The Asian and Australasian Journal of Plant Science and Biotechnology ©2010 Global Science Books

Direct Multiple Shoot Regeneration of *indica* Rice (*Oryza sativa*) Var. 'Rasi'

Gunnaiah Raghavendra • Gollarahalli Kenchappa Kumaraswamy • Boregowda Ramya • Hakkare Swamidatta Sandesh • Kalenahalli Narasimhamurthy Yogendra • Nanjappa Deepak • Paramanahally Hanumanthegowda Ramanjini Gowda*

Department of Biotechnology, University of Agricultural Sciences, GKVK, Bangalore-560065, India

Corresponding author: *ramanjini@yahoo.com

ABSTRACT

Tissue culture of *indica* rice cultivars is difficult compared to *japonica* cultivars as they are recalcitrant to *in vitro* culture. *In vitro* culture of rice through an intervening callus stage not only poses the problem of somaclonal variation but also takes a very long time to regenerate. Rapid regeneration of *indica* rice variety 'Rasi' was carried out from the shoot basal portion, which produced multiple shoots on Murashige and Skoog (MS) medium containing thidiazuron (TDZ) and 6-benzyl amino purine. MS medium with 4 mg/L TDZ produced 11 shoots/explant with 93.3% regeneration efficiency. Shoots rooted on MS medium with 0.5 mg/L α-naphthalene acetic acid and were transferred to hardening medium containing equal volumes of sterilized sand and vermicompost within 45 days of culture. *In vitro* grown plants were uniform and no somaclonal variation was observed.

Keywords: cytokinins, *indica* rice, MS medium, rapid regeneration, rice tissue culture, somaclonal variation
Abbreviations: BAP, 6-benzyl amino purine; **MS**, Murashige and Skoog; **NAA**, α-naphthalene acetic acid; **TDZ**, thidiazuron

INTRODUCTION

Rice is staple food for large part of the World's human population, especially in East and South East Asia. It provides 27% of dietary energy and 20% of dietary protein in the developing World (Anonymous 2004). China is the highest producer with 187.4 million ones followed by India (144.57 million tones) and Indonesia (57.1 million tones) (FAOSTAT 2009). Even though world rice production is increasing every year, pests and diseases, adverse physiological and environmental factors, affect its productivity and quality. Rice is the World's single largest market for agrochemicals (Anonymous 2004). The cost of agrochemicals used for control of various pests and diseases and the loss due to their damage adds up to billions of dollars each year.

Tissue culture has played significant role in crop improvement in maintenance and development of haploids, male sterile lines and self-incompatible lines, deriving somaclonal variants, etc. This method is also used to get pathogen-free plants and to rescue rare plant species and genetic improvement through transformation technology. Thus, tissue culture is supplementing the conventional breeding programs and crop improvement through biotechnology.

Genetic engineering has now made it is possible to develop resistant rice varieties for various biotic and abiotic stresses. Bt rice has been field evaluated in China and India and has shown resistance against several insect pests – the striped stem borer, yellow stem borer, pink stem borer, leaf-folder, and green semi-looper. Transgenic rice has also been developed for other genes like *Xa21* for bacterial leaf blight resistance, Chitinase (Chi11) for sheath blight resistance, PPT for herbicide resistance, PEPC with enhanced photosynthetic efficiency (C4 rice), ORF-2 for resistance to rice yellow mottle virus (RYMV), DREB for drought tolerance and TPSP for salt tolerance (Datta 2004). Biofortification of rice is also gaining importance with rice engineered with genes for β-carotene biosynthesis (Golden rice) (Ye *et al*. 2000) might be able to reduce vitamin A deficiency and iron has been enhanced in *indica* rice by introducing the *ferritin* gene driven by the endosperm-specific promoter Vasconcelos *et al*. 2003).

Transformation of *indica* rice is limited to few cultivars because the majority of *indica* varieties are recalcitrant to *in vitro* response. Even though there have been a few reports of successful transformation of *indica* rice by *Agrobacterium* (Aldemita and Hodges 1996; Rashid *et al.* 1996; Nayak *et al.* 1997; Zhang *et al*. 1997; Khanna and Raina 1999, 2002; Mohanty *et al*. 2002). These transformation conditions either showed low transformation efficiency or were applicable to only limited *indica* varieties because of low regeneration efficiency. Establishment of highly efficient and rapid *in vitro* regeneration system for *indica* rice varieties will accelerate the use of transformation technology in breeding programs. Within *indica* subspecies, significant variations of *in vitro* culture response still exist in different genotypes (Oinam and Kothari 1993; Seraj *et al.* 1997; Khanna and Raina 1998). Though, the callus induction was good enough in above studies, regeneration of shoots was difficult and somaclonal variation was observed due to repeated sub cultures. Hence, in this study, an experiment was carried out to regenerate shoots directly from shoot basal portion without intervening callus stage. Three days germinated shoot basal portion as explant was used for direct multiple shoot regeneration at higher concentrations of cytokinins.

