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Full length Research paper

Studies on *Agrobacterium* mediated genetic transformation of rice (*Oryza sativa* L.) for drought tolerance using Dreb 1 gene

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Improving crops that are better adapted to abiotic and biotic stresses is gaining importance for sustainable food production in the present era of rapid climate change. The present investigation was carried out to optimize genetic transformation method in rice (*Oryza sativa L*. cv. Lalat) employing *Agrobacterium* strains GV3107 harbouring binary vector pCAMBIA2300 with rd29A::DREB1 gene construct. De-husked mature seeds of rice cv. 'Lalat' treated with Bavistin (1%) for 10 minutes followed by 0.1% $HgCl_2$ + Tween-20 for 6 minutes resulted better asceptic culture with the maximum survival percentage. Incorporation of phytohormone 2, 4-D (2.5 mg/l) in the modified MS medium induced profuse, white, friable, embryogenic calli. The best *in vitro* regeneration from calli was obtained in the modified MS medium supplemented with BAP, NAA and Kinetin. The modified MS medium supplemented with 2.0 mg/l BAP, and 0.2 mg/l NAA was able to induce the maximum multiple shoots and organogenesis. The maximum number of multiple shoots (9.75) was recorded in modified MS medium supplemented with 2.0 mg/l BAP and 0.2 mg/l NAA within 30.75 days of culture. For root induction NAA was found to be the most effective phytohormone and effective rooting was recorded on modified MS medium supplemented with 1.0 mg/l NAA and 0.2 mg/l BAP within 25 to 30 days after culture of shoots on rooting medium. Various factor influencing transformation of rice callus and multiple shoots were optimized such as co-cultivation period (3 days) and bacterial inoculum density (0.6-0.8 at 600 nm), acetosyringone concentration (200 µM) and Kanamycin sensitivity (120 mg/l). The transformation efficiency based on Kanamycin selection was found to be 8.97 % whereas the transformation frequency on the basis of the PCR was found to be 2.56%.

Key Words: *Agrobacterium tumefaciens*, DREB1A, genetic transformation, *Oryza sativa,* abiotic stress.

INTRODUCTION

Rice (*Oryza sativa L.* (2n=24)) is the second most important staple food for more than half of the world's population. In India, rice occupies 21% of the total cropped area. Rice is an anomaly among the domesticated cereals – tropical C3 grass that evolved in semi-aquatic, low-radiation habitat. As such, rice carries an odd portfolio of tolerances and susceptibilities to abiotic and biotic stresses as compared to other crops. The *indica* varieties are widely grown in tropical, subtropical and temperate regions and occupy 80% of the rice cultivation areas in the world (Ayres and Park, 1994) and being the staple food for more than 3 billion people, predominantly in developing countries (Guyer *et al.,* 1998).

Intensification of agriculture with appropriate agro-technologies has taken place in many parts of the world and that led to an increase in global food grain production from approximately 850 million tons in 1960 to 2350 million tons in 2007 (Godfray *et al*., 2010). It has been projected that global food production must increase by 70% by 2050 to meet the demand of growing global population (Varshney *et al*., 2011). Food security is under challenge in most parts of the world owing to competing claims for land, water, labour, energy and capital for industrialization and urbanization, which impose more

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pressure to produce more food per unit of land (Godfray *et al*., 2010). At the same time global climate change is likely to increase the problems day by day and leads to food insecurity, hunger and malnutrition for millions of people in developing countries (Nelson *et al*., 2010; Sivakumar and Ndiangui, 2007). The global temperatures are increasing 2.5–4.3^{0}C by the end of the century, with significant effects on food production (Parry *et al*., 2009, Nelson *et al*., 2010, Lobell, *et al*., 2008) and malnutrition. It has been projected that rising global temperatures between 1981 and 2002 reduced the yields of major cereals by $5 billion per year (Lobell. and Field, 2007). In addition to the challenge of extremes temperature abiotic stress like hot, cold and drought or water stress as well as flooding associated with climatic variability, the incidence and severity of biotic stresses such as pests, diseases, insect and the attack of alien weed species are also expected to be greater. Cropping systems at greatest risk include wheat and rice (*Oryza sativa*) in South and Southeast Asia and maize in Southern Africa (Lobell *et al*., 2008, Hyman *et al*., 2008, Wassmann *et al*., 2009, Varshney *et al*., 2011).

In India, rice is mostly grown in monsoon climates under rainfed conditions that are subject to intermittent submergence (water depths of 0.5 to 1 m that cover the foliage), drought, and in coastal regions, salinity which affect the productivity, quantity and quality of rice. Upon exposure to the stresses, many genes are induced and their products are thought to function as cellular protectants of stress-induced damage (Bray, 2000; Thomashow, 1999; Shinozaki and Yamaguchi-Shinozaki, 1999). Studies on the expression of stress-regulated genes in Arabidopsis (*Arabidopsis thaliana*) have demonstrated the presence of several stress-response path-ways involving genes that act either in an abscisic acid (ABA)-dependent or an ABA-independent manner (Shinozaki and Yamaguchi-Shinozaki, 1997).

