PARTIAL DESICCATION OF SCUTELLUM-DERIVED RICE CALLUS IMPROVES *AGROBACTERIUM*-MEDIATED TRANSFORMATION

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(Received on Date: 7th July 2016 Date of Acceptance: 26th August 2016)

ABSTRACT

Rice varieties of the *indica* subspecies are more difficult to transform than those of the *japonica* subspecies. Here, we report that partial desiccation of rice callus significantly improves *indica* rice transformation efficiency. Four different binary vectors with hygromycin plant selection marker (*hph*) were mobilized in *Agrobacterium tumefaciens* LBA4404 (pSB1) and used to transform scutellum-derived rice callus. Without desiccation, 6.65 % calli formed transgenic hygromycin-resistant shoots. In comparison, 36-hr desiccated rice calli yielded hygromycin-resistant shoots in 11.76 % calli. Partial desiccation of the rice calli improved transformation efficiency by 77 % and transgenic shoots regenerated much faster upon desiccation of calli.

Keywords: *Indica* rice, partial desiccation, somatic embryogenesis, scutellum-derived callus

No: of Figures : 2 **No:of Tables: 1** **No: of References:26**

INTRODUCTION

Cereals such as rice, wheat and maize together account for 60 % of the world's food production, of which rice itself is the principal food for 50 % of the world's population. Among these cereals, rice is highly amenable to genetic manipulation through *Agrobacterium*-mediated transformation and by particle bombardment (Tyagi and Mohanty, 2000). Among these two methods, *Agrobacterium*-mediated transformation is more preferred because of the precision of T-DNA transfer and low copy number T-DNA integration. The success of genetic transformation is highly dependent on efficient *in vitro* regeneration of plants.

Somatic embryogenesis, a common process for regeneration of plants in rice, facilitates regeneration of genetically modified plants. The first successful regeneration of shoots from seed-derived rice callus was reported by Nishi *et al*. (1968). It has been reported that in rice tissue culture, choice of a totipotent explant, growth hormone combination, culture conditions like temperature, light, darkness and physical treatments play crucial roles in successful regeneration of shoots (Rueb *et al*., 1994).

Compared to explants such as shoot apex, leaf base and immature embryos (Manimaran *et al*., 2013), scutellum-derived rice callus is the most suitable source for transformation in *indica* varieties (Kumar *et al*., 2005). In rice callus-based transformation, selection and regeneration processes take 3-4 months to generate a transgenic plant. It has been reported that *indica* rice varieties show a lower regeneration potential as compared to the *japonica* rice varieties (Abe and Futsufara, 1984). One of the reasons for low regeneration frequency observed in *indica* rice variety is the prolonged tissue sub-culturing which results in browning of callus (Manimaran *et al*., 2013). Many efforts have been made to develop transgenic *indica* rice (Datta *et al*., 2000; Sridevi *et al*., 2008). These studies suggested that optimization of callus induction and regeneration are critical for the development of transgenic plants in *indica* rice varieties.

Maturation of somatic embryos and their regeneration to plantlets are dependent on physical and chemical stresses. Partial desiccation has been reported to promote somatic embryo differentiation and shoot development in soybean, wheat (Rance *et al*., 1994), sugarcane (Kaur and Gosal, 2009) and rice (Chand and Sahrawat, 2001). Tsukahara and Hirosawa (1992) observed that dehydration of cell suspension-derived calli of *japonica* rice for 24 hr increased shoot regeneration from 5 to 47 %. Ikram-ul-Haq *et al*. (2009) reported that a combination of chemical desiccation (through 3 % maltose and 3 % sorbitol supplementation in regeneration medium) and physical dehydration of callus prior to regeneration promotes higher regeneration in *indica* rice. Therefore, the present study was performed to determine the effect of desiccation on regeneration of shoots from transformed *indica* rice callus.

MATERIALS AND METHODS

Plant materials and methods

Mature rice seeds (*Oryza sativa* L. subsp. *indica* cv Pusa Basmati1) were dehusked manually. Surface sterilization of dehusked seeds was done as described by Vijayachandra *et al*. (1995).