MATERIALS AND METHODS

The seeds of *indica* rice variety 'Rasi' – a popular high yielding variety in south India were procured from the National Seed Corp., G.K.V.K., Bangalore. The dehisced seeds were surface sterilized by treating with 70% ethanol for one minute and then washed with sterile water. Ethanol washed seeds were then treated with 4% sodium hypochlorite and a drop of Tween-20 solution for 45 min on a slow motion shaker. The treated seeds were washed with sterile water for 3-4 times inside laminar airflow chamber.

Received: 13 July, 2009. Accepted: 13 August, 2009.

Short Communication

Sterilized seeds were placed on 9 mm Petri dishes containing MS basal medium with 0, 2, 4, 6 and 8 mg/L thidiazuron (TDZ) and 4, 6 and 8 mg/L 6-benzyl amino purine (BAP). Twenty seeds were placed on each plate with four replications. Seeds were incubated in growth chamber at 25°C under white fluorescent tubes with light intensity of 2000 lux and a 16-hr photoperiod until 0.5-1.0 cm seedlings had grown. At this stage, seedlings were separated from the endosperm, shoot tip and roots were completely cut off leaving a 5-10 mm thick basal portion. This explant was placed on fresh medium with the same treatments mentioned above respectively. Multiple shoots arose from the thick basal portion after 15-20 days of culture. Shoot clumps were separated and individual shoots were placed on basal medium with 0.5 mg/Lα-naphthalene acetic acid (NAA) for rooting. After 2-3 weeks of rooting, individual seedlings were transferred to pots containing equal volumes of sterilized sand and vermicompost. The plants were hardened in the greenhouse at 28°C and 80% relative humidity. Watering was done at regular intervals and pesticides were not sprayed as any pests and diseases were not observed. All statistical analysis was performed by ANOVA F test at p=0.05 using MS-excel.

RESULTS AND DISCUSSION

The potential of callus induction and regeneration in rice tissue culture depends on a number of factors, such as the genotype of the donor plant, the type and physiological status of the explant, the composition and concentration of the basal salt, organic components and plant growth regulators in the culture medium. Among these factors, genotypic difference is the most important. Visarada and Sarma (2002) noted that even though *indica* varieties produce callus early, i.e. within 7-10 days of culture, the callus was compact and proliferation was slow. On transfer to regeneration medium, redifferentiation of callus was observed instead of regeneration, as in *japonica* cultivars. Over a long sub-culture period, a rapid decline in regeneration was observed (Visarada and Sarma 2002). In many *indica* cultivars, recovery of transgenic plants is difficult as cells accessible for gene transfer may not be suitable for plant regeneration (Komari *et al*. 1998). Rapid *in vitro* regeneration from the shoot basal portion without an intervening callus stage would overcome the regeneration problems incurred in culture of *indica* varieties.

Multiple shoot induction

In this experiment, highest percent of multiple shoot induction was observed in 4 mg/L TDZ (93.33%) followed by 6 mg/L BAP (91.67%) and 8 mg/L BAP (88.33%) (**Table 1**). More than 80% of the shoots regenerated into whole plants and were readily transferred to soil. The regeneration efficiency only ranged from 0-12% in 21 *indica* cultivars tested through callus-mediated *in vitro* culture (Beena 2006). Maximum regeneration (14.88%) was observed from callus of popular Phillipine varieties on N6 media (Mahmuda and Nenita 2005). Even in Basmati varieties, maximum regeneration reported is 69% in 'Basmati 370', which responds well to *in vitro* culture (Muhammed *et al*. 2008).

Multiple shoots (more than two shoots/explant) were observed in treatments with 4 and 6 mg/L TDZ and 4, 6 and 8 mg/L BAP (**Table 1, Fig. 1**). The average number of shoots/explant regenerated on each treatment were 11 and 8 shoots/explant, respectively in 4 and 6 mg/L TDZ and 6, 8, 6 shoots/explant, respectively in 4, 6 and 8 mg/L BAP. In a similar experiment of multiple shoot induction in *indica* rice using shoot apical meristem as explant on MS media with 4 mg/L TDZ, maximum of 6 shoots per explant were recorded (Yookongkaew *et al*. 2007).