The recent advances in plant tissue culture and recombinant DNA technology have made it possible to develop genetic engineered crops tolerant to abiotic and biotic stress. The present investigation aims at the development of an efficient protocol for regeneration of transformed cell to whole plants. The genetic transformations on rice crop cv. ‘Lalat’ were achieved using *Agrobacterium* strain GV3107 containing pCAMBIA2300 binary vector. These vectors contain rd29 promoter and DREB1 gene for drought tolerance. The transcription factors that binding to DRE element are designated as DREBs; that encoded AP2 domain-containing transcription factors and regulate the expression of stress-related genes in an ABA-independent manner (Shinozaki and Yamaguchi-Shinozaki, 1997 & 1999). In the past, several transgenic crops have been engineered using C- repeat binding factors (CBFs) and other TFs without yield penalty (Century *et al*., 2008, Yang *et al*., 2010). Transgenic rice plants over expressing Arabidopsis CBF3/DREB1 or ABF3 TF showed improved tolerance to drought and salinity without growth retardation (Pellegrineschi *et al*., 2004; Ito *et al*., 2006, Oh *et al.*, 2005). However, only few crops such as rice (Oh *et al*., 2009), maize and canola (*Brassica napus*), expressing the desired TF and other genes, have been tested under real field stress conditions (Yang, *et al*., 2010, Varshney *et al*., 2011). Genetically-modified foods have the potential to solve many of the world's hunger and malnutrition problems, and to help protect and preserve the environment by increasing yield and reducing reliance upon chemical pesticides and herbicides. However, we must proceed with caution to avoid causing unintended harm to human health and the environment as result of our enthusiasm for this powerful technology.

MATERIALS AND METHODS

Plant material and Bacterial strain

Rice seeds (*Oryza sativa L*. ssp. *indica* cv. Lalat) were obtained the Breeder Seed Production unit of Orissa University of Agriculture and Technology, Bhubaneswar, Odisha, India and used for *in vitro* culture for *Agrobacterium* mediated genetic transformation for drought tolerance using DREB 1 gene. *Agrobacterium* strain GV3107 containing pCAMBIA2300 binary vector **(**Fig. 1**)** which contains the DREB1 gene and rd29 promoter construct (IARI, New Delhi) was cultured in yeast extract maltose agar (YEMA) medium containing Kanamycin (50 mg/l) and Rifampicin (10 mg/l) and used for genetic transformation of rice calli.

Callus induction

The de-husk mature seeds of rice cv. ‘Lalat’ were initially washed with mild detergent and then treated with Bavistin (1%) for 10 minutes followed by 0.1% $HgCl_2$ + Tween-20 for 6 minutes. The seeds were then properly washed thrice with sterile distilled water to remove all the traces of disinfectants and blot dried on sterilized filter paper. These sterilized seeds were cultured on modified MS (MMS) medium containing (2.0, 2.5, 3.0, and 4.0 mg/l) 2,4-dichlorophenoxyacetic acid (2,4-D), 300 mg/1 casein hydrolysate, 500 mg/l Proline, 30 g/1 sucrose and 0.8% agar, pH 5.7. The explants were maintained in dark at 25±2ºC for 4 weeks. To evaluate the effects of NAA, BAP and Kinetin on shoot regeneration of the calli, three-week-old calli were used as explants. Calli were cut into small pieces (5 mm in diameter) and cultured on modified MS medium (MMS) in combination with (0.2, 0.5, 1.0, 2.0, and 3.0 mg/l) BAP and (0.2, 0.5, 1.0, 2.0, and 3.0 mg/l) Kinetin. The cultures were then maintained under 16 hours photoperiod (light intensity 20 µmole $m^{-2}s^{-1}$). The elongated shoots were transferred to modified MS medium