Callus induction and *Agrobacterium*-mediated transformation

The sterilized seeds were incubated on a callus induction medium (CIM) [Murashig and Skoog (MS) salts, 500 mg L^{-1} proline, 3 % sucrose (w/v), and 2.25 g L^{-1} Phytagel, pH=5.8] in a tissue culture room set at 16 hr light (100 $\mu En^{-2}s^{-1}$) and 8 hr dark photoperiod at 25 °C. After 21-day incubation in CIM, the scutellum-derived calli were subcultured and incubated on fresh CIM for 4 days. *Agrobacterium*-mediated transformation of scutellum-derived rice callus was performed as described earlier by Sridevi *et al*. (2003). The 4-day subcultured embryogenic calli were infected by immersing them in the *Agrobacterium* culture for 15 min and then the calli were transferred to the co-cultivation medium (CIM supplemented with 10 g L^{-1} glucose, 3 g L^{-1} Phytagel and 100 µM acetosyringone) for 3 days in dark. The co-cultivated calli were washed twice with liquid CIM and finally rinsed with liquid selection medium (SM) (CIM supplemented with 250 mg L^{-1} cefotaxime). Rinsed calli were placed in darkness on the solid (SM) (CIM supplemented with 4 g L^{-1} Phytagel, 50 mg L^{-1} hygromycin and 250 mg L^{-1} cefotaxime). After 14 days, one more round of culturing for 21 days was done in SM.

Partial desiccation

Following two rounds of incubation on the SM, 15 to 17 calli were placed in a sterile Petri plate on a Whatman #1 filter disc. The Perti plates were sealed with the Micro-pore tape and kept for 36 hr in an incubator in darkness at 28 ± 1 °C.

Plantlet regeneration

After the desiccation treatment, the calli were transferred to the shoot regeneration medium (RM1) [MS medium supplemented with kinetin (3 mg L^{-1}), naphthaleneacetic acid (1.5 mg L^{-1}), Phytagel (6 g L^{-1}), hygromycin (40 mg L^{-1}) and cefotaxime (250 mg L^{-1})]. After 14 days of incubation in darkness, the calli were transferred to light for shoot development. For complete development of shoots, the calli were subcultured on RM 2 ([MS medium supplemented with kinetin (3 mg L^{-1}), naphthaleneacetic acid (1.5 mg L^{-1}), Phytagel (4 g L^{-1}), hygromycin (40 mg L^{-1}) and cefotaxime (150 mg L^{-1})] at 21-day intervals till distinct shoots emerged.

Southern blot analysis

Total plant DNA was extracted from fresh rice leaves using cetyltrimethylammonium bromide (Rogers and Bendich, 1988) and estimated in a fluorometer using Hoechst dye 33258. Plant DNA (2.5 µg) was digested with an appropriate restriction enzyme and electrophoresed in a 0.8 % agarose gel in 1X Tris-borate-EDTA (TBE) buffer. After depurination, denaturation and neutralization, DNA was transferred to the Zetaprobe nylon membrane (Biorad, Hercules, USA). The probe DNA was labelled with [a-^{32}P]dCTP (Board of Radiation and Isotope Technology, Mumbai, India) using Megaprime™ DNA labelling kit (GE Health care UK Limited, Little Chalfont, UK). Hybridization and washes were done as described by Ramanathan and Veluthambi (1995).

Results and Discussion

Induction of embryogenic calli with high regeneration potential is one of the major requirements for the development of transgenic rice. Embryogenic callus formation from the scutellum part of the seed (Fig. 1A) was visible during 7-10 days in CIM (Fig. 1B). Maximum callus proliferation was observed after 19-21 days (Fig. 1C). It has been reported by Katiyar *et al*. (1999) that the CIM supplemented with 2, 4-D efficiently induces unorganised callus tissue in rice. Phytagel (2.25 g L^{-1}) was found to be a suitable gelling agent for callus induction. The calli formed on the CIM were white and had compact and nodular texture (Fig. 1C).

In this study, 21-day-old scutellum-derived callus (Fig. 1C) was preincubated for 4 days and then was used as an explant for transformation. The calli were co-cultivated independently with *A. tumefaciens* LBA4404 (pSB1) harbouring four different binary plasmids- pCAMBIA1301, pPZP101-*hph*, pCAMBIA0390-*hph* and pCAMBIA0390-*hph*-hp*MSUFL1*(fig.1D). All binary vectors had *hph* as the selectable marker. The plasmid pSB1 harbours *vir*B, *vir*G and *vir*C genes of the supervirulent Ti plasmid pTiBo542 which improves rice transformation by *Agrobacterium* (Komari *et al*., 1996).

