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ORIGINAL ARTICLE

Exploring a Rapid Genetically Stable Direct Regeneration System In Rice (*Oryza sativa* L.)

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ABSTRACT
Majority of indica varieties are recalcitrant to in vitro culture. MS media fortified with 500mg/l casein hydrolysate and 250mg/l adenine disulphate under hormonal supplementation of 0.5mg/l PBZ (paclobutrazol) + 1.5mg/l TDZ (thiodizuron) +1.0mg/l BAP (6-Benzylaminopurine) followed by 2.5mg/lBAP + 0.2mg/l NAA induced higher frequency (71%) of direct multiple shoots. MS with 0.5mg/l PBZ + 1.5mg/l TDZ +1.0mg/l BAP was efficient for induction of profuse multiple shoots within seven days; whereas, 2.5mg/l BAP + 0.2mg/lNAA resulted excellent multiple shoot induction response (82.2%) with profuse rooting. Complete plantlets were hardened and transferred to greenhouse for establishment with survival rate of 85.3%. The plantlets did not reveal any somaclonal variation. Such a rapid in vitro direct regeneration system can be suitably used for genetic transformation in upland indica rice.
***Key words**: Direct regeneration, multiple shoots, callus induction, plant regeneration, rice*

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Abbreviations: MS: Murashige and Skoog; **BAP**: 6-benzyl amino purine; **NAA**: α-naphthalene acetic acid; **TDZ**: thidiazuron, **PBZ**: Paclobutrazol

INTRODUCTION

Rice (*Oryza sativa* L., family- Graminae and subfamily -Oryzoideae) is the staple food for more than half of the world's population. Rice kernels provide 49% of the calories and 39% of the protein in the human diet [1]. To keep pace with the alarming increase in human population, it has to be increased to 50% by 2025 [2]. Productivity of rice has already reached plateau and as such the scope of conventional breeding is limited to offer any major stride to reduce yield gap between potential yield and realized yield. However, innovative breeding approach integrating in vitro culture technique can be a way forward for genetic improvement of yield, quality traits and stress resistance.

In vitro culture has significant role in rapid multiplication of rare plant species and production of transgenic plants. PBZ is a growth retardant and inhibitor of gibberellins. MS media fortified with 40mg/l PBZ increased the production of plantlets and reduced the number of dead leaves and height of shoots in sugarcane [3]. While, TDZ is the best synthetic purine cytokinin for *in vitro* regeneration and *in vivo* multiplication of plant species recalcitrant to propagation. TDZ is much effective in concentrations 10 to 1000 times less than other phytohormones [4]. TDZ at lower concentration (0.01 and 0.1mM) was effective on different varieties of sugarcane and can be used as alternative for BAP and Kinetin [5]. Explants cultured in BAP when transferred to TDZ fortified medium proliferate significantly and induce healthy plantlet regeneration.

Transgenic rice has been developed against several insect pests like the striped stem borer, yellow stem borer, pink stem borer, leaf folder, and green semi-looper. Genetic transformation for resistance to bacterial leaf blight (Xa21 gene), sheath blight (chitinase gene 'Chi11'), yellow mottle virus (ORF-2 gene), drought stress (DREB gene) salt stress (TPSP gene) and enhanced photosynthetic efficiency (PEPC gene in C4 rice) has been successful in rice[6]. Besides, genetically modified biofortified product e.g., golden rice to combat vitamin A deficiency [7] and iron rich indica rice by incorporation of ferritin gene driven

by the endosperm-specific promoter [8] have been reported. But, the pace of progress in indica rice is highly limited due to very low transformation efficiency. Regeneration efficiency is affected by a number of factors including the genotype, the type and physiological status of the explants, composition of the culture medium, plant growth regulators and culture conditions [9]. Within indica sub-species, significant variations of *in vitro* culture response still exist in different genotypes [10]. 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 develop rapid plantlet regeneration directly from embryos without intervening callus stage. In this context, establishment of highly efficient and rapid in vitro regeneration system for indica rice varieties will accelerate the use of genetic transformation in breeding programmes.