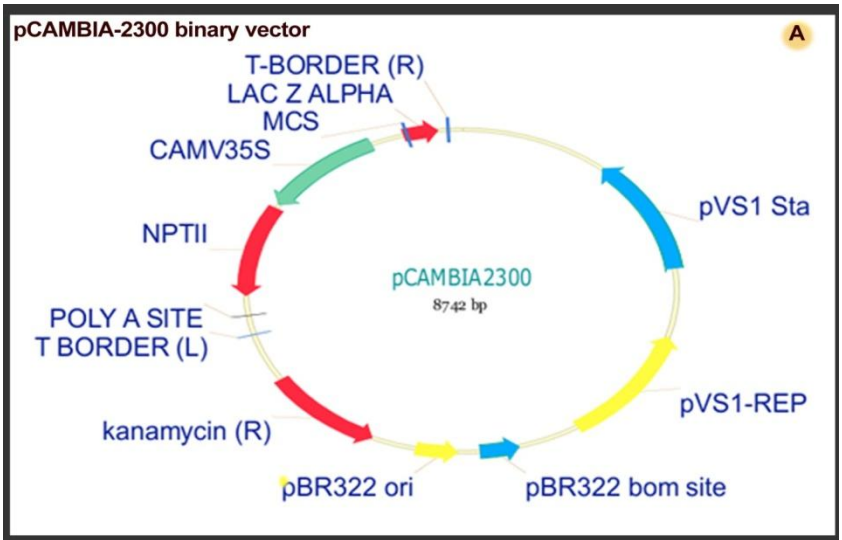

Fig. 1: Schematic map of pCAMBIA-2300 binary vector harboring a marker gene NPT-II and DREB1A gene of interest with rd29A promoter

supplemented with different concentrations of NAA (0.2, 0.5, 1.0 mg/l) for rooting. Number of shoots producing roots and types of roots produced were recorded after 20 days of culture. Rooted shoots were transferred to pots containing soil: sand: FYM (1:1:1) mixture for hardening in the green house.

Effect of antibiotics on plant regeneration

Surface-sterilized seeds of rice cv. 'Lalat' were induced to form calli on modified MS medium containing 2,4-D (2.5mg/l), Casein hydrolysate (300 mg/l), Proline (500 mg/l), sucrose (30 g/1), agar (0.8%) at pH 5.7. Primary calli obtained after 3 weeks of culture were then transferred to the regeneration medium (MMS medium) containing ((0.2 to 3.0) mg/l BAP, (0.2 to 1.0) mg/l NAA, 30 g/1 sucrose and 0.8% (w/v) agar, pH (5.7)) supplemented with antibiotics. To determine the effects of the antibiotics on callus regeneration, Kanamycin, Carbenicillin and Cefotaxime were added to the regeneration medium at concentrations of 0, 50, 100, 150, 200 and 250 mg/1. The effective concentrations of Kanamycin were determined. The antibiotic Kanamycin was used as selectable marker for the selection of transformed plants therefore it is essential to find out sensitivity of antibiotics on the explants. All kinds of antibiotics were added to the regeneration medium after autoclaving. Calli were then cultured at 25±2ºC under 16 hour photoperiod (light intensity 20 µmole $m^{-2}s^{-1}$). The regeneration percentage and number of shoots per regenerating callus were evaluated after 4 weeks of culture.

To determine the lethal dose of Kanamycin during the seed-germination stage, callus induction stage, multiple shooting stage, sterilized seeds and calli of non-transformed rice cv. 'Lalat' were cultured on modified MS medium supplemented with phyto-hormone 2 mg/l BAP, 0.2 mg/l NAA, 0.2 mg/l Kinetin along with 30 g/l sucrose, 0.8% (w/v) agar at pH 5.7**)**. Cultures were maintained at 25±2ºC under 16 hour photoperiod (light intensity 20 µmole $m^{-2}s^{-1}$). After 14-20 days of culture the survival rates of non-transformed rice seeds were determined. The lowest concentration that caused the death of all non-transformed rice plants was chosen for the selection of transformants.

Co-cultivation

Agrobacterium tumefaciens. strain GV3107 carrying pCAMBIA2300 binary vector contained rd29A::DREB1 gene constructs **(**Fig. 1**)** was used for the transformation of rice calli and multiple shoots. Bacterial cultures were grown on environmental shaker at 28ºC for 16-18 hours until OD (at 600nm) = 1.0. The explants were soaked in *Agrobacterium* suspension for 20 minutes on rotary shakers, they were then incubated on liquid plant growth medium (LPGM) for 3 days. After co-cultivation, the calli and multiple shoots were washed thoroughly in sterile

double distilled water containing 600 mg/l Cefotaxime, 200 mg/l Carbenicillin and 80 mg/l Kanamycin. After washing co-cultivated explants were transferred to the modified MS medium supplemented with 600 mg/1 Cefotaxime and 200 mg/1 Carbenicillin and 80 mg/l Kanamycin. The surviving calli and explants were then transferred to the regeneration medium supplemented with the same selective agents. After 45 days of culture, regenerating plants with well developed roots were potted and grown in greenhouse.

