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GENOTYPIC RESPONSE TO MATURED EMBRYO-DERIVED CALLUS INDUCTION AND PLANT REGENERATION IN HIGH YIELDING UPLAND RICE (O*ryza sativa* L.)

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ABSTRACT

An efficient and reproducible protocol is required to achieve high frequency transformation from transformed calli. In this paper, high frequency plant regeneration from mature embryo-derived embryogenic calli of recalcitrant indica rice cultivars ('Khandagiri', 'Sahbhagidhan' and 'Mandakini') is reported. Maximum frequency of embryogenic and nodular callus (73.5%) followed by plant regeneration (88.5%) was obtained in cv. 'Khandairi'. Further, 'Sahbhagidhan' (82.3%) and 'N22 Sel' (75.3%) showed higher organogenic plantlet regeneration than 'Mandakini' (70.8%), but reverse was the case for somatic embryogenic plant regeneration. Rice cv. 'Annada' and 'Vandana' exhibited poor organogenic and somatic embryogenic callusing and plantlet regeneration response. Therefore, organogenic and somatic embryogenic plant regeneration have their own genetic basis and these two phenomena might be under separate genetic control in various genotypes. The present high throughput plant regeneration system can be amenable for genetic improvement through *in vitro* mutagenesis and *Agrobacterium*-mediated genetic transformation in upland rice.

Key words: Callus induction, genotypic response, *Oryza sativa*, plant regeneration, mature embryo.

INTRODUCTION

Rice (*Oryza sativa* L., 2n = 24, family *Poaceae*) is one of the world's most important staple cereal food crop. It feeds over half of the global population. India is the 2nd populous country in the world which out of the present global population of 7.3 billion houses more than 1.37 billion people (*www.worldpopulationreview.com*). The current status of food production in India is 283.37 million tonnes (2018-2019) and it is likely to increase by 25.3% (355 million tonnes) to feed estimated 1.5 billion people by 2030 (*http://www.consultancy.in*). This is indeed a challenging task. About 8% of the global rice-growing area is in uplands (Saito *et al.,* 2018) and nearly two-third of lies in Asia alone. India has vast stretches of marginal uplands which mostly remain fallow even during rainy season. Average grain yield of upland rice is as low as 2.1 t ha^{-1} in Sub-Saharan Africa and around 2.65 t ha^{-1} in India (Tsujimoto *et al.,* 2019; Jaganmohan, 2020). The cultivation of upland rice can reduce the demand for irrigation water by 50-70% as compared to the lowland and irrigated rice. Therefore, there is a need to reorient breeding strategy to increase food production. Genetic improvement of rice for drought tolerance through conventional breeding is slow owing to the limited genetic variation, low heritability of seed yield and uncertainty in drought timing, intensity and duration. Rice cultivars 'Mandakini', 'Sahbhagidhan', 'N22 selection' (a pure line sel. of N22),

'Annada' and 'Vandana' are known to moderately thrive under moisture stress, but seed yield is not satisfactory. 'Khandagiri', an improved upland rice variety, though retains high yield potential is extremely sensitive to drought stress. In this context, *in vitro* culture can play a significant role to develop climate resilient drought tolerant rice cultivars for upland situation through genetic transformation and *in vitro* mutagenesis.

An efficient regeneration system is a prerequisite for successful genetic transformation and creation of productive genetic variants through *in vitro* mutagenesis. Many researchers have attempted *in vitro* genetic manipulation of rice for enhancing the productivity, nutritive value and tolerance to biotic and abiotic stresses using mature dehusked seed culture (Hiei and Komari, 2008; Sahoo *et al*., 2011; Azizi *et al*., 2015; Abiri *et al*., 2017). However, its application is limited by plant genotype and hormonal supplementation in the medium (Yaqoob *et al*., 2021) for sustained growth of calli, subsequent plant regeneration and survival as fertile plants. It is often difficult to induce embryogenic calli and regenerate plants from these callus cultures especially those belonging to Indica subspecies (Tripathy *et al.*, 2018). Besides, within the Indica group, there are significant variations in *in vitro* culture responses among different genotypes (Khanna and Raina, 1998). Azizi *et al.* (2015) showed poor callus induction from seed, proliferation and regeneration frequency in two Malaysian cultivars, namely MARDI's quality rice 74 (MRQ74) and MRQ50. Thus, recalcitrant nature of Indica subspecies seems to be a major limiting factor in transfer of valuable genes (Toenniessen, 1991). To achieve this, selection of genotype with efficient callus induction and plant regeneration ability is absolutely necessary. Therefore, the present investigation aims at the identification of suitable high yielding cultivar(s) for efficient embryogenic callus formation and subsequent rapid plant regeneration *in vitro* amenable for follow-up step for genetic improvement through *in vitro* mutagenesis and *Agrobacterium*-mediated genetic transformation (Hoque and Mansfield, 2004).