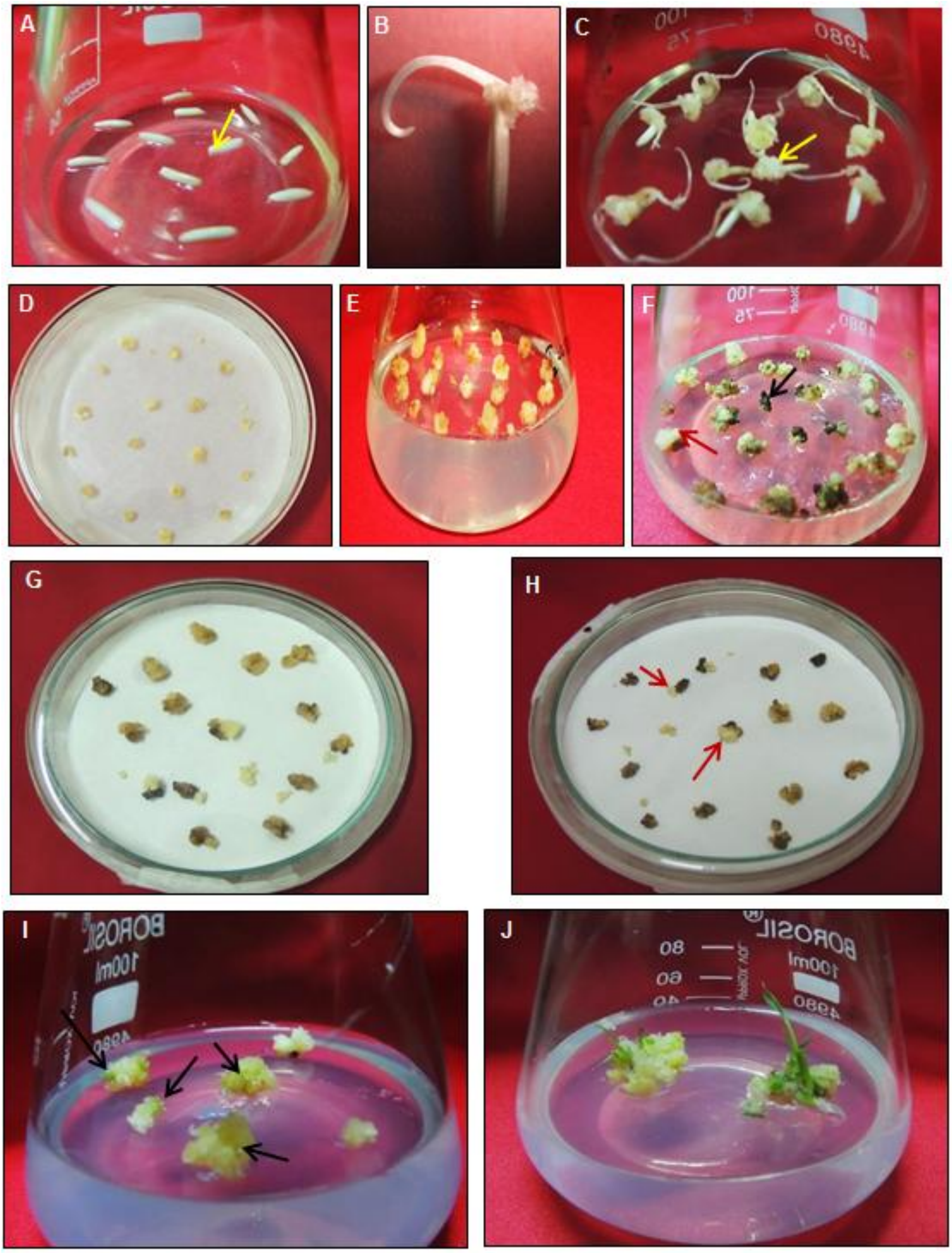

Figure 1. Callus induction, somatic embryogenesis and plant regeneration in transformed *indica* rice (PB1). (A) Surface-sterilized mature rice seeds placed on a callus induction medium (arrow indicates the scutellum region of the seed). (B) Induction of embryogenic callus from seeds 10 days after inoculation on callus induction medium (CIM). (C) Scutellum-derived rice calli (21 days after inoculation of seeds on CIM, indicated by an arrow). (D) Co-cultivation of calli with *Agrobacterium*. (E) Transformed calli grown after co-cultivation on selection medium (SM) containing 50 mg L $^{-1}$ hygromycin. (F) Transformed calli grown on SM containing 50 mg L $^{-1}$ hygromycin (red arrow indicates transformed callus and black arrow indicates untransformed callus) after first round of selection. (G) A Petri plate containing transformed calli before desiccation. (H) A Petri plate containing transformed calli after 36-hr of desiccation. (I) Development of shoot buds on desiccation-treated calli on regeneration medium (RM1) (indicated by arrows) after 17-19