Isolation of Plasmid DNA

Plasmid *DNA* containing the gene construct was isolated from *Agrobacterium* by alkali lysis method (Sambrook and Russell, 2001). Single *Agrobacterium* colony was picked up aseptically using sterile inoculation needle and was grown overnight in 10 ml YEM medium containing Kanamycin (50 mg/l), Gentamycin (10 mg/l) and Rifampicin (10 mg/l) in sterile conical flask. Overnight grown culture was transferred to centrifuge tube and centrifuged for 20 min at 10,000 rpm (25^0C). The supernatant was removed and cell pellet was dried to which 200 µl ice cold suspension buffers was added and pellet was dissolved by vortexing. Freshly prepared lysis buffer (200µl) was added, and stored in ice. After 5 min 200 µl of 1.5 M pottassium-acetate was added and mixed well (not by overtaxing) and stored in ice for 10 minutes. Lysate was centrifuged for 20 min at 12,000 rpm. Supernatant was transferred to next tube. Equal volume of phenol-chloroform was added, vortexed and centrifuged for 10 min at 10,000 rpm (4^0C). Aqueous upper layer was transferred to fresh tube. DNA was precipitated by adding 600 µl of isopropanol and kept overnight at -20°C temperature. Suspension was centrifuged at 12,000 rpm for 20 min, (4^0C) and DNA pellet was dried. Pellet was washed with 1 ml of 70 percent ethanol and pellet was dried completely. The pellet was dissolved in 30 µl of TE buffer. Then 2 µl of RNAse was added and incubated at 37°C for 1 hour. Purified sample was stored at -20° C temperature for further use.

Isolation of Plant Genomic DNA

Genomic DNA from putative transformants and control plants was isolated following the method described by Edwards *et al*., 1991. Plant tissue was macerated in pestle and mortar with liquid nitrogen for 2 minutes. The powder was transferred to the micro centrifuge tubes and pre-warm extraction buffer (65^0C) (Tris-HCl (pH 7.5) - 200mM, NaCl - 250mM, EDT - 2.5mM, SDS - 0.5%) was added. The samples were incubated at 60-65^0C for 1 hour in water bath. After 1 hour the samples were cooled at room temperature and 0.6 vol. of Chloroform:Isoamyl alcohol (24:1) was added with intermittent shaking for 5-6 min. The content was centrifuged at 12,000 rpm for 20-25 minutes at room temp. The supernatant was transferred to the fresh micro centrifuge tube by adding equal volume of child Iso-propanol and incubated at room temperature for 2 minutes and again centrifuged at 12,000 rpm for 10 min. Pellet was dried, washed with 70% ethanol and suspended in 100 µl 1X TE buffer to which 2-4 µl RNAse (1 mg/ml) was added and incubated at 37 ºC in water bath for half an hour. DNA was precipitated using 1/10th volume of 3M sodium acetate, ethanol and incubated over night at 4°C. The solution was centrifuged at 12,000 rpm for 2 min and pellet was dried again and was suspended in 50 µl 1X TE.

Molecular Analysis

The detection of transgene was carried out following PCR (AB Systems, Perkin Elmer) analysis using gene specific primer: forward primer 5'ACAGAGGAGTTCGTCGGAGA3', reverse primer 5'GAGTCTCCAAGCCGAGTCAG3'. The PCR conditions were as follows: denaturation at 94ºC for 2 min followed by 35 amplification cycles (94ºC/ 1 min, 58ºC/1min, 72ºC/1 min) and final extension step at 72ºC for 10 min. The PCR products were separated in 1.5 % agarose gel containing 0.8 % Ethidium bromide and visualized in Geldoc system.

RESULTS AND DISCUSSION

Callus induction and regeneration

Agrobacterium mediated genetic transformation of rice and subsequent regeneration are dependent on several factors. Among them, the choice of explants, hormonal composition of the medium used, nutritional supplements and culture condition highly affects the callusing and regeneration (Lin *et al*., 1995; Katiyar *et al*., 1999). Hiei *et al*. (1994) reported that scutellum derived callus was the most amenable explants for *Agrobacterium* mediated genetic transformation. In the present study, mature rice seeds were used to obtain scutellum derived calli and the maximum callus induction frequency (96%) was obtained on modified MS medium containing 2, 4-D (2.5 mg/l) **(Plate 1A-C and Table 1)**. Some reports recommended 2, 4 D (6 mg/l) (Aldemit and Hodges, 1996; Lin and Jhang, 2005) however most reports recommended 2, 4-D (2.5 mg/l) for callus induction which is in accordance with our results too. The similar findings were reported by Saharan *et al.* (2004), Ignacimuthu and Arockiasamy (2006) and Wani *et al.* (2010). They advocated that MS medium containing 2.5mg/l 2, 4-D had produced profuse, loose friable calli. Rachmavati and Anzai (2004) and Carsono and Yoshid (2006) also

Table 1. Effect of media and plant growth regulators(PGRs) on callus induction of rice cultivar 'Lalat.'

