High frequency plant regeneration from embryogenic callus of a popular *indica* rice (*Oryza sativa* L.)

Alagarsamy Karthikeyan, Shunmugiah Thevar Karutha Pandian and Manikandan Ramesh

Department of Biotechnology, Alagappa University, Karaikudi 630 003, India

ABSTRACT

The aim of the study is to establish a routine procedure for high frequency plant regeneration from *in vitro* raised embryogenic callus of abiotic salt sensitive *indica* rice (*Oryza sativa* L.) cultivar ADT 43. The effect of synthetic auxin 2,4-D on callus induction was optimized to achieve high frequency plant regeneration from fresh embryogenic callus without further subculture. Friable, nodular and creamish-white embryogenic callus cultures were raised from mature rice seeds on LS medium supplemented with 2.5 mg L⁻¹ 2,4-D and 1.0 mg L⁻¹ thiamine-HCL. Plant regeneration was achieved by the 24 days old embryogenic callus on MS medium supplemented with 1.0 mg L⁻¹ BAP and 1.5 mg L⁻¹ NAA. *In vitro* regenerated plants with multiple tillers and roots were transferred to sterile soil and maintained in the growth chamber. The regenerated plants exhibited normal growth and were phenotypically similar to plants maintained in the garden. Using the present protocol, 25-30 plantlets were regenerated from 50 individual mature seed derived callus within two to three months. This protocol has the potential for large-scale production of elite plants after genetic transformation. [Physiol. Mol. Biol. Plants 2009; 15(4) : 371-375] E-mail : aduindi.india@gmail.com

Keywords : *Oryza sativa*, 2,4-D, Regeneration, Mature seed, Embryogenic callus

INTRODUCTION

Rice consumers are increasing at the rate of 1.8 % every year without any systematic increase in production. It is estimated that rice production has to be increased by 50 % by 2025 to feed the ever-growing world population (Khush and Virk, 2000). This requires acceleration in rice production with less land and water. Solving these problems entails development of the rice varieties with higher yields, excellent grain quality, and resistance to various biotic and abiotic stresses. In order to genetically engineer the local rice varieties to useful traits (particularly tolerance to abiotic salt stress conditions in salt sensitive ADT 43), it is essential to optimize reproducible protocol for high frequency plant regeneration from easily available explants like mature seed embryos.

In general, genetic improvements are believed to be difficult in graminaceous species due to their

recalcitrance to *in vitro* manipulations. However these constraints were overcome by culturing the calli on nutrient media with moderate concentrations of 2,4-D and subsequent plant regeneration (Vasil, 1994). 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* raised cultures (Khanna and Raina, 1998). Despite a large number of reports on rice tissue culture, there is significant genotype-dependence, and *in vitro* regeneration of *indica* rice is still a challenging task (Kumria *et al.*, 2000). There are several successful reports on regeneration from explants such as leaf blade (Yan and Zhao, 1982), root tips (Sticklen, 1991), leaf sheath/leaf sections (Wernicke and Milkovits, 1984), immature panicles (Shu and Wei, 1980), mature seed embryos (Ramesh and Gupta, 2006), immature embryo (Koetje *et al.*, 1989), coleoptile (Oinam and Kothari, 1995), anthers (Genovesi and Magill, 1982), stem nodes (Furuhashi and Yatazuva, 1964), seedling derived mesocotyl segments (Mascarenhas *et al.*, 1975), leaf segments (Henke *et al.*, 1978) and leaf bases (Ramesh *et al.*, 2009).

Correspondence and reprint requests : Manikandan Ramesh
Telephone : 04565-225215; Fax : (+4565) 225 202

However, protocols for high frequency plant regeneration have not been reported in this popular local *indica* rice variety (ADT 43) in a shorter duration using embryogenic callus cultures. In Tamil Nadu, rice is cultivated in 1.52 million hectares of which ADT 43 alone constitutes nearly 21 % (Selvaraj and Ramasamy, 2006). In order to develop abiotic tolerant popular *indica* rice varieties through biotechnology, it is essential to develop efficient and reproducible regeneration system from cultured tissues at high frequency. In the present communication, a reliable and reproducible protocol for *in vitro* plant regeneration from 24 days old mature seed derived callus cultures is reported, which can be exploited to raise transgenic plants using vector- mediated /direct gene delivery approaches.