Among the hormones, TDZ responded better than BAP in regeneration efficiency as well as maximum number of multiple shoots (**Table 1**). This was in accordance with a report in which the action of synthetic cytokinin TDZ induced more shoots than BAP in *indica* rice cv. 'Jaumala' (Gairi and Rashid 2004). TDZ is active at lower concentrations than the amino purine cytokinins and found to be less susceptible to plant degrading enzymes than endogenous cytokinins (Huetteman and Preece 1993). TDZ at 4 mg/L induced 5-7 shoots per explant in Thai *indica* varieties using shoot apical meristen as explant. No significant differences were observed among the 13 Thai *indica* varieties they tested (Yookongkaew *et al*. 2007).

Table 1 Multiple shoots regeneration from shoot basal culture of *indica* rice var. 'Rasi'.

TDZ + BAP (mg/L)	№ of explants	№ of explants producing multiple shoots	% of multiple shoot production	№ of shoots/ explant
T_0 (0+0)	20	0.00	0.00	1.00
T_1 (2+0)	20	0.00	0.00	1.00
T_2 (4+0)	20	18.67	93.33	10.67
T_3 (6+0)	20	17.33	86.67	8.00
T_4 (0+4)	20	14.00	70.00	5.67
T_5 (0+6)	20	18.33	91.67	8.00
T_6 (0+8)	20	17.67	88.33	6.67
CV%		3.55	3.55	11.18
SEm		0.36	1.78	0.53
CD 1%		1.01	5.07	1.52

TDZ: thidiazuron, BAP: 6-benzyl amino purine

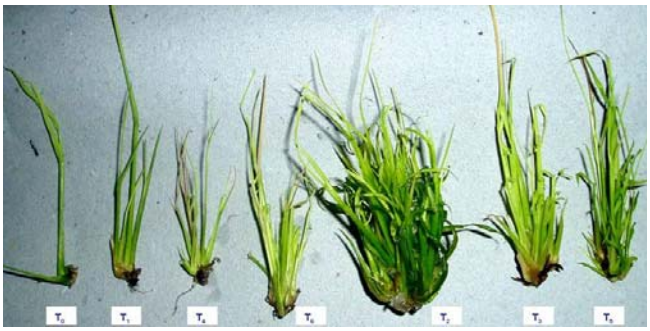

Fig. 1 Multiple shoots regeneration from shoot basal culture of *indica* rice variety 'Rasi' at different concentrations of TDZ and BAP. T_0: MS0 MS media with no hormones; T_1: MS0 + 2 mg/L TDZ; T_2: MS0 + 4 mg/L TDZ; T_3: MS0 + 6 mg/L TDZ; T_4: MS0 + 4 mg/L BAP; T_5: MS0 + 6 mg/L BAP; T_6: MS0 + 8 mg/L BAP.

Rapid regeneration

Shoot growth was also faster in MS media with 4 mg/L TDZ in which 5-6 cm shoot growth was observed within 15 days of culture in 'Rasi'. In contrast, the regeneration of rice plants through callus intervention took minimum of 14 weeks for 'Rasi' with 2-3 healthy plants/explant (Table 2) (Raghavendra 2007). Even in recalcitrant varieties like 'Basmati 370' and 'Basmati 371', 21-28 days-old calli of took 27-30 days to regenerate into a complete plant (Muhammed *et al*. 2008) while the shoot basal portion as explant regenerated into rice plantlets which rooted profusely (**Fig. 2**) and could be transferred to soil within 45-60 days.

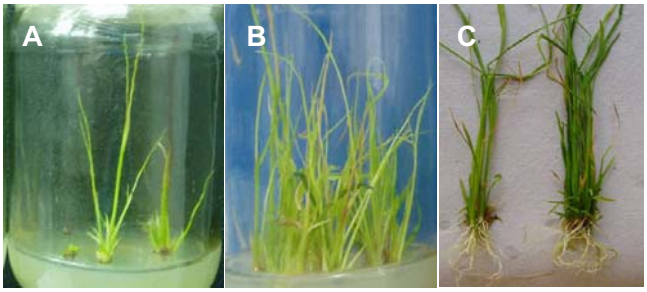

Fig. 2 ***In vitro*** **regeneration of** ***indica*** **rice variety 'Rasi'.** (**A**) Direct multiple shoot regeneration from shoot basal portion; (**B**) Shoot regeneration from callus; (**C**) Rooting of individual shoots.