Treatments	Plants Growth Regulators		Percent Response of Explants (%)	Mean Days for Callus Induction	Nature of Callus
	Kinetin mg/l	2,4-D mg/l			
T_1	0.5	1.0	79.0(62.73)	36.25	Long shoots, very less compact, yellow mass
T_2	0.5	1.5	84.0(66.42)	35.25	Long shoots, less compact yellow mass produce
T_3	0.0	2.0	88.0(69.73)	33.50	Shoots, friable , white yellow mass produce
T_4	0.5	2.5	92.0(73.57)	30.50	Loose, Friable, and yellow mass produce
T_5	0.0	2.5	96.0(78.46)	28.75	Loose, friable, white mass produce
T_6	0.5	3.0	76.0(60.67)	37.25	Shoots, friable, less white mass produce
T_7	1.0	3.0	74.0(59.34)	38.09	Long Shoots, white yellow mass produce
T_8	0.0	3.0	80.0(63.43)	35.00	Loose, friable, less white mass
T_9	0.5	3.5	64.0(53.13)	39.25	Friable, brown yellow mass
T_{10}	0.5	4.0	50.0(45.00)	41.00	Compact, brown, mass produce
CD(0.05)			01.76	02.41	
CV			05.85	04.419	

*Figures within parenthesis are arcsine transformed values.

Table 2. Indirect organogenesis from callus and effects of plant growth hormones in rice cultivar 'Lalat'.

Treatments	Growth Regulators			Percent of calli inducing shoots	Mean Days for Initiation of Shoots	Average no. of Shoot Produce
	Kinetin mg/l	BAP mg/l	NAA mg/l			
T_1	0.0	1.5	0.5	55.0(47.87)	36.00	05.50
T_2	0.0	2.0	0.5	85.0(67.21)	34.00	08.50
T_3	0.0	2.0	0.2	97.5(80.90)	30.75	09.75
T_4	0.0	3.0	0.5	75.0(60.00)	37.50	07.50
T_5	1.5	0.0	0.5	70.0(56.79)	37.75	07.00
T_6	1.5	0.0	1.0	62.5(52.24)	38.75	06.25
T_7	1.5	0.0	0.2	55.0(47.87)	39.50	05.50
T_8	2.0	0.0	0.5	75.0(60.00)	39.75	07.50
CD(0.05)				0.8755	3.129	1.3

reported the maximum callus induction on MS medium supplemented with 2 mg/l 2, 4-D**.** To study the time required for obtaining embryogenic calli, mature seeds were incubated on the callus induction medium for 14, 25 and 30 days and the calli obtained were then sub-cultured on the same but fresh medium for 4 days. Twenty eight days followed by four days were found to be the best time period for maximum callusing and callus induction frequency was 96.0% **(Table 1)**. The resulting calli were white, yellowish, nodular, compact and highly

Table 3. Effects of plant growth regulators on induction of roots from shoots of rice cultivar 'Lalat'.

Treatment	Plants growth hormones			Avg. no. of shoots producing roots	Percent response	Types of roots
	BAP	NAA	Kinetin			
T1	0.5	0.5	0.5	2.50	25.00(30.00)	Very less no. of shoots
T2	0.2	0.5	0.2	4.75	47.50(43.28)	Less, no of shoots
T3	0.5	0.0	0.0	8.25	82.50(65.27)	Long, multiple ,
T4	0.2	1.0	0.0	9.75	97.50 (80.90)	Long, multiple branching, thick
T5	0.0	0.5	2.0	9.00	90.00(71.57)	Long, single, thin.
T6	0.0	1.0	0.2	7.50	75.00(60.00)	Long , thin
CD (0.05)				1.37		

prolific with 10.24 mm diameter **(Plate 1C)**.

The greatest intricacy one would encounter in rice is recalcitrant monocotyledonous species that exhibits natural resistance to *Agrobacterium* infection and *Agrobacterium* mediated genetic transformation through tissue culture, shoot regeneration and organogenesis from the scutellum derived rice calli. Regeneration or organogenesis is highly dependent on genotype. Therefore, before using the above calli in genetic transformation, it was necessary to standardize or confirms its regeneration ability. The best regeneration frequency (97.50%) was observed with modified MS medium supplemented with 2.0 mg/l BAP and 0.2 mg/l NAA (Table 2 and Plate 1D-F). This medium induces 9.75 number multiple shoots and organogenesis within 30.75 days of culture. The similar findings were reported by Tripathi *et al*. (2010), Ignacimuthu and Raveendar (2011) where they had used 2 mg/l BAP, 0.5 to 1 mg/l NAA, along with basal MS media.. In order to study the regeneration efficiency, 28.75 days old calli were treated under dark and light for two weeks. The calli incubated under light turned to green after 20 days but regeneration frequency was very poor compared to the dark treated ones. The dark incubated calli took four weeks to turn green. Green shoot buds with well-developed leaf like structures were obtained after 30-40 days of culture.