MATERIALS AND METHODS

Healthy and disease free seeds of *indica* rice (*Oryza sativa* L.) cultivar ADT 43 collected from Tamil Nadu Rice Research Station, Aduthurai, Tamil Nadu, India were manually dehusked and surface sterilized with 70% (v/v) ethanol for 60 sec followed by 0.1% mercuric chloride for 4 min and thoroughly rinsed five times in sterile distilled water. Surface sterilized seeds were blot dried with sterile Whatman No.1 filter paper and aseptically cultured on Linsmaier and Skoog's medium, 1965 supplemented with different concentrations of 2,4-dichlorophenoxyacetic acid (0.5 - 4.5 mg L⁻¹), 1.0 mg L⁻¹ thiamine-HCL and 3.0 g L⁻¹ gelrite with a pH of 5.8. The cultures were incubated at 26±2 °C in total darkness. After 24 days of inoculation, callus tissues induced from the mature seeds were excised and transferred to MS medium for regeneration. Only embryogenic sectors were used for the regeneration experiments.

About 5-7 mm diameter sized embryogenic calli was transferred to Murashige and Skoog's medium, 1962 supplemented with different concentrations of benzylamino purine and naphthalene acetic acid (Table 2). The cultures were kept under 16 hr light (cool white fluorescent light tubes providing irradiance of 50 mmol m⁻² s⁻¹) and 8 hr dark cycle at 26±2 °C. Complete rooted plantlets (more than 8 cm in length after 28 days in regeneration medium)) were removed without any traces of agar, and transferred to plastic culture boxes containing autoclaved mixture of red soil, sand and vermiculite (1:1:1). Initially, the boxes were covered with perforated thin polythene bags for one week to prevent desiccation and to maintain the humidity. After two weeks, plantlets were transferred to pots containing

a mixture of 1:1 ratio of garden soil and vermiculite and maintained in garden for maturity.

The callus induction percentage was calculated after 24 or 26 days by counting the number of seeds forming callus as a percentage of cultured seeds. For fresh weight analysis from 3 replications (each 40-50 seeds), 5 explants with calli were randomly selected; mean and standard errors were calculated. Regeneration frequency was calculated as the number of regenerable embryogenic callus pieces producing plantlets with both shoot and root divided by the number of callus pieces inoculated.

RESULTS

In vitro embryogenic callus induction

In order to study the time requirement for obtaining regenerable embryogenic calli, seeds were incubated in the medium for 24 days. In another set of experiment, seeds were incubated in the medium for 24 days, followed by sub culturing for another 16 days before using them for regeneration experiments. Among the different concentrations of 2,4-D tested, the percent response of callus induction on LS medium supplemented with 2.5 mg L⁻¹ was high (74.2 %) than on medium with 2.0 mg L⁻¹ (66.1 %) and 3.0 mg L⁻¹ (54.9 %). It was also observed that 2.5 mg L⁻¹ 2,4-D was ideal concentration for further proliferation of embryogenic callus in the sub cultured medium. The

Table 1. Effect of 2,4-D on *in vitro* callus induction from mature seed explants of *indica* rice cv. ADT 43 on LS medium

Concentration of 2,4-D (mg L ⁻¹)	Percentage of callus induction	Fresh weight of callus (in mg) Mean±SE
0.5	38.5	38.5±0.86
1.0	44.0	44.0±0.37
1.5	50.6	50.6±0.54
2.0	66.1	66.1±0.61
2.5	74.2	78.2±1.81
3.0	54.9	54.9±0.9
3.5	51.5	51.5±0.83
4.0	46.5	46.5±0.77
4.5	39.6	39.6±0.39

Values are statistically significant (P <0.05)

Fig. 1. Different stages of embryogenic callus induction from mature seed explants and plant regeneration in *indica* rice cultivar ADT 43. (a) Intact dehusked seeds of ADT 43 on LS medium with 2.5 mg L^{-1} 2,4-D; (b) Callus induction after 14 d of culture initiation; (c) Friable white to yellow embryogenic callus sub cultured on fresh medium after 24 days; (d) Embryogenic callus transferred to MS + 1.0 mg L^{-1} BAP and 1.5 mg L^{-1} NAA emerging green shoot buds after 12-14 days; (e) Complete plantlet regeneration after 41 days; (f) Hardened ADT 43 plants.

