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Evaluation and Validation of Rice (*Oryza sativa* L.) Genotypes for Tissue Culture Response from Mature Embryos

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A B S T R A C T

The biotechnological intervention to enhance crop productivity via genetic transformation mainly depends upon an efficient regeneration protocol. Japonica, Indica and Basmati varieties of rice vary highly in their regeneration capabilities. Establishment of a highly efficient regeneration system for mature embryos in rice will enable transformation of foreign genes in rice. In this study, 12 genotypes of rice representing japonica, indica and basmati varieties were assessed for their calli and green plant regeneration frequencies. The mature seeds of the genotypes were inoculated on MS media supplemented with auxin (2,4-D @ 2.5 mg/l), cytokinin (kinetin@ 0.5mg/l) and 3% sucrose. They exhibited variable calli induction and green plant regeneration frequencies. Calli regeneration frequency ranged from 100% - 20%, highest being in GS-88, Kamad and PS-3 (100%) and lowest in Kohsar (20%). Highest regeneration frequency was obtained in K-332 and Kamad. Among all the varieties K-332, GS-88 and Kamad performed well in respect of callus induction and green plant regeneration. The plants were successfully acclimatized and transferred to pots.

Introduction

"Rice (*Oryza sativa* L.) is a source of food to over three and a half-billion people and in Asia alone, around two billion people obtain more than 50% of their dietary calories from rice and rice products (*Caicedo et al.*, 2007)". Globally the crop is grown over 167.24 mha area with the production of 769.65 million tonnes (http://www.fao.org/faostat/, 2017). Genetic and molecular make-up of rice is under active investigation and it is considered as a model monocot system due to its relatively small genome size of 389 Mb, ease

of transformation, well known genetics (Sahi *et al.,* 2006), availability of a dense physical map and molecular markers (Wu *et al.,* 2002), full-genome transcription profiling using high density oligonucleotide tiling microarrays (Li *et al.,* 2006) together with its complete genome sequence (Sasaki *et al.,* 2005).

Since the landmark breakthrough of sequencing of rice genome, considerable progress has been achieved in the area of genetic engineering, functional genomics and annotation of genes of agronomic importance (Shimamoto and Kyozuka, 2002; Bajaj and Mohanty, 2005; Ge *et al.,* 2006). Validation and standardization of *in vitro* tissue culture technique is a prerequisite for working on such advance areas pertaining to plant transformation (Jan *et al.,* 2001) and functional genomics.

Diverse rice germplasm of temperate origin is being maintained at MRCFC, Khudwani, SKUAST-Kashmir. Mostly the germplasm has been characterized for cold tolerance, earliness, agronomic traits and for resistance to biotic stresses (Najeeb *et al.,* 2015).

Some of the important rice accessions were selected for studying their response and amenability to tissue culture.

Tissue culture response is usually characterized by high genotype to protocol interaction (Ge *et al.,* 2006) and is affected by the number of factors such as composition of basal media, explant, culture conditions, combinations of plant growth regulators (PGRs) and the genotype (Azria and Bhalla, 2000). Therefore, it becomes necessary to standardize a procedure on a set of genotypes with respect to standard media conditions and procedures. The objective of our study was to evaluate the genotypic variation in callus formation and regeneration capacity of rice genotypes under tissue culture.

Materials and Methods

Plant material

Pure seeds of 12 experimental genotypes procured from MRCFC, Khudwani, SKUAST- Kashmir were used as source of mature embryos for callus induction and plant regeneration (Table 1). The study was conducted at Plant Tissue Culture Lab, Faculty of Horticulture, SKUAST- Kashmir, J&K, India during the years 2018 and 2019.

Seed sterilization

Mature healthy seeds of all the genotypes were carefully dehusked with a sharp scalpel, disinfected through washing with water using few drops of Tween 20 for 5 minutes.

Seeds were surface sterilized with mercuric chloride (0.1% w/v) for 10 mins followed by 5 washes with autoclaved distilled water under the laminar flow hood.

Callus induction media

Murashige and Skoog (1962) (MS) media was used for callus induction. In this study, MS media supplemented with 2,4-D @ 2.5 mg/l and kinetin@ 0.5mg/l which proved as a best combination in an earlier study conducted by Wani *et al.,* (2011) was used. Sucrose (3%) was used as a sole carbon source. pH of media was adjusted at 5.8 by adding 1.0 N HCl with the help of pH meter. The media was solidified with 0.8% agar and autoclaved at 121°C for 15 minutes at 15 psi. Media was poured in the petriplates under laminar air flow. Sterilized seeds were placed on the callus induction media and the petriplates were incubated in culture room with 16 hours of light and 8 hours of dark cycle at 25 ± 1^{o}C. Incubation was continued till emergence and growth of calli for a period of 2-3 weeks in culture room.

Regeneration and hardening media

Regeneration media contained full strength MS, 3% sucrose and growth hormones BAP (2 mg/l) and NAA (0.5 mg/l) and Kinetin (0.5 mg/l). pH of the media was adjusted to 5.7 and solidified with 8g/l agar. Media was autoclaved for 20 min at 121°C.Calli induced from seeds were transferred to regeneration media. After calli regeneration plantlets were transferred to plastic cups containing autoclaved pre-soaked vermiculite and perlite medium, in the ratio of 1:1 inside the growth chamber.

80% RH and 28°C temperature was maintained inside the growth chamber. After hardening for 15 days in growth chamber, plants were transferred to the soil in the mud pots and were kept in green house and maintained at 30°C with 70%relative humidity for the period of 30 days and later transferred to field in open air conditions.

Data analysis and statistics

The experiment was laid in Complete Randomized Design (Gomez and Gomez, 1984).Each of the 12 genotypes was represented by 14 seeds per petri plate and such two plates per genotype (corresponding to two replications) comprised of one experimental unit.

Observations were recorded on callus induction and green plant regeneration at appropriate time. The Callus induction frequency (CIF) was obtained from the number of calli against the total number of explants inoculated. