. The texture of the callus surface was nodular bearing a number of globular structures approximately 1 mm in diameter, whereas the surface of callus cultured on medium with 2, 4-D alone was smooth. According to Jain et al. (1995), initiation of callus is influenced by several factors such as explants type and plant growth regulators particularly auxins and cytokinins. Therefore, it is concluded that the media containing combination of 1 mg/L 2,4-D and 10 mg/L NAA or 5-10 mg/L 2,4- D alone can be used for initiation of fine callus culture of indica rice, MR232.

Table 1: Callus induction (%) of *indica* rice when cultured on MS media supplemented with different combination s of NAA and 2,4-D.

2,4-D(mg/L)	*Callus induction* (%)					
	NAA (mg/L)					
	0	1	5	10	20	50
0	0	0	0	0	0	0
1	78.8±6	68±7	91±7	95±8	55±6	34±3
5	96±11	43±6	71±10	65±5	45±7	24±2
10	97±9	32±8	34±4	22±6	11±1	0
20	43±4	21±4	5±0.5	7±0.8	0	0

Callus obtained from the above experiments were sub-cultured onto the fresh media in order to induce further for the development of embryogenic callus. After 3-4 weeks of the sub-cultured period, most treatments showed more callus browning, desiccation and necrotic. All the treatments that were supplemented

with 2-4, D produced higher percentage of browning ranging from 45-67% (Table 2). Although with 2, 4-D treatments, globular callus produced compact with very little embryogenic formation, but still turned into brown colour and watery when retaining for a longer period on the same medium. Similar results were observed in banana with globular non-embryogenic secondary calli which turned brown within 4-8 months after cultured in medium containing 2,4-D alone (Strosse at al., 2006). In general, callus cultured on media containing 2, 4-D continuously for 6 to 8 weeks tend to have a negative effect on the callus development. Therefore, using 2, 4-D alone was not the best treatment to induce embryogenic callus in rice variety and also due to a higher browning response after sub-culture.

The combination of 2, 4-D and NAA produced browning after sub-culture even though the percentage were lower (5-32%) when compared with the application of 2, 4-D alone. Moreover, the combinations of 2, 4-D and NAA at concentrations of 1-5 mg/L and 5-20 mg/L, respectively, were the most effective for embryogenic callus formation with no browning effect. The compact callus from these treatments turned yellowish in color and had variable proliferation rates. Our results showed that combination of 1 mg/L 2, 4-D and 5-10 mg/L 2, 4-D not only showed higher in callus induction but also no browning formation at all. Callus browning formation increased as 2, 4-D increased (10-20 mg/L) in combination with 50 mg/L NAA. Hence, we have shown that combination of 1 mg/L 2, 4-D and 10 mg/L NAA was the most effective for the induction of fine calli without browning appearance. The results from this study showed that concentrations and combinations of hormones greatly influence the proliferation of callus. Similar findings were reported in MR 219 rice, that the capacity of callus proliferation was greatly influenced by the medium manipulation during sub-culture using selected hormones (Syaiful et al., 2009).

Table 2 : Effect of percentage of browning (%) on callus after sub-culture on medium supplemented with different concentration of NAA and 2,4-D. (-) ; no callusing.

2,4-D (mg/L)	NAA (mg/L)					
	0	1	5	10	20	50
0	-	-	-	-	-	-
1	45±6	5±1	0	0	0	35±6
5	51±	0	0	0	0	23±4
10	55±7	27±3	19±3	23±2	21±5	-
20	67±8	32±7	0	0	-	-

Performance of calli on combination media containing of 2, 4-D and NAA were observed. The frequency of embryogenic calli produced was recorded after 6 weeks in culture as shown in Table 3. All the combinations of 2, 4-D and NAA or 2, 4-D alone induced embryogenic callus after 6 weeks in culture at ranged 11-65%. The highest embryogenic callus obtained in medium supplemented 10 mg/L NAA and 1 mg/L 2, 4-D with 65%. Most of the callus remained friable, compact and globular, and indicated that they were embryogenic in nature. Embryogenic calluses grew very well and therefore were used for subsequent experiments.

Table 3: Frequency of embryogenic callus (%) obtained after cultured on MS containing different concentration of NAA and 2, 4-D for 6 weeks.