Table 2. Effect of BAP and NAA on *in vitro* plant regeneration from mature seed derived embryogenic callus of *indica* rice cv. ADT 43 on MS medium

S.No	Hormone Concentration (mg L^{-1})		Efficiency of complete plant regeneration Mean $\pm$ SE		Number of shoots/callus culture Mean $\pm$ SE
	BAP	NAA	24 day old callus	40 day old callus	
1.	0.5	0.05	49.03 $\pm$ 0.50*	41.11 $\pm$ 0.21*	2.33 $\pm$ 0.57*
2.	1.0	0.5	60.40 $\pm$ 0.81**	56.83 $\pm$ 0.77**	5.31 $\pm$ 1.52**
3.	1.0	1.5	86.0 $\pm$ 0.60	74.3 $\pm$ 0.80	8.66 $\pm$ 1.15
4.	1.5	1.5	Nil	Nil	Nil
5.	2.0	2.0	Nil	Nil	Nil
6.	2.5	2.5	Nil	Nil	Nil

Values are statistically significant ($P < 0.05$)

*Shoot with stunted root; ** Stunted root with shoots unable to regenerate in to complete plant

highest callus growth in terms of mean fresh weight (78.2 ± 1.81) was observed in medium supplemented with 2.5 mg L^{-1} 2,4-D (Table 1). Most of the seed explants exhibited the initiation of callus after 8–10 days from the scutellar region (Fig. 1B) and developed compact, nodular creamish-white embryogenic calli after 24 days of culture initiation (Fig. 1C). As expected, seeds cultured on medium with lowest (0.5 mg L^{-1}) and the highest concentrations of 2,4-D (4.5 mg L^{-1}) showed lower induction of callus growth (38.5 ± 0.86 and 46.5 ± 1.00). In addition, higher concentrations of 2,4-D caused browning in most of the initiated callus and affect further proliferation in the sub-cultured medium.

In vitro plant regeneration

Among the various combinations of BAP and NAA tried, only medium fortified with 1.0 mg L^{-1} BAP and 1.5 mg L^{-1} NAA showed the highest shoot regeneration frequency of $86 \pm 0.6 \%$ and $74.3 \pm 0.80 \%$ from 24 and 40 days old callus cultures respectively (Table 2). The emergence of numerous green shoot buds was observed on the 11th day of incubation. Most of the shoot buds were developed in to shoots after 16–18 days (Fig. 1D) and complete plants with multiple tillers and roots after 28 days (Fig. 1E). In all the combinations tried, it was observed that 24-days old callus cultures uniformly showed higher regeneration frequency in all three replications as against the 40 days old cultures. Each callus clump developed at least 8–10 shoots (8.66 ± 1.15) and numerous roots after 28 days of incubation in regeneration medium under 16 hr light and 8 hr dark photoperiod at $26 \pm 2^\circ\text{C}$ (Table 2). Most of the other combinations of BAP and NAA tried developed only rooting.

Acclimatization

The regenerated plantlets with well-developed roots were transferred to polypropylene boxes containing an autoclaved mixture of red soil, sand and vermiculite in growth chamber remain healthy and erect (Fig. 1F). The polythene covers were removed after 2 weeks of hardening in the growth chamber. About 80 % of the hardened plants recovered well after 21 days and were successfully established in pots.