The balance of auxin and cytokinin in the medium plays very important role in the determination of the morphological fate of callus (Christianson and Warnick, 1985). The well develop multiple shoots are transferred to rooting medium (MMS) supplemented with NAA. The highest percentage (97.50%) root regeneration from shoots was recorded in modified MS medium supplemented with 0.2 mg/l BAP and 1.0 mg/l NAA (Table 3 and Plate 1G). Similarly Khan *et al.* (2007) reported that MS basal salts containing 1 mg/l NAA produced better rooting in rice. These results are in accordance with the research findings of Ignacimuthu and Arockiasamy (2006) and Nazim-Ud-Dowl *et al.* (2008) and they had reported that MS basal medium supplemented with 0.5mg/l NAA induced maximum roots. This finding is also in congruent with the findings of Hossain *et al.* (2009). They reported that basal MS medium supplemented with 0.5mg/l NAA produced better rooting in rice.

Genetic transformation of rice calli and screening of transformants

Agrobacterium tumefaciens strain GV 3107 was transformed with pCAMBI 2300 binary vector according to the protocol described by Holsters *et al*. (1978). Transformed colonies were selected on YEM medium supplemented with 50 mg/l Kanamycin and 10 mg/l Rifampicin and then confirmed by colony PCR. The transformed colonies resulting 188 bp fragments from rd29A:DREB1 gene specific primers were selected as positive colonies and they were used for transformation of rice calli. The temperature of incubation, duration of co-cultivation with *Agrobacterium*, concentration and the composition of bacteriostatic agent and duration of selection and concentration of antibiotic selection marker are the other factors reported which affect *Agrobacterium* mediated transformation of rice **(**Pollock *et al*., 1983; Dillen *et al*., 1997; Katiyar *et al*., 1999). To find the optimum conditions for co-cultivation, different concentration of acetosyringone and the duration of co-cultivation were tested. Therefore it is essential to find out the lethal dose of Kanamycin for the selection of transformed plants. The extent of proliferation of embryogenic calli and multiple shoots at different concentration of Kanamycin was evaluated. The effect of various concentrations of Kanamycin and other antibiotics on survivability of explants is represented in Table 4**.** The lethal dose of Kanamycin observed for control

Table 4. Effect of Kanamycin on the control explants of rice cultivar 'Lalat'.

Treatments	Kanamycin concentration mg/l	Explants survival after 20 days	Percent survival
T_1	Control	20.00	100.00(90.00)
T_2	40	16.50	82.50(65.27)
T_3	60	13.75	68.75(56.01)
T_4	80	7.50	37.50(37.76)
T_5	100	2.25	11.25(19.59)
T_6	120	0.00	0.00(0.00)
CD 0.05		1.833	

Table 5. Effect of co-cultivation of period on the survivability of explants.

Sr. No.	Duration of co-cultivation days	No. of explants cultured	Avg. no. of explants alive after co-cultivation	Percent survival (%)	Agrobacterium growth
1	6	20	7.75	35.00(36.27)	+++
2	5	20	12.50	57.32(49.20)	++
3	4	20	16.25	77.85(61.89)	++
4	3	20	18.75	89.00(70.63)	+
CD (0.05)			2.56		
SEM			0.83	3.21	

*co-cultivation medium contains 200µM acetosyringone.

Table 6. Sensitivity of *Agrobacterium tumefaceu*m L. to various levels of Cefotaxime/Carbenicillin on the callus and multiple shoots induction of explants.

Sr. No.	MMS+2.0 mg/l BAP+0.2mg/l NAA +Cefotaxime/ Carbenicillin mg/l	Reappearance of Agrobacterium in explants		Percent reappearance response		Growth of *Agrobacterium*	
		Cefotaxime	Carbenicillin	Cefotaxime	Carbenicillin	Cefotaxime	Carbenicillin
1	0	20.00	20.00	100.00(90.0)	100.00	++++	++++
2	100	18.50	17.50	92.50(74.11)	87.60	++	++
3	200	15.50	17.25	77.50(61.68)	86.25	++	++
4	400	12.25	15.75	61.25(51.50)	78.75	++	++
5	600	8.50	9.50	42.50(40.69)	47.50	+	+
6	600+200+ no sucrose	0.50	0.50	02.50(09.10)	02.50	+	+
7	600+200+80mg/l Kanamycin no sucrose	0.00	0.00	00.00(0.00)	0.00	-	-