Table 2 Comparison of direct multiple shoot regeneration and regeneration with callus intervention of *indica* rice var. 'Rasi'.

Characteristics	Direct multiple shoot regeneration	Regeneration with callus intervention
Days taken for complete plant regeneration	45-55 days	105-110 days
Total number of subcultures	3	6-8
No. of plants regenerated per explant	8-11	2-3
Somoclonal variation	Not observed	20-30%
Use in *Agrobacterium*-mediated transformation	Possible	Possible
Use in biolistic transformation	Not possible	Possible

Somaclonal variation

Multiple shoots were 5-6 cm long within 15-20 days of culture and required only two subcultures before transfer to hardening medium (**Table 2**). The plants were uniform and no variation was observed among the plants that were raised by separating the multiple shoots. In callus-mediated regeneration, the risk of somaclonal variation and problems in transgene inheritance and stability of transgene expression is high (Bregitzer and Tonks 2003). It has also been observed that the incidence of genetic mutations and somaclonal variation was low in plants regenerated from shoots due to the absence of tissue dedifferentiation steps that are common in the initiation of callus and somatic embryo cultures (Hirochika 1993). Culture of shoot apices can be combined with either *Agrobacterium*-mediated transformation or particle bombardment (Gould and Magallanes-Cedeno 1998; Zapata *et al.* 1999; Cho *et al*. 2003; Goldman *et al*. 2003). This rapid method can be used for testing the functions and transient expression of genes (Yookongkaew *et al.* 2007).

CONCLUSION

Rice plants regenerated *in vitro* from the shoot basal portion without an intervening callus stage could be regenerated in a short time and transferred to soil within 5-8 weeks of culture. Direct regeneration of multiple shoots from the shoot basal portion can maintain genetic stability once transgenic plants are obtained and a large population of plants can be obtained in short time. This rapid regeneration system can also be used for *Agrobacterium*-mediated transformation.

ACKNOWLEDGEMENTS

The authors are thankful to Kirk House Trust, U.K., for providing research grants to carry out the research.

REFERENCES

Aldemita RR, Hodges TK (1996) *Agrobacterium tumefaciens* mediated transformation of *japonica* and *indica* rice varieties. *Planta* **199**, 612-617

Anonymous (2004) Rice is life - international year of rice - 2004. *NARC Newsletter (Rice Special Issue)*, 8pp

Beena C (2006) Genetic effect on the regeneration of the calli of rice. *Indian Journal of Crop Science* **1**, 207-208

Bregitzer P, Tonks D (2003) Inheritance and expression of transgenes in barley. *Crop Science* **42**, 1303-1308

Cho MJ, Choi HW, Okamoto D, Zhang S, Lemaux PG (2003) Expression of green fluorescent protein and its inheritance in transgenic oat plants generated from shoot meristematic cultures. *Plant Cell Reports* **21**, 467-474

Datta SK (2004) Rice biotechnology: A need for developing countries. *AgBioForum* **7 (1&2)**, 31-35

FAOSTAT (2009) http://www.fao.org/site/339/default.aspx

Gairi A, Rashid A (2004) TDZ-induced somatic embryogenesis in non-responsive caryopses of rice using a short treatment with 2,4-D. *Plant Cell, Tissue Organ Culture* **76**, 29-33

Goldman JJ, Hanna WW, Fleming G, Ozias-Akins P (2003) Fertile transgenic pearl millet [*Pennisetum glaucum* (L.) R. Br.] plants recovered through microprojectile bombardment and phosphinothricin selection of apical meristem-, inflorescence-, and immature embryo-derived embryogenic tissues. *Plant Cell Reports* **21**, 999-1009

Gould H, Magallanes-Cedeno M (1998) Adaptation of cotton shoot apex culture to *Agrobacterium*-mediated transformation. *Plant Molecular Biology Reporter* **16**, 1-10

Hirochika H (1993) Activation of tobacco retrotransposons during tissue culture. *EMBO Journal* **12**, 2521-2528

Huetteman CA, Preece JE (1993) Thidiazuron: a potent cytokinin for woody plant cell tissue culture. *Plant Cell, Tissue and Organ Culture* **33**, 105-121

Khanna HK, Raina SK (1999) *Agrobacterium*-mediated transformation of *indica* rice cultivars using binary and superbinary vectors. *Australian Journal of Plant Physiology* **26**, 311-324