MATERIALS AND METHODS

Mature seeds of *Oryza sativa* cv. Khandagiri were dehusked manually and these were immersed in sterile distilled water for 20 mins. The kernels were surface sterilized with 1% bavistin (w/v) for 15 mins followed by washing with sterile distilled water. Further, the kernels were treated with 0.1% $HgCl_2$ solution (containing a drop of Tween 20) for 6 minutes followed by intermittent shaking (x5) for 5 mins with sterile distilled water and blot dried on sterile filter paper under aseptic condition. Then the sterilized seeds were aseptically cultured in MS [11] medium supplemented with Adenosine disulphate (ADS) (0.25g/l), Casein hydrolysate (CH) (0.5g/l), sucrose (30g/l), various concentrations and combination of 6-Benzyl aminopurine (BAP) (1.0-6.0 mg/l), Thidiazuron (TDZ)(1.0-6.0 mg/l), Naphthalene acetic acid (NAA) (0.2 mg/l) and Paclobutrazol (PBZ) (0.5 mg/l). The pH of the media was maintained to 5.7-5.8 using 0.1N NaOH or 0.1N HCl and then 0.8% agar was added to the media. 20ml. of molten media was transferred to 25 x 150 mm glass test tubes and were plugged with non-absorbent cotton. The cultures were sterilized at 121°C and 15 psi for 20 mins. Three kernels were placed on each glass culture tube per treatment (hormone combination) in each replication and the experiment was laid out in CRD with twenty four replicates. The cultures were incubated in culture room at 25+ 1°C under white fluorescent tubes with light intensity of 2000 lux and a 16-hr photoperiod for four weeks. Genomic DNA of direct regenerants was isolated as per Dellaporta *et al*. [12] and genetic fidelity test was performed using RAPD profiling.

RESULTS AND DISCUSSION

Direct morphogenesis from explants without an intervening callus phase results true to the type progenies and thus, minimizes somaclonal variation. In this context, direct regeneration of plantlets may be also rewarding for its use in genetic transformation. A quick, effective and reproducible direct regeneration system can serve the purpose. However, tissue polarity, the orientation of the explant in culture, size of explant, and concentration and growth regulators play a significant role in determining the direct regeneration potential. In the present investigation, an attempt was taken to induce direct regeneration of multiple shoots from dehusked kernels in primary culture. MS media supplemented with BAP and TDZ alone at 1 to 6mg/l as well as their combination and that with PBZ was tried to assess direct shoot regeneration response in cv. Khandagiri (Table 1). TDZ at 4-5mg/l responded better than BAP with moderate shoot growth as also supported by [13, 14]. BAP elicited poor direct shooting at lower concentration and even addition TDZ with 1.0-1.5m/l BAP did not show encouraging result in terms of percentage response, number of micro shoots/ explants and shoot growth. However, addition of 0.5mg/l PBZ with 1.0 -1.5mg/l TDZ + 1.0- 1.5mg/l BAP induced high direct regeneration response(39.2-71%) within seven days with profuse multiple shoots(3.8 - 5.3) and faster shoot growth, and it was maximum (71%, 5.3) in MS + 0.5mg/l PBZ with 1..5 mg/l TDZ + 1.0 mg/l BAP (Fig. 1a, Table 1). However, Raghavendra *et al.* [14] reported higher per cent of multiple shoot induction in 4mg/L TDZ (93.33%) followed by 6 mg/L BAP (91.67%) and 8 mg/L BAP (88.33%). In contrast, the regeneration of rice plants through callusing usually would require about 14 weeks. 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 [15]. Mahajan *et al*. [16] reported maximum multiple shoot induction frequency in MS media supplemented with BAP (0.5mg/l) and Kinetin (0.5mg/l). In contrast, TDZ responded most efficiently even in low concentration than BAP for rapid plantlet regeneration and multiple shoot formation [14]. Puhan *et al*. [17] reported best direct multiplication of shoots in rice variety Basmati 370 and EIC-005 (Pusa Basmati) in MS basal media and MS medium supplemented with 0.5mg/l BAP showed. Differential response of above hormones might be due to endogenous level of hormone and physical growth factor in addition to temperature and photoperiod [18].

Table 1. Response of different phytohormones for direct regeneration in rice.

Phyto-hormone	Concentration (mg./l)	% Response	No. of microshoots /explants	Shoot growth
BAP	1.0	0.3	0.2	+
	2.0	0.5	0.3	+
	3.0	3.1	1.2	+
	4.0	10.2	2.0	++
	5.0	15.2	2.2	++
	6.0	28.6	3.2	+++
TDZ	1.0	2.5	1.2	+
	2.0	8.8	2.6	++
	3.0	11.9	3.2	+++
	4.0	18.2	4.5	++++
	5.0	16.5	4.0	++++
	6.0	10.3	2.9	++
BAP + TDZ	1.0 + 1.0	2.7	1.1	+
	1.0 + 1.5	3.1	1.5	+
	1.5 +1.0	3.2	2.2	++
	1.5 + 1.5	3.4	2.9	++
BAP + TDZ + PBZ	1.0 + 1.5 + 0.5	71.0	5.3	+++++
	1.5 + 1.0+ 0.5	39.2	3.8	++++
	1.5 + 1.5 + 0.5	42.3	4.7	++++