DISCUSSION

In the present study, we succeeded in obtaining short term protocol for high frequency plant regeneration from mature seed derived embryogenic callus cultures in order to use them for genetic transformation experiments as

done previously in oats (Matsuda *et al.*, 1998). Development of simple method to regenerate highly fertile plants in a relatively short period of time is desirable to achieve genetic modification in crop plants like rice. It was demonstrated that 1–5 mm segments of leaf base of 3–4 days old rice seedlings were most suitable for the induction of callus as compared to any other seedling derived organ (Henke *et al.*, 1978). In the present study using 8 different concentrations (0.5 – 4.5 mg L^{-1}) of 2,4-D, only 2.5 mg L^{-1} showed maximum callus induction and mean fresh weight. The stimulatory effect of 2,4-D at $11.3 \mu\text{M}$ on callus induction from immature basal portion of rice leaf segment (Abdullah *et al.*, 1986) and $9.0 \mu\text{M}$ in inducing regenerable calli from leaf blade segments of rice (Yan and Zhao, 1982) was successfully demonstrated. Similarly 2,4-D at concentrations between 9.0 and $11.3 \mu\text{M}$ was found to be satisfactory in calli induction from leaf segments of African perennial rice, *Oryza longistaminata* (Boissot *et al.*, 1990) and coleoptile segments of *indica* rice (Oinam and Kothari, 1995). 2,4-D at concentrations between 4.52 and $11.3 \mu\text{M}$ facilitated embryogenic callus induction in somatic tissues of *indica* and *japonica* rice varieties (Kavi Kishore and Reddy, 1986). Only the first leaf segments were totipotent and produced embryogenic calli with the ability to regenerate complete plantlets. Similar observations were reported in other cereal food crops such as oat (Gless *et al.*, 1998), barley (Becher *et al.*, 1992) and wheat (Haliloglu, 2006). 2,4-D at 2.5 mg L^{-1} was found to be satisfactory in inducing regenerable calli from mature seed explants. The present results are consistent with the earlier observation that 2 mg L^{-1} of 2,4-D respond better on callus induction (Tyagi *et al.*, 2007). In all varying concentrations, necrosis of callus was visible with increasing concentration of 2,4-D. In our observation, any callus that turned brown during subculture were found to be unsuitable for regeneration. With low concentration callus induction was poor.

The stimulatory effect of BAP in combination with NAA has been reported to facilitate regeneration in rice callus cultures (Boissot *et al.*, 1990). The present investigation revealed that, BAP at 1.0 mg L^{-1} and NAA 1.5 mg L^{-1} were found optimum for complete plant regeneration from mature seed derived 24 days old callus. The presence of high concentration of BAP was instrumental for generating more than 8 shoots per callus clump. However, regeneration was not observed in other higher concentrations of auxins and cytokinin. Addition of higher concentrations of BAP ($>1.0 \text{ mg L}^{-1}$) and NAA ($>1.5 \text{ mg L}^{-1}$) was not beneficial because they facilitate only rooting even after 30 days in culture medium.

The findings evidence an efficient protocol for *in vitro* plant regeneration from mature seed derived callus cultures of popular local *indica* rice cultivar ADT 43 has been achieved. The present protocol provides a rapid regeneration system, which could be conveniently used for producing genetically modified plants via *Agrobacterium* – mediated and biolistic transformation approach.

ACKNOWLEDGEMENTS

This work was supported by a research grant from University Grants Commission, Govt. of India, New Delhi. AK is grateful to University Grants Commission for the award of UGC Research Fellowship in Sciences for Meritorious Students (RFSMS). The authors gratefully acknowledge the use of the Bioinformatics Infrastructure facility, Alagappa University funded by the DBT, Ministry of Science and Technology, Government of India (No. BT/B1/04/055/2001).