days, following desiccation. (J) Formation of shoots from somatic embryos on RM2 after 25-30 days, following desiccation

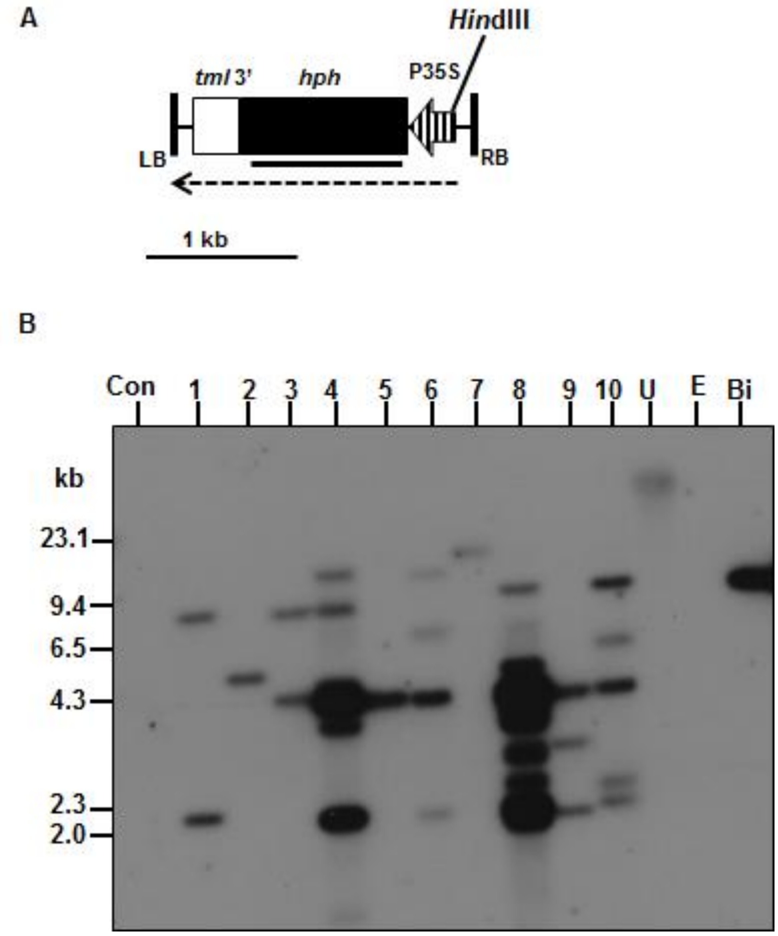

Figure 2. Southern blot analysis of rice plants transformed with *Agrobacterium tumefaciens* LBA4404 (pSB1, pPZP101-*hph*). (A) T-DNA of the binary plasmid pPZP101-*hph*. The left border (LB) junction fragment (>1.7 kb, the distance between the *Hin*dIII site and LB), marked with a broken line with an arrow, will hybridize to the *hph* probe (marked with a bold line). *P35S*, *Cauliflower mosaic virus* 35S promoter; *hph*, hygromycin phosphotransferase gene; *tml 3'*, tumour morphology large polyadenylation signal; LB, left T-DNA border; RB, right T-DNA border. Scale (1.0-kb) is marked. (B) Southern blot analysis of T_0 rice plants transformed with the binary plasmid pPZP101-*hph*. Hybridization was done with the [a-^{32}P]dCTP-labelled *hph* probe. Plant DNA (2.5 µg) from 10 T_0 transgenic plants (lanes 1 to 10) and a control, untransformed plant (Con) was digested with *Hin*dIII. The binary plasmid pPZP101-*hph* (lane Bi, 50 pg), digested with *Hin*dIII, was used as a positive control. The positions of the λ/*Hin*dIII fragments are marked