2,4-D (mg/L)	NAA (mg/L)					
	0	1	5	10	20	50
0	0	0	0	0	0	0
1	23±2	21±3	41±6	65±3	43±7	11±2
5	25±4	32±6	45±4	49±4	23±3	3±1
10	45±5	13±3	15±1	19±2	9±3	0±0
20	14±3	5±0.5	0	0	0	0

Somatic embryogenesis

Proliferated embryogenic callus were transferred to medium supplemented with different ABA and gelrite agar concentrations with different time incubations in order to investigate their potential for somatic embryogenesis. Histological studies confirmed the identity of potential somatic embryos. Sections of embryogenic callus revealed different stages of embryoids on the surface, but the basal portion of the embryoids remained embedded in the parental calli. Highly meristematic cells were seen actively dividing to form compact masses. The intense metabolic and mitotic activity at these sites caused them to expand and emerge rapidly from the callus. The cells of somatic embryos were exhibited dense cytoplasm and reduced number of vacuoles. The multiplication of somatic embryos cells resulted in highly differentiated cells. After 4-6 weeks on the treatment, some meristematic cells acquired embryonic characteristics, such as high nucleoplasmic ratio, dense cytoplasm and a large nucleus with a large and densely stained nucleolus (Fig.1 [B, C]). Most of callus cultured on 9-15 g/L gelrite with ABA and incubated at 4-8 weeks the production of somatic embryos. Percentage of whitish somatic embryos derived from embryogenic callus is shown in Table 4. Generally, the somatic embryos produced were ranging between 11-87%. Whitish embryos initiated on media containing 10 mg/L ABA, supplemented with 9 g/L gelrite and incubated for 4weeks showed the highest (87%). No somatic embryos were produced on media containing 3 g/L gelrite due to browning effect after culturing for 4 weeks. Even though 3 g/L agar is a common concentration used in tissue culture work it is not recommended in the process of somatic embryogenesis for the indica rice variety MR232. Since the combination of 6-9 g/L gelrite agar, 5-10 mg/L ABA and incubation for 4-8 weeks produced good embryos formation, therefore these treatments were used in the following experiments for regeneration of rice plantlets.

Table 4: Percentage (%) of whitish somatic embryos formation derived from per 3 gm of embryogenic callus

	ABA 1 mg/L Weeks				ABA 5 mg/L Weeks				ABA 10 mg/L Weeks			
Agar (gram)	2	4	6	8	2	4	6	8	2	4	6	8
3	0	0	0	0	0	0	0	0	0	0	0	0
6	0	0	0	0	0	0	41±5.2	47±5.9	0	71±9.1	69±8	31±5.6
9	0	0	25±3.4	34±3.4	0	15±3.6	54±4.4	37±8.4	0	87±7.8	81±5	51±7.8
12	0	0	23±5.2	21±6.1	0	29±5.1	41±9.2	27±4.2	14±2.1	65±3.2	59±7	48±5.2
15	0	0	11±1.1	4±0.6	0	18±1.6	14±2.6	17±3.3	9±2.4	45±4.1	34±4	24±4.7

Table 5: Number of rice plantlets/per 3 g embryos obtained after pre-treated with ABA then transferred on regeneration medium.

MS basal (without NAA)						
	ABA 5 mg/L Weeks			ABA 10 mg/L Weeks		
Agar (gram)	4	6	8	4	6	8
6	0	0	0	2±0.40	3±0.25	3±0.18
9	0	0	0	1±0.10	2±0.15	4±0.17
12	0	0	0	0	0	1±0.12
15	0	0	0	0	0	0

MS with NAA						
	ABA 5 mg/L Weeks			ABA 10 mg/L Weeks		
Agar (gram)	4	6	8	4	6	8
6	0	3±0.40	1±0.15	2±0.22	5±0.10	9±0.89
9	0	2±0.40	4±0.81	1±0.11	8±0.11	10±1.11
12	0	0	5±0.79	1±0.08	6±0.78	8±0.78
15	0	0	3±0.22	1±0.07	1±0.08	1±0.10