MATERIALS AND METHODS

The present study was carried out in the Department of Agricultural Biotechnology, College of Agriculture, OUAT, Bhubaneswar (India) during the year 2017. Mature healthy dehulled kernels of rice cv. 'Mandakini', 'Khandagiri', 'Sahbhagidhan', 'N22 Sel'., 'Annada' and 'Vandana' (received from EB I Section, Department of Plant Breeding & Genetics, OUAT, Bhubaneswar) were washed with 2% carbendazim (w/v) for 30 min. and surface sterilized with 70% ethanol for 2 min followed by washing (2x) with sterilized distilled water with a drop of Tween 20 with continuous shaking for 10 min. The seeds were then treated with 0.1% (w/v) $HgCl_2$ solution for 6 min, followed by rinsing (5x) with sterile distilled water and blot-dried on sterilized filter paper before inoculation on culture medium. Sterilized seeds were aseptically cultured in modified CIM medium (MS basal with 3% sucrose, 0.3% agar + 0.2% phytagel, 500 mg L^{-1} casein hydrolysate, 150 mg L^{-1} proline and 2.5 mg L^{-1} 2,4-dichlorophenoxyacetic acid + 0.5mg L^{-1} kinetin) for callus induction (Tripathy, 2021). Calli induced from the respective genotypes were transferred to R medium (MS basal with 2.5% sucrose, 0.3% agar + 0.2% phytagel, 500 mg L^{-1} casein hydrolysate, 500 mg L^{-1} adenine sulphate: ADS, 150 mg L^{-1} proline, and 2.0 mg L^{-1} 6-benzyl amino purine + 0.5 mg L^{-1} naphthalene acetic acid) to study genotypic plant regeneration response (Tripathy, 2021). The pH of above media was adjusted to 5.7. The 20 mL. molten medium was transferred to each glass culture tube (25 x 150 mm) and plugged with non-absorbent cotton before autoclaving at 121°C and 1.05 kg cm^{-2} for 15 min. The culture tubes were incubated in culture room at 25$\pm$1°C in dark till 4 weeks for callus induction and follow-up growth. The calli of each genotype were incubated in culture room 4 weeks for plant regeneration at

25±1°C under white fluorescent tube light intensity of 2000 lux and 16-hr photoperiod. The *in vitro* grown plantlets were then transferred to pot mixture (peatmoss: perlite, 2:1) in a green house.

The experiment was laid out in complete randomized design with 24 replicates for callus induction and plantlet regeneration. Observations were recorded for callus induction frequency (CIF %), relative growth of callus (score: + for poor growth to +++++ for excellent growth), extent of necrosis (% necrotic calli), embryogenic callusing response (% embryogenic calli induced), morphogenetic potential (% calli formed plantlets) and plant establishment. Data sets were analyzed statistically using Duncan's multiple range test (Duncan, 1955).

RESULTS AND DISCUSSION

Genotypic response for callus induction

The key factors for enhancing successful regeneration are genotypes, tissue source of explants, combination and concentration of growth regulators, and culture condition. In present study, 1500 dehusked kernels of each of the six rice genotypes were cultured in callus induction medium (CIM) containing 2.5 mg L^{-1} 2,4-D + 0.5 mg L^{-1} Kn and other media recipes used which was reported to induce fast growing creamy, embryogenic and nodular calli with minimum necrosis in upland rice (Tripathy *et al.*, 2018). Abiri *et al.* (2017) reported reproducible protocol of Malaysian rice cv. 'MR219' through somatic embryogenesis using 2 mg L^{-1} 2,4-D, 2 mg L^{-1} kinetin, 50 mg L^{-1} L-proline, 100 mg L^{-1} casein hydrolysate and 30 mg L^{-1} potassium metasilicate. The genotypes showed varied responses to callus induction and quality of calli formed (Table 1). In cv. 'Khandagiri' out of 1500 seeds plated, 1347seeds (89.8%) formed calli within 9 days of primary culture. Of the calli formed, 73.5% (990 seeds) showed the formation of globular somatic embryos on calli surface. In case of cv. 'Mandakini', out of 1500 seeds plated 1209 seeds (80.6%) formed calli and of the calli formed, 542 (44.8%) showed globular embryogenic regions. As the calli grew about 2 weeks old, these usually developed necrosis sooner or later depending on the source genotype. Calli from cv. 'Annada' and 'Vandana' were unable to maintain growth and eventually became necrotic; whereas the calli from cv. 'Khandagiri' and 'N22 Sel' were actively dividing as shown by the extent of callus growth. Response to callus induction was excellent (89.8%) in cv. 'Khandagiri' and it was fairly high (>80%) in cv. 'N22 Sel', 'Sahbhagidhan' and 'Mandakini'. The rice cv. 'Annada' and 'Vandana' gave moderate callusing response (72-74%). Rice cv. 'Khandagiri' induced maximum somatic embryogenic response and such calli continued to produce somatic embryos even with repeated sub-