20explants were cultured into each replication, under each treatment.
++++: Prominent growth of *Agrobacterium*
++*:* Moderate growth of *Agrobacterium*
+: Slight growth of *Agrobacterium*
-: No growth of *Agrobacterium*

plant was 120 mg/l. The antibiotic Kanamycin at concentration of 100 mg/l was used for primary selection of putative transformed whereas 120 mg/l Kanamycin concentration used for the final selection of putative transformants. In this work, proliferation medium containing 50 mg/l Kanamycin was found to be most suitable for selecting transformed cells. The effect of Kanamycin on the control explants of rice cultivar was present in Table 4. The similar findings were also reported by Khan *et al.* (2007), Hossain *et al.* (2009),

Table 7. *in vitro* Calli and Shoot transformation studies using rd 29A::DREB1A gene for 'Lalat' cultivars.

No of Explants	No of shoots obtained after co-cultivation	No of shoots obtained on kanamycin 20 days after inoculation	No of plantlets transformed to growth chamber	Transformation frequency (%) (based on kanamycin selection	PCR +ve	Transformation frequency (%) Based on molecular analysis
80	78	7	7	8.97%	2	2.56%

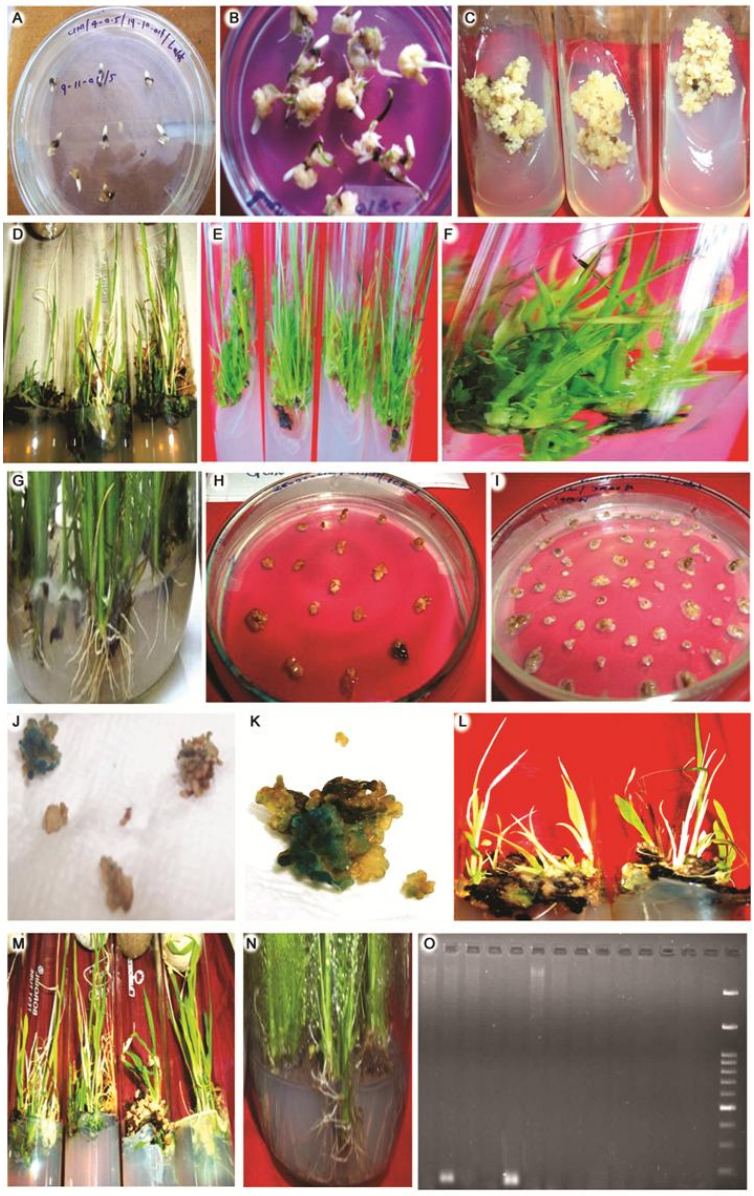

Plate 1. Genetic transformation and regeneration of rice (*Oryza sativa* L.) cv. 'Lalat' using DREB 1 gene

Ratnayake and Hettiarachchi (2010), where they use 50mg/l kanamycin concentration for the selection of putative transformants explants.