Table 1 Effect of desiccation on regeneration of hygromycin-resistant shoots from scutellum-derived rice calli transformed with *Agrobacterium tumefaciens* LBA4404 (pSB1) harbouring different binary plasmids. Hygromycin (50 mg L^{-1}) was used for selection in selection medium SM and 40 mg L^{-1} was used for selection in regeneration medium (RM). [Mean ± SD (n=6)]

Binary plasmid used for transformation	**Non-desiccation**			**Desiccation**		
	Number of calli used	**Number of calli regenerated on Hyg***	**Transformation %**	**Number of calli used**	**Number of calli regenerated on Hyg***	**Transformation %**
pCAMBIA1301	150	9.0	6.0	150	16.0	10.6
pCAMBIA1301	150	11.0	7.3	150	15.0	10.0
pPZP101-*hph*	250	19.0	7.6	250	29.0	11.6
pPZP101-*hph*	250	15.0	6.0	250	31.0	12.4
pCAMBIA0390-*hph*	100	7.0	7.0	100	12.0	12.0
pCAMBIA0390-*hph*-hp*MSUFL1*	100	6.0	6.0	100	14.0	14.0
Mean	166.6	11.16	6.65	166.6	19.5	11.76
Mean ± SD			6.65 ± 0.73			11.76 ± 1.4

Two rounds of selection were done. Transformed calli were first kept in the SM for 14 days and then transferred to SM for a 21-day period. In the presence 50 mg L^{-1} hygromycin, untransformed calli turned brown (Fig. 1E), and eventually stopped proliferation and turned black (Fig. 1F). However, the transformed calli continued to proliferate slowly and remained white (Fig. 1F).

In each of the six independent transformation experiments, 15 to 17 transformed calli were kept for partial desiccation in a Perti plate for a period of 36 hr at 28 ± 1 °C (Fig. 1G). After 36-hr desiccation stress, milky white tissues of the callus (Fig. 1H) were dissected from the dark brown callus mass with the help of sterile forceps and placed on RM1 with hygomycin (40 mg L^{-l}) and cefotaxime (250 mg L^{-1}) for 21 days.

In this work, calli subjected to desiccation stress and those without desiccation stress were used in six independent transformation experiments (Table 1). *A. tumefaciens* LBA4404 (pSB1) harbouring the binary plasmids pCAMBIA1301, pPZP101-hph, pCAMBIA0390-*hph*, pCAMBIA0390-*hph*-hp*MSUFL1* were used for transformation. All binary vectors harboured *hph* as the plant selectable marker. In the calli which were not subjected to desiccation, 6.65 % calli developed hygromycin-resistant shoots (Table 1). On the other hand, in those calli subjected to desiccation, 11.76 % calli developed hygromycin-resistant shoots (Table 1). The values are significantly different at 5 % level. The results indicated that partial desiccation of calli, prior to the regeneration step, increased transformation efficiency by 77 %. In addition to higher transformation efficiency, the shoots regenerated from the transformed calli much faster in the desiccated calli in comparison to the non-desiccated calli.

Rance *et al*. (1994) observed that partial desiccation of mature embryo-derived calli accumulated two additional proteins one day after dehydration treatment. This result indicates that desiccation triggers a rapid biochemical change in the calli. Dehydration treatment of scutellum-derived rice callus improved the germination rate of somatic embryos and vigour of plant regeneration by promoting the accumulation of nutrients and storage proteins which are used by embryos during germination (Mariani *et al*., 2000). Recent studies revealed that desiccation activates the of late embryogenesis abundant (LEA) protein genes, which provides protective molecules to the embryo tissue during osmotic stress (Bartels *et al*., 2005). The *indica* rice varieties differ from *japonica* rice in the level of osmotic response towards the desiccation treatment. It has been observed that low water content of rice callus, cultured on a medium containing mannitol and a high concentration of gelling agent, was the vital factor for the regeneration of plants. Sah *et al*. (2014) observed that a combination of agar (8.0 g L^{-1}) and Phytagel (2.0 g L^{-1}) enhanced regeneration in *japonica* rice. Studies indicated that a 48-hr desiccation treatment showed significant increase in regeneration when compared with 24-hr treatment (Vennapusa *et al*., 2015; Saharan *et al*., 2004).