REFERENCES

- Abdullah R, Cocking EC and Thompson JA (1986). Efficient plant regeneration from rice protoplasts through somatic embryogenesis. *Biotechnology*. 4: 1087-1090.
- Becher T, Haberland G and Koop H (1992). Callus formation and plant regeneration in standard and micro explants from seedlings of barley (*Hordeum vulgare* L.). *Plant Cell Rep.* 11: 39-43.
- Boissot N, Valdez M and Guiderdoni E (1990). Plant regeneration from leaf and seed-derived calli and suspension cultures of the African perennial wild rice, *Oryza longistaminata*. *Plant Cell Rep.* 9: 447-450.
- Furuhashi K and Yatazuva M (1964). Indefinitely growing rice callus in tissue culture. *Kagaku*. 34: 623.
- Genovesi AD and Magill CW (1982). Embryogenesis in callus derived from rice microspores. *Plant Cell Rep.* 1: 257-260.
- Gless C, Lorz H and Jahne – Gartner A (1998). Establishment of a highly efficient regeneration system from leaf base segments of oat (*Avena sativa* L.). *Plant Cell Rep.* 17: 441-445.
- Haliloglu K (2006). Efficient regeneration system from wheat leaf base segments, *Biol. Plant.* 50: 326-330.
- Henke RR, Mansas MA and Constantin MJ (1978). Organogenesis and plantlets formation from organ and seedling derived calli of rice (*Oryza sativa* L.). *Physiol. Plant.* 44: 11- 14.
- Kavi Kishor PB and Reddy GM (1986). Regeneration of plants from long-term cultures of *Oryza sativa* L. *Plant Cell Rep.* 5: 391-393.
- Khanna HK and Raina SK (1998). Genotype X culture medium interaction effects on regeneration response of three indica rice cultivars. *Plant Cell Tiss. Org. Cult.* 52:145-153.
- Khush GS and Virk PS (2000). Rice Breeding: Achievement and future strategies. *Crop Improv.* 27: 115-144.
- Koetje DS, Grimes HD, Wang YC and Hodges TK (1989). Regeneration of indica rice (*Oryza sativa* L.) from primary callus derived from immature embryos. *J. Plant Physiol.* 135: 184-190.
- Kumria R, Waie B, Pujni D and Rajam MV (2000). Biotechnology of Rice: Present limitations and future prospects, *Plant Cell Biotech. Mol. Biol.* 1: 1-12.
- Linsmaier EM and Skoog F (1965). Organic growth factor requirements of tobacco tissue cultures. *Physiol. Plant.* 18:100-127.
- Mascarenhas AF, Pathak M, Hendre RR and Jagannatha V (1975). Tissue culture of maize, wheat, rice and sorghum. Initiation of viable callus and root cultures, *Indian J. Exp. Biol.* 13: 103-105.
- Matsuda Y, Bushnell WR, Somers DA and Rines HW (1998). Rapid embryogenesis and plant regeneration of target tissue derived from juvenile shoots for transformation of oat by microprojectile bombardment, *Plant Biotechnol.* 15: 139-143.
- Murashige T and Skoog FA (1962). Revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.* 15: 473-497.
- Oinam GS and Kothari SL (1995). Totipotency of coleoptile tissue in indica rice (*Oryza sativa* L. cv. CH1039). *Plant Cell Rep.* 14: 245-248.
- Ramesh M and Gupta AK (2006). Genetic transformation of embryogenic callus cultures of indica rice using particle bombardment and selection of stably transformed hygromycin resistant plants. *Asian J. Biotechnol. Env. Sci.* 18: 617-622.
- Ramesh M, Murugiah V and Gupta AK (2009). Efficient *in vitro* plant regeneration via leaf base segments of indica rice (*Oryza sativa* L.). *Indian J. Exp. Biol.* 47: 68- 74.
- Selvaraj KN and Ramasamy C (2006). Drought, agricultural risk and rural income case of water limiting rice production environment. *Economic Politic Weekly*, 2739.
- Shu LH and Wei JY (1980). Diversities of young rice panicles *in vitro* at different developmental stages. *J. Wuhan Univ.* 1: 94-100.
- Sticklen MB (1991). Direct somatic embryogenesis from rice mature root, *J. Plant Physiol.* 138: 577-580.
- Tyagi H, Rajasubramaniam S and Dasgupta I (2007). Regeneration and *Agrobacterium*- mediated transformation of a popular indica rice variety ADT 39. *Curr. Sci.* 93: 678-683.
- Vasil IK (1994). Molecular improvement of cereals. *Plant Mol. Biol.* 25: 925-937.
- Wernicke W and Milkovits L (1984). Developmental gradients in wheat leaves. Response of leaf segments in different genotypes cultured *in vitro*. *J. Plant Physiol.* 115: 49-58.
- Yan CJ and Zhao QH (1982). Callus induction and plantlet regeneration from leaf blade of *Oryza sativa* L. subsp indica. *Plant Sci. Lett.* 25: 187-189.