Frequency of Kanamycin resistant calli obtained following each variation in co-cultivation conditions was taken as the transformation frequency. The presence of 200 µM acetosyringone in co-cultivation medium and co-cultivation for 3 days were found to be the most suitable

for optimum transformation **(**Table 5**)**. The similar findings were reported by Kumari *et al*. (2007) and they opined that callus induction medium containing 200µM acetosyringone and co-cultivation for 3 days had increased frequency of transformation. Ignacimuthu and Raveendar (2011) and Tran *et al.* (1999) reported that selection medium supplemented with 20 mM acetosyringone enhanced the transformation efficiency**.** Nazim *et al.*, (2008) and Rahman *et al.,* (2011) observed that increase in co-cultivation period had increased the transient GUS expression in co-cultivated explants. Regeneration and analysis of transformants compared to the non-transformed calli regeneration frequency of 97.50%, transformed calli showed very low regeneration frequency (2.56%) (Table 6). Kumar *et al.* (2005) reported transformation efficiency of 4.6% - 5.5% and 6.4% - 7.3% for two recalcitrant elite *Indica* rice cultivars. Formations of embryogenic calli were enhanced with the use of high percentages of gelling agent (phytagel) and maltose as carbon source (Kumar *et al*., 2005).

The selection of transformed cells was carried out in selection medium containing suitable effective antibiotics. Selection medium is important part of genetic transformation. In the present study, different concentrations of antibiotics such as Cefotaxime, Carbenicillin, and Kanamycin were used in modified MS medium for selection of transformants. The concentration varied from 100 mg/l to 600 mg/l. The best results were obtained from from modified MS medium without sucrose supplemented with 600 mg/l Cefotaxime, 200 mg/l Carbenicillin and 80 mg/l Kanamycin (Table 6 and Plate 1H-I). These findings are in correlation with the results of Ratnayake and Hettiarachchi (2010), who had reported the use of N_6B_5 medium supplemented with Hygromycin (50 mg/L), Cefotaxime (500 mg/L) as selection medium. Bacteriostatic agent, Cefotaxime was used to prevent *Agrobacterium* growth after co-cultivation. It has been reported that the use of high concentration of bacteriostatic agents may reduce the regeneration ability of the calli as they structurally resemble auxins. Saharan *et al.* (2004), Ignacimuthu and Arockiasamy (2006), Pipatpanukul *et al.* (2004) also reported that MS medium supplemented by 250 mg/l Cefotaxime, and 50 mg/l Hygromycin concentration was efficient to kill the *Agrobacterium.* However Khan *et al.* (2007) used N_6 medium with 1000 mg/l Cefotaxime to inhibit the growth of *Agrobacterium*.

Further, in combination with other callus inducing hormones such as 2, 4-D may cause loss in regeneration potential (Lin *et al*., (1995); Okkels and Pederson, 1998). The green color areas appeared on the middle of the calluses after 3 weeks and when transferred to regeneration medium later developed into tiny shoots. For the root induction NAA was found to be the most effective phytohormone. *In vitro* regenerated shoots with defined stems were cultured on MMS rooting medium for vigorous root development before transferring them into soil (Plate 1L-N). After 15-20 days, well-developed root system was observed in the plantlets. The plantlets transferred to soil survived under the normal environmental conditions and grew to maturity. As per the gene construct, Kanamycin is employed for selection of transformed cells and explants.

For the molecular analysis, the total genomic *DNA* was isolated from the plantlets germinated in the selection medium (Kanamycin 120 mg/l). Then the PCR reaction was carried for the amplification of the DREB1 gene, and the expected two band of 188 bp was obtained (Plate 1O & Table 7) which shows presence of transgene in the putative transformants obtained from the selection medium (Kanamycin 120 mg/l).

Acclimatization of transformed plants in the green house

After co-cultivation transformed explants was allowed to regenerate on modified MS medium supplemented with different concentration of nutrients, growth promoters, and carbohydrate source. The modified MS medium supplemented 2.0 mg/l BAP, 0.2 mg/l NAA and 0.2 mg/l Kinetin and also selective agent's 120-100 mg/l Kanamycin **(**Table 6 & 10B**)**. The acclimatization of regenerated transformed plants was done in green house under control temperature (28-30 ^{0}C) and humidity (80-90 %). Aldermit and Hodges (1996) also reported that Mannose resistant rice plants were transferred to soil and grown in the green-house at 29 ^{0} C (day), 21 ^{0}C (night) and 80% relative humidity. The *in vitro* plantlets were removed from the culture vessels and then the roots of the plantlets were washed properly to remove growth medium attached with the roots. Then plantlets were kept in the small glass beaker for the establishment of roots, and submerging only the root portion along with fresh water for one or two days. Then plantlets were transferred to the sterile soil in the pot, after one day plants were transferred into the green house for acclimatization. About 80% of plantlets were survived in the greenhouse after one month of transfer. Transformation efficiency based on Kanamycin selection was found to be 8.97 % whereas the transformation frequency on the basis of the PCR was found to be 2.56% **(**Table 7**)**. The present findings will considerably facilitate to open up new avenues for the development of transgenic plants of better quality and resistant to different biotic and abiotic stress.

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