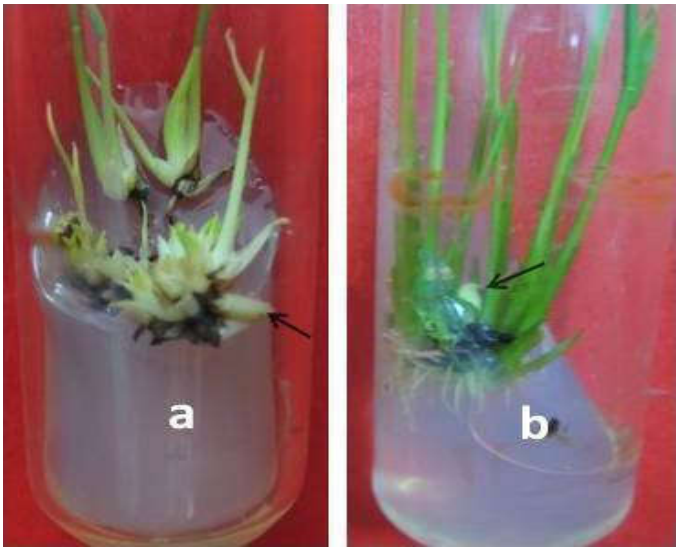

Fig 1. Direct plantlet regeneration without callusing in primary culture of rice cv. Khandagiri. a- Direct multiple shoot formation in MS + 0.5mg/l PBZ + 1.5mg/l TDZ + 1.0mg/l BAP , b- Direct multiple shoot regeneration with profuse rooting in MS + 2.5mg/l BAP + 0.2mg/l NAA. Arrow: rice kernel used as explants in primary culture.

Simultaneous direct shoot formation with rooting in primary culture seems to offer added advantage for rapid multiplication of plantlets. Therefore, lower concentration of NAA (0.2m/l) with 2.0-3.0mg BAP/TDZ was tried to induce rooting along with multiple shoot formation in primary culture (Table 2). MS supplemented with 2.5mg/l BAP + 0.2mg/l NAA resulted excellent direct shoot regeneration response (82.2%) with healthy profuse rooting (12.2) (Fig. 1b) and those were readily transferred to soil within 5 weeks of culture. In contrast, regeneration efficiency via callusing has been reported to be about 12-14.9% only in indica rice [19, 20]. In the present investigation, TDZ with NAA revealed feeble response to shoot and root formation, while, BAP at 2.5mg/l with lower concentration of TDZ (0.5mg/l) + 0.2mg/l NAA elicited satisfactory multiple shoot regeneration response with moderate root growth. Healthy plantlets were initially hardened in sterile distilled water for three days in the culture room and then transferred to polystyrol pots (containing sterilized FYM, red soil, vermiculite in 1:2:1 ratio) in greenhouse for establishment which revealed survival rate of 85.3%.

Table 2. Direct shoot regeneration with rooting in rice cv. Khandagiri.

Phyto-hormone	Concentration (mg./l)	% Response for shooting	No. of roots /explants	Root growth
BAP + NAA	2.0 + 0.2	69.1	7.5	+++
	2.5 + 0.2	82.2	12.2	+++++
	3.0 + 0.2	71.4	8.4	+++
TDZ + NAA	2.0 + 0.2	3.4	0.7	+
	2.5 + 0.2	5.6	1.2	+
	3.0 + 0.2	4.8	0.8	+
BAP + TDZ + NAA	2.5 + 0.5 + 0.2	85.5	9.5	+++
BAP+TDZ+PBZ+NAA	2.5+0.5+0.5+0.2	70.2	3.8	++

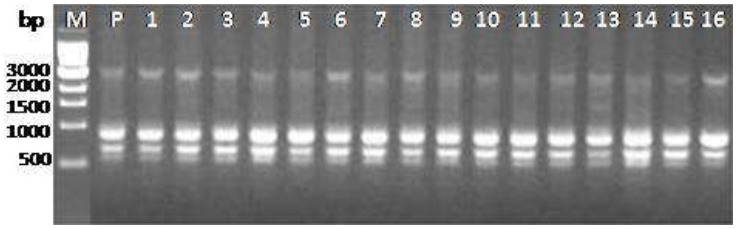

Fig 2. RAPD profile of the parent variety 'Khandagiri' (Lane: P) and in vitro direct regenerated plantlets (Lane:1-16) using primer OU 3.

In the present investigation, the PCR amplification was carried out by five selected RAPD primers. Genetic fidelity test showed absence of any variation in the banding pattern which clearly indicates genetic stability among direct regenerants (Fig. 2). Such regeneration system may be suitably used for genetic transformation in rice.

CONCLUSION

Efficient and reproducible regeneration system is a pre-requisite for stable genetic transformation. A large number of multiple shoots were regenerated in vitro from embryo in a short time without tissue de-differentiation via callus formation and the plantlets were transferred to soil within 5 weeks of culture. Such *in vitro* derived plants maintained genetic stability without any somaclonal variation and hence, amenable for Agrobacterium-mediated genetic transformation in rice.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

AUTHORS CONTRIBUTION

Swapan K. Tripathy designed, conducted the experiment and prepared the manuscript with the help of M. Maharana

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