In the first phase of regeneration in this work, the calli were kept in darkness for 14 days and transferred to light for 7 days. Regeneration in darkness is an important step because somatic embryogenesis is promoted in the presence of auxins which are quickly degraded under light (Arzate-Fernandez *et al*., 1997). Within 17-18 days after incubation in RM1, green shoot buds started appearing in desiccated calli (Fig. 1I) and subsequently, shoots emerged after 7-10 days in RM2 (Fig. 1J). However, in non-dehydrated calli, initiation of shoot regeneration started only 45-49 days after desiccation in the RM2. The findings in this report show that partial desiccation of the calli, which improves regeneration, significantly improved *Agrobacterium*-mediated transformation of *indica* rice.

Southern blotting was performed to check the integration of T-DNA in the hygromycin-resistant plants regenerated from the desiccated calli. Ten representative plants transformed with *A. tumefaciens* LBA4404 (pSB1, pPZP101-*hph*) were taken for the analysis (Fig. 2A). Plant DNA (2.5 µg) was digested with *Hin*dIII and Southern blot analysis was done with the *hph* probe. DNA from all 10 hygromycin-resistant plants (1 to 10) displayed hybridization of junction fragment longer than 1.7 kb (Fig. 2B). The results confirm the transgenic nature of the hygromycin-resistant plants.

CONCLUSION

In comparison to *japonica* rice cultivars, *indica* rice cultivars are more difficult to transform. This study shows that the recalcitrant nature of *indica* rice variety for *Agrobacterium*-mediated transformation can be effectively overcome by partial desiccation of calli prior to the shoot regeneration step. In addition to a 77 % increase in transformation efficiency, partial desiccation of calli also increased the rate at which hygromycin-resistant shoots emerged. Thus, desiccation of callus emerges as an important physical stress to improve transformation efficiency of *indica* rice.

REFERENCES

Abe T, Futsufara Y. Varietal difference of plant regeneration from root callus tissues in rice. Japan J Breed 1984; 34: 147-155.

Arzate-Fernandez A. M, Nakazaki T, Okumoto Y, Tanisaka T. Efficient callus induction and plant regeneration from filaments with anther in lily (*Lilium longiflorum* Thunb.). Plant Cell Rep 1997; 16: 836-840.

Bartels D. Desiccation tolerance studied in the resurrection plant *Craterostigma plantagineum*. Integr Comp Biol 2005; 45: 696-701.

Chand S, Sahrawat A. K. Stimulatory effect of partial desiccation on plant regeneration in *indica* rice (*Oryza sativa* L.). J Plant Biochem Biotechnol 2001; 10: 43-47.

Datta K, Koukolikova-Nicola Z, Baisakh N, Oliva N, Datta S. K. *Agrobacterium*-mediated engineering for sheath blight resistance of *indica* rice cultivars from different ecosystems. Theor Appl Genet 2000; 100: 832-839.

Ikram-ul-Haq, Chang-Xing Z, Mukhtar Z, Jaleel C. A, Azooz M. M. Effect of physical desiccation on plant regeneration efficiency in rice (*Oryza sativa* L.) variety super basmati. J Plant Physiol 2009; 166: 1568-1575.

Katiyar S. K, Chandel G, Singh P, Pratibha R. Genetic variation and effect of 2, 4-D on *in vitro* plant regeneration in *indica* rice cultivars. Oryza 1999; 36: 254-256.

Kaur A, Gosal S. S. Desiccation of callus enhances somatic embryogenesis and subsequent shoot regeneration in sugarcane. Indian J Biotechnol 2009; 8: 332-334.