Table 1: Genotypic response of various rice genotypes for callus induction in modified CIM medium**

Rice genotypes	Days first callus observed	Callus induction frequency* (%)	Growth of callus***	No. of necrotic calli (%)	Frequency of somatic embryogenic calli (%)
Mandakini	10.8	80.6 ± 0.73 [c]	+++	5.0	44.8[b]
Khandagiri	9.0	89.8 ± 0.98 [a]	+++++	2.8	73.5[a]
Sahbhagi Dhan	10.0	84.7 ± 1.00 [b]	++++	0.0	34.7[c]
N22 Sel.	8.6	85.6 ± 0.72 [b]	+++++	0.0	36.0[c]
Annada	15.2	74.4 ± 0.71 [d]	++	20.0	14.0[e]
Vandana	12.0	72.0 ± 0.55 [d]	+++	10.5	26.4[d]

*The values are mean ± S.E; Means followed by the same letter within columns were considered not significantly different at $p \leq 0.05$. **CIM medium: MS basal with 3% sucrose, 0.3% agar + 0.2% phytagel, 500 mg L^{-1} casein hydrolysate, 150 mg L^{-1} proline and 2.5 mg L^{-1} 2,4-D + 0.5 mg L^{-1} Kn.

***Growth of calli: poor (+), low (++), moderate (+++), good (++++) and excellent (+++++).

culture in the same medium. In contrast, cv. 'Annada' showed poor response (14.0%). Varying response to callus induction has been reported in Malaysian wetland rice varieties 'MR220', 'MR220-CL2', 'MR232' and 'Bario' to the tune of 76, 94, 85 and 42%, respectively, using MS + 3 mg L^{-1} 2,4-D and 30 g L^{-1} maltose (Mustafiz and Wagiran, 2018).

Puhan and Siddiq (2013) tested *in vitro* culturability of 12 rice accessions representing Indica, Japonica, aromatic and wild groups in MS + 0.5 mg L^{-1} BAP, of which cv. 'Basmati 370' gave better response to callusing. Evangelista *et al.* (2009) stressed the importance of callusing ability, structure of the callus, and regeneration capability as basis for determining suitability of *in vitro* culture. The genotype 'LX 278' was the most amenable to tissue culture, forming 78.8% of embryogenic calli; while cv. 'PSB Rc 28' and 'PSB Rc 58' were unable to sustain growth and often differentiated roots (55.3%) and became necrotic (76.9%). Besides, Roy *et al.* (2012) reported differential response of genotypes in MS medium supplemented with 2 mg L^{-1} 2,4-D for inducing high amount of embryogenic calli.

Genotypic response for plant regeneration

Genotypic differences in callus induction, subsequent callus growth and plant regeneration in rice have not been fully understood. Several laboratories have enunciated regeneration of plants from various rice explants (Chen *et al.*, 1985, Datta *et al.*, 1990; Islam *et al.*, 2005), However, the quality of callus, embryogenic response and regeneration potential are said to be influenced by the genotype.

In present study, shoot buds appeared on callus within 2 weeks of culture in R medium. The percentage of calli regenerating plantlets and their survivability varied with cultivar. About 82-85% of organogenic calli of cv. 'Khandagiri' and 'Sahbhagi Dhan' transferred to R medium resulted in efficient plant regeneration (Table 2). These genotypes also showed significantly high number of shoots per responsive calli (10.6-12.8) indicating good regenerability through organogenic plant regeneration. Rice cv. 'Mandakini' and 'N22 Sel' had moderate organogenic response. Plants regenerated from the above cultivars, except 'N22 Sel', resulted in >80% survival on transfer to pot mixture in greenhouse. Roy *et al.* (2012) reported differential responses to indirect organogenesis in rice cv. 'Khitish', 'IR-64', 'IR-36', 'IR-72', 'PNR-546', 'Swarna', 'Taraori' 'Basmati' and 'Gobindabhog'.