ISSN: 2075-6240
Available Online: www.journal-phytology.com

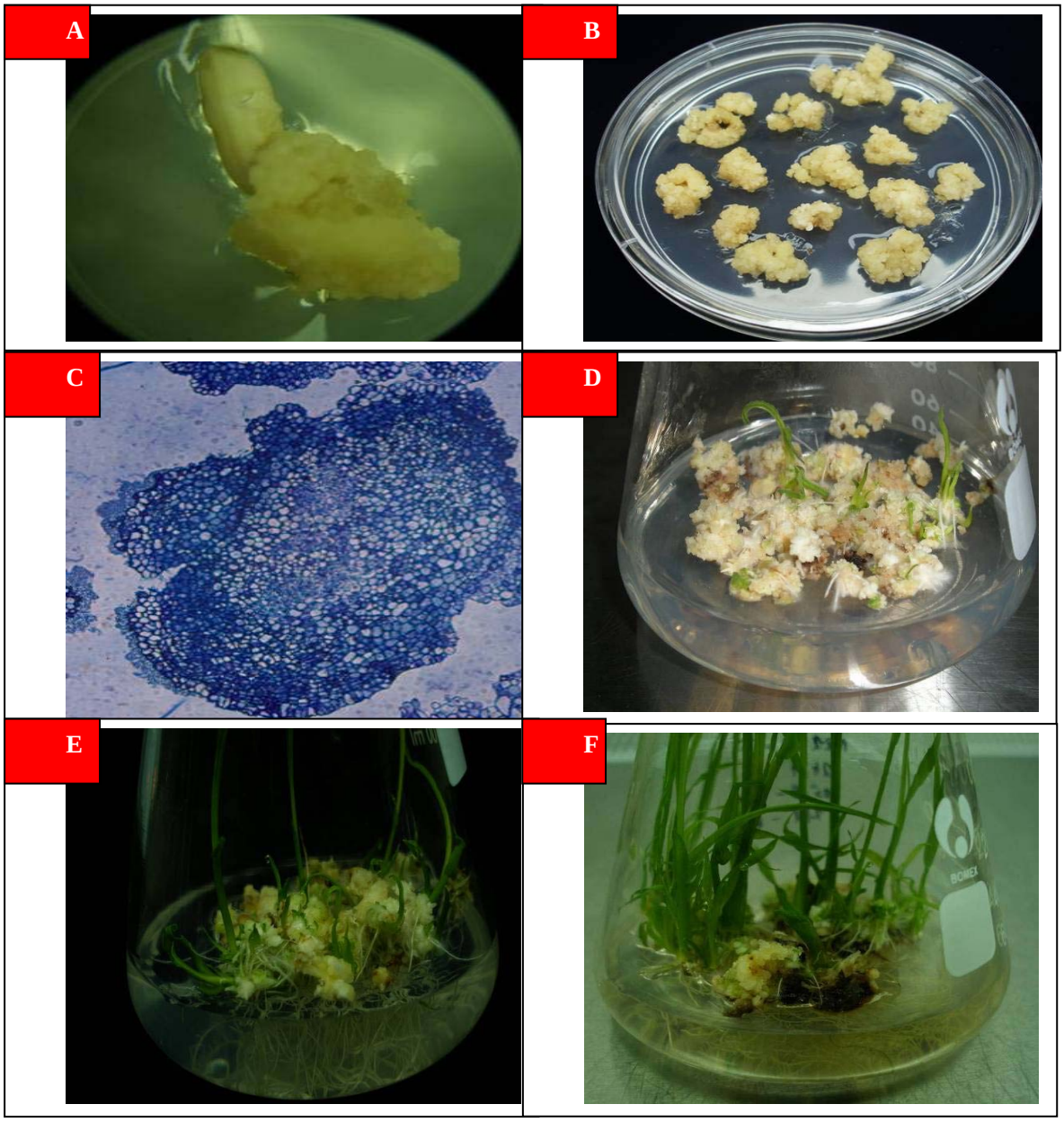

Fig. 1: Plantlet regeneration from callus derived from *in vitro* cultured of embryos indica rice. (A) Callus formation from embryos of rice on callus induction medium with 2,4-D after 3 weeks of culture; (B) Initiation of embryogenic callus after 6 weeks of culture on medium with 1 mg/L 2, 4-D and 10 mg/L NAA; (C) Histological observation of somatic embryogenesis in early stage globular pro-embryo with associated vacuolated cells; (D) Somatic embryo initiation, turned to green and started to regenerate plantlets after 2-3 months of culture; (E) Plantlets elongation on pre-regeneration medium contaning 0.5 mg/L NAA; (F) More plantlets regeneration after subsequence sub-culture on basal MS medium.

Plant regeneration

The somatic embryos form previous experiments were transferred onto MS medium with or without hormones for further development into plantlets. The number of plantlets produced is shown in Table 5. The embryos from each treatment were observed until the development into whole plants. Results showed that the embryos cultured on MS medium without NAA were mostly weak in growth. In addition, the number of rice plantlets produced within a month of culture were less (1-4 plantlets) than those cultured on

medium with NAA. Media with NAA produced more plantlets ranging between 1-10 plantlets. In addition, the highest number of plantlets was produced from media supplemented with hormones and pre-treated earlier with 10 mg/L ABA, 9 g/L gelrite and incubated for 8 weeks. The regeneration of plantlets was increased during subsequent sub-cultures on basal MS medium. The development of the plantlets is shown in (Fig. 1[D-F]). This medium containing hormones is effective in inducing higher number of shoots for indica rice. Similarly, the presence of BAP and NAA was found to be most suitable for somatic embryo germination in rice MR 219 (Syaiful et al., 2009). They reported that 82.5% of the somatic embryos were germinated into the seedlings upon transferring the somatic embryos on the modified MS medium with 2 mg/L BAP and 0.05 mg/L NAA. However, some have been reported that medium without growth regulators is a typically the medium used for inducing somatic embryogenesis in many plants, including the wetland monocots, *Phragmites australis* (Straub et al., 1988) and *Sporobolus virginicus* (Straub et al., 1992). Our results showed that the addition of cytokinin hormones can be beneficial in increasing the number of regenerated rice plantlets.