Komari T, Hiei Y, Saito Y, Murai N, Kumashiro T. Vectors carrying two separate T-DNAs for co-transformation of higher plants mediated by *Agrobacterium tumefaciens* and segregation of transformants free from selection markers. Plant J 1996; 10:165-174.

Kumar K. K, Maruthasalam S, Loganathan M, Sudhakar D, Balasubramanian P. An improved *Agrobacterium*-mediated transformation protocol for recalcitrant elite *indica* rice cultivars. Plant Mol Biol Rep 2005; 23: 67-73.

Manimaran P, Kumar G. R, Reddy M. R, Jain S, Rao T. B, Mangrauthia S. K,

Sundaram R. M, Ravichandran S, Balachandran S. M. Infection of early and young callus tissues of *indica* rice BPT 5204 enhances regeneration and transformation efficiency. Rice Sci 2013; 20: 415-426.

Mariani T. S, Miyake H, Takeoka Y. Improvement of direct somatic embryogenesis in rice by selecting the optimal developmental stage of explant and applying desiccation treatment. Plant Prod Sci 2000; 3: 114-123.

Nishi T, Yamada Y, Takahashi E. Organ redifferentiation and plant restoration in rice callus. Nature 1968; 219: 508-509.

Ramanathan V, Veluthambi K. Transfer of non-T-DNA portions of the *Agrobacterium tumefaciens* Ti plasmid pTiA6 from the left terminus of T_L-DNA. Plant Mol Biol 1995; 28: 1149-1154.

Rance I.M, Tian W, Mathews H, de Kochko A, Beachy R. N, Fauquet C. Partial desiccation of mature embryo-derived calli, a simple treatment that dramatically enhances the regeneration ability of *indica* rice. Plant Cell Rep 1994; 13: 647-651.

Rogers S. O, Bendich A. J. Extraction of DNA from plant tissues. In: Gelvin SB, Schilperoort RA, Verma DPS (eds) Plant molecular biology manual. Kluwer, Dordrecht 1988. pp A6/1–10.

Rueb S, Leneman M, Schilperoort R. A, Hensgens L. A. M. Efficient plant regeneration through somatic embryogenesis from callus induced on mature rice embryos (*Oryza sativa* L.). Plant Cell Tissue Organ Cult 1994; 36: 259-264.

Sah S. K, Kaur A, Sandhu J.S. High frequency embryogenic callus induction and whole plant regeneration in *japonica* rice cv. Kitaake. J Rice Res 2014; 2:2 http://dx.doi.org/10.4172/jrr.1000125.

Saharan V, Yadav R. C, Yadav N. R, Chapagain B. P. High frequency plant regeneration from desiccated calli of *indica* rice (*Oryza sativa* L.). Afr J Biotechnol 2004; 3: 256-259.

Sridevi G, Parameswari C, Sabapathi N, Raghupathy V, Veluthambi K. Combined expression of chitinase and β-1,3-glucanase genes in *indica* rice (*Oryza sativa* L.) enhances resistance against *Rhizoctonia solani*. Plant Sci 2008; 175: 283-290.

Sridevi G, Sabapathi N, Meena P, Nandakumar R, Samiyappan R, Muthukrishnan S, Veluthambi K. Transgenic *indica* rice variety Pusa Basmati 1 constitutively expressing a rice chitinase gene exhibits enhanced resistance to *Rhizoctonia solani*. J Plant Biochem Biotechnol 2003; 12: 93-101.

Tsukahara M, Hirosawa T. Simple dehydration treatment promotes plantlet regeneration of rice (*Oryza sativa* L.) callus. Plant Cell Rep 1992; 11: 550-553.

Tyagi A. K, Mohanty A. Rice transformation for crop improvement and functional genomics. Plant Sci 2000; 158: 1-18.

Vennapusa A. R, Vemanna R. S, Rajashekar R. B. H, Babitha K. C, Kiranmai K, Nareshkumar A, Sudhakar C. An efficient callus induction and regeneration protocol for a drought tolerant rice *indica* genotype AC39020. J Plant Sci 2015; 3: 248-254.

Vijayachandra K, Palanichelvam K, Veluthambi K. Rice scutellum induces *Agrobacterium tumefaciens vir* genes and T-strand generation. Plant Mol Biol 1995; 29: 125-133.