The ability to form embryogenic calli in callus culture is an important consideration in genetic transformation studies as each somatic embryo induced is a single cell event and has capacity to regenerate into complete plantlet (Gandonou *et al.*, 2005b). In present study, the erstwhile mentioned 73.5% embryogenic callus formed by 'Khandagiri' (Table 1) indicates comparatively higher chance of regenerating plants than other genotypes. Indeed, on verification, the embryogenic calli of cv. 'Khandagiri', when placed in modified regeneration R medium, formed complete plantlets with

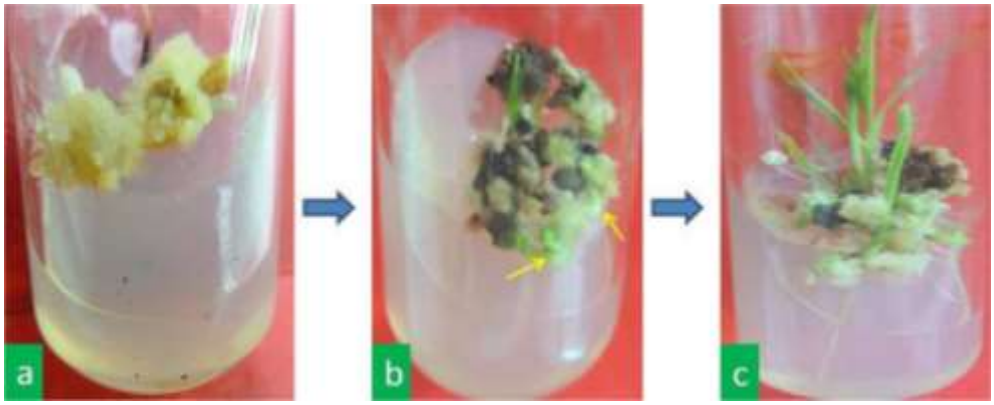

Fig. 1: Organogenic primary callus culture (a), shoot bud formation (b) and plant regeneration (c) in rice cv. Khandagiri

Table 2: Genotypic response for plantlet regeneration in R medium**

Rice genotypes	Organogenic response			Somatic embryogenic response		
	Per cent Response*	No. of shoots/ responsive callus	Surviv-ability (%)	Per cent Response*	No. of shoots/ responsive callus	Surviv-ability (%)
Mandakini	70.8 ± 0.72[b]	5.6	86.1	76.8 ± 1.01[b]	4.6	83.0
Khandagiri	85.6 ± 1.01[a]	12.8	88.0	88.5 ± 0.17[a]	5.8	70.0
Sahbhagi Dhan	82.3 ± 1.07[a]	10.6	82.3	70.2 ± 1.05[b]	3.5	80.1
N22 Sel.	75.3 ± 0.72[b]	7.8	75.2	62.5 ± 0.76[c]	3.8	65.0
Annada	44.0 ± 0.35[d]	4.3	52.8	20.6 ± 0.43[e]	1.1	30.8
Vandana	54.8 ± 0.61[c]	5.2	63.0	32.2 ± 0.18[d]	1.5	38.4

Means followed by the same letter within columns were considered not significantly different at $P \leq 0.05$.
*Values are mean + S.E.; ** MS basal with 2.5% sucrose, 0.3% agar + 0.2% phytagel, 500 mg L^{-1} casein hydrolysate, 500 mg L^{-1} ADS, 150 mg L^{-1} proline, and 2.0 mg L^{-1} BAP + 0.5 mg L^{-1} NAA).

highest frequency (88.5± 0.17) as well as higher number of shoots callus^{-1} (5.8). However, it mostly resulted in the formation of numerous albino plantlets. Azria and Bhall (2000) revealed somatic embryogenic plant regeneration of 4 Australian rice varieties but these varied in the number of shoots produced per callus. We were able to mitigate albinism using 500 mg L^{-1}ADS doze as suggested) in R medium which produced profuse green plantlets with sporadic albino micro-tillers. Rice cv. 'Mandakini', 'Sahbhagidhan' and 'N22 Sel' resulted in moderate regenerability (62-76%) from somatic embryogenic origin. Among all genotypes, cv. 'Annada' showed poor somatic embryogenic response (20.6 ± 0.43%). Varying response to plantlet regeneration (40-82%) has also been reported