4. Conclusion

The present study confirmed the production of embryogenic calli, somatic embryos as well as regeneration of indica rice, MR 232 variety was established. Improvement of somatic embryogenesis system could be used to improve quality of plant materials and application in the genetic engineering efforts on this Malaysian potential and new indica rice variety.

References

1. Asit, B.M., Aparna, M., and Biswas, A. 2003. Somatic embryogenesis in root derived callus of *Indica* rice. Plant Tissue Culture, 13(2): 125-133.

2. Barandiaran, X., Di Pietro, A., and Martin, J. 1999. Biolistic transfer and expression of a *uidA* reporter gene in different tissues of *Allium sativum* L. Plant Cell Reports, 17: 737–741.

3. Jain, S., Varshney, A., and Kothari, S.L. 1995. Embryogenic callus induction and efficient plant regeneration in prosomillet. Cereal Research Communication, 29: 313–320.

4. Khaleda, L., and Al-Forkan, M. 2006. Genetypic variability in callus induction and plant regeneration through somatic embryogenesis of five deepwater rice (*Oryza sativa* L.) cultivars of Bangladesh. African Journal of Biotechnology, 5 (16): 1435-1440.

5. Khanna, H.K., and Raina, S.K. 1998. Genotype_culture media interaction effects on regeneration response of three indica rice cultivars. Plant Cell, Tissue and Organ Culture, 58: 145–153.

6. Mikami T., and Kinoshita, T. 1988. Genotypic effect on the callus formation from different explants of rice (*Oryza sativa* L.). Plant Cell, Tissue and Organ Culture, 12:311-314.

7. Murashige, T. and Skoog, F. 1962. A revised medium for rapid growth and bioassay with tobacco tissue cultures. Plant Physiology, 15: 473-497.

8. Seraj, Z.I., Islam, Z., Faruque, M.O., Devi, T. and Ahmed, S. 1997. Identification of regeneration potential of embryos derived calluses from various *indica* rice varieties. Plant Cell, Tissue and Organ Culture, 48: 9-13.

9. Straub, P.F., Decker, D.M and Gallagher, J.L. 1992. Characterization of tissue culture initiation and plant regeneration in *Sporobolus virginicus* (Gramineae). American Journal of Botany, 79:1119-1125.

10. Straub, P.F., Gallagher J.L and Decker D.M. 1988. Tissue culture and long-term regeneration of *Phragmites australis* (Cav.).

Trin.ex. Steud. Plant Cell, Tissue and Organ Culture, 15: 73-78.

11. Strosse H., Schoofs, H., Panis B., Andre E., Reyniers , K. and Swennen R. 2006. Development of embryogenic cell suspensions from shoot meristematic tissue in bananas and plantains (*Musa s*pp). Plant Science, 170:104-112.

12. Syaiful, B.P., Siti, N.A.A., Maheran, A. A., Sariah, M., and Othman, O. 2009. Somatic embryogenesis from scutellar embryo of *Oryza sativa* L. var. MR 219. Pertanika Journal of Tropical Agriculture Science, 32 (2): 185-194.

13. Tang, W., and Newton, R.J. 2003. Genetic transformation of conifers and its application in forest biotechnology. Plant Cell Reports, 22: 1-15.

14. Thengane, S.R., Deodhar, S.R., Bhosle, S.V. and Rawak, S.K. 2006. Repetative somatic embryogenesis and plant regeneration in *Garcinia indica* Choiss. In Vitro Cellular and Development Biology Plant, 42:256-261.

15. Xiaojia, Ge., Zhaohui ,Chu., Young, J.L., and Shiping, W. 2006. A tissue culture system for different germplasms of indica rice. Plant Cell Reports, 25: 392-402.

16. Zhang, J., Feng ,Q., Jin, C., Qiu, D., Zhang, L., Xie, K., Yuan, D., Han, B., Zhang Q., and Wang, S. 2005. Features of the expressed sequences revealed by a large-scale analysis of ESTs from a normalized cDNA library of the elite indica rice cultivar Minghui 63. Plant Journal, 42: 772-780.