Khanna HK, Raina SK (2002) Elite *indica* transgenic rice plants expressing modified Cry1Ac endotoxin of *Bacillus thuringiensis* show enhanced resistance to yellow stem borer (*Scirpophaga incertulas*). *Transgenic Research* **11**, 411-423

Khanna HK, Raina SK (1998) Genotype and culture media induction effects on regeneration response of three *indica* rice cultivars. *Plant Cell, Tissue and Organ Culture* **52**, 145-153

Komari T, Hiei Y, Ishida Y, Kumashiro T, Kubo T (1998) Advances in cereal gene transfer. *Current Opinion on Plant Biology* **1**, 161-165

Lin YJ, Zhang Q (2005) Optimizing the tissue culture conditions for high efficiency transformation of *indica* rice. *Plant Cell Reports* **23**, 540-547

Mahmuda KM, Nenita VD (2005) Callus induction and plant regeneration from rice epicotyl. *Plant Tissue Culture* **15 (1)**, 51-56

Mohanty A, Kathuria H, Ferjani A, Sakamoto A, Mohanty P, Murata N, Tyagi AK (2002) Transgenics of an elite *indica* rice variety Pusa Basmati 1 harbouring the *codA* gene are highly tolerant to salt stress. *Theoretical and Applied Genetics* **106**, 51-57

Muhammed Tariq, Gower Ali, Fazal Hadi, Shakeel Ahmed, Nasir Ali, Ali Shah (2008) Callus induction and *in vitro* plant regeneration of rice (*Oryza sativa* L.) under various conditions. *Pakistan Journal of Biological Science* **11 (2)**, 255-259

Nayak P, Basu D, Das S, Basu A, Ghosh D, Ramakrishnan NA, Ghosh M, Sen SK (1997) Transgenic elite *indica* rice plants expressing CryIAc-endotoxin of *Bacillus thuringiensis* are resistant against yellow stem borer (*Scirpophaga incertulas*). *Proceedings of National Academy of Sciences USA* **94**, 2111-2116

Oinam GS, Kothari SL (1995) Totipotency of coleoptile tissue in indica rice (*Oryza sativa* L. cv. CH 1039). *Plant Cell Reports* **14**, 245-248

Raghavendra G (2007) Transformation of *indica* rice variety 'Rasi' with *GUS* and *Glucanase* genes. MSc thesis, University of Agricultural Sciences, Bengalure, India, 128 pp

Rashid H, Yokoi S, Toriyama K, Hinata K (1996) Transgenic plant production mediated by *Agrobacterium* in *indica* rice. *Plant Cell Reports* **15**, 727-730

Reddy VS, Leelavathi S, Sen SK (1985) Influence of genotype and culture medium on microspore callus induction and green plant regeneration in anthers of *Oryza sativa*. *Plant Physiology* **63**, 309-314

Seraj, ZI, Islam Z, Faruque MO, Devi T, Ahmed S (1997) Identification of the regeneration potential of embryo derived calluses from various *indica* rice varieties. *Plant Cell, Tissue and Organ Culture* **48**, 9-13

Tang W (2003) Additional virulence genes and sonication enhance *Agrobacterium tumefaciens*-mediated loblolly pine transformation. *Plant Cell Reports* **21**, 555-562

Vasconcelos M, Datta K, Oliva N, Khalekuzzaman M, Torrizo L, Krishnan S, Oliveira M, Goto F, Datta SK (2003) Enhanced iron and zinc accumulation in transgenic rice with the ferritin gene. *Plant Science* **164**, 371-378

Visarada KBRS, Sarma NP (2002) Qualitative assessment of tissue culture parameters useful in transformation of *indica* rice. *Current Science* **82**, 343-346

Ye X, Al-Babili S, Klöti A, Zhang J, Lucca P, Beyer P, Potrykus I (2000) Engineering the provitamin A (β-carotene) biosynthetic pathway into (carotenoid free) rice endosperm. *Science* **287**, 303-305

Yookongkaew N, Srivatanakul M, Narangajavana J (2007) Development of genotype-independent regeneration system for transformation of rice (*Oryza sativa* ssp. *indica*). *Journal of Plant Research* **120**, 237-245

Zhang J, Xu RJ, Elliott MC, Chen DF (1997) *Agrobacterium*-mediated transformation of elite *indica* and *japonica* rice cultivars. *Molecular Biotechnology* **8**, 223-231