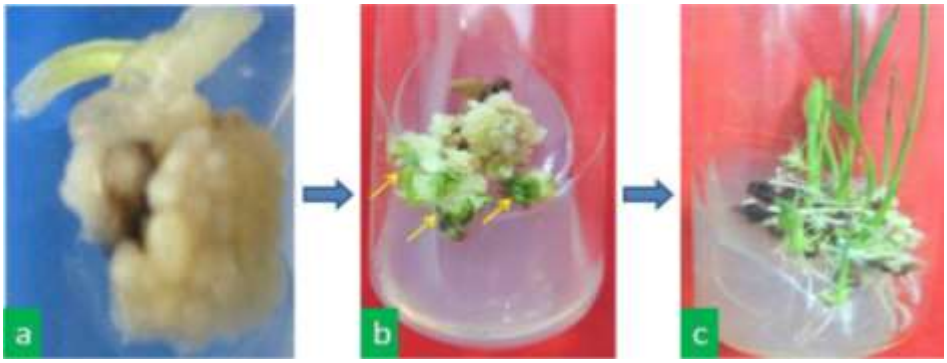

Fig. 2: Somatic embryogenic primary callus culture(a), somatic embryo germination(b) and complete plant regeneration with well-developed profuse rooting(c) in cv. 'Khandagiri'

Fig 3: Rice cv. 'Khandagiri' plant establishment in pot mixture

in four Malaysian rice varieties ('MR220', 'MR220-CL2', 'MR232' and 'Bario') using MS medium supplemented with a combination of 2 mg L^{-1} BAP (6-benzylaminopurine), 2 mg L^{-1} Kn (Kinetin) and 0.5 mg L^{-1} NAA (Mustafiz and Wagiran, 2018). Besides, polyamines reportedly play vital role on somatic embryogenesis and cv. 'ASD16' exhibited high regeneration potential than cv. 'ADT43' and 'IR 64' (Sundararajan *et al.*, 2020). Saharan *et al.* (2004) achieved a maximum of 63% shoot regeneration frequency in rice cv. 'HKP 46'. Similarly, Tariq *et al.* (2008) reported 4-8 plantlets callus^{-1} with regeneration frequencies of 61-69% in 'Basmati' varieties. However, Verma *et al.* (2011) obtained reproducible and highest somatic embryogenic response with 0.4 µM 2,4-D + 2.0 µM Kn in rice cv. 'Govind' (69.03%), and in cv. 'Pusa Basmati-1' (67.77%) with 0.4 µM 2,4-D + 0.4 µM Kn and on hormone-free MS medium in cv. 'Jaya' (65.99%).

In present study, cv. 'Sahbhagidhan' (82.3 ± 1.07%) and 'N22 Sel' (75.3± 0.72%) showed higher organogenic plantlet regeneration than cv. 'Mandakini' (70.8 ± 0.72%), but reverse was the case for somatic embryogenic plant regeneration (Table 2). This indicates that organogenic and somatic embryogenic plant regeneration have their own genetic basis and these two phenomena might be under separate genetic control in some genotypes. Half strength modified MS medium was shown to initiate healthy rooting with few laterals. However, MS with NAA @ 1.0 mg L^{-1} with 0.2 mg L^{-1} BAP resulted in better rhizogenic response (84.6 ± 1.05%) and the excised shoots developed profuse normal rooting within a week's period.

The plantlets when transferred to pot mixture (peat moss: perlite, 2:1), successfully acclimatized in greenhouse under partial shade (Fig. 3). The plants regenerated from first few callus cultures were phenotypically normal and fertile. While, those derived from long term callus cultures revealed genetic variation in one or more characters. Thus, the *in vitro* protocol formulated in this study may be suitably used for *Agrobacterium*-mediated genetic transformation and *in vitro* mutagenesis in upland rice.

Conclusion: A number of *in vitro* culture approaches are now available for genetic manipulation in rice. However, each technique requires a highly responsive base material (genotype) and optimized protocol including artificial media composition, hormonal supplementation and culture conditions for efficient callus induction and reproducible rapid plant regeneration. The high throughput somatic embryogenic plant regeneration system developed in rice cv. 'Khandagiri' may be amenable for *Agrobacterium*-mediated genetic transformation and *in vitro* mutagenesis. Further, the genotype-dependent somatic and organogenic plantlet regeneration in the present set of rice varieties elucidated separate inherent genetic control for such different modes of morphogenic potential.

Conflict of interest: It is declared that there is no conflict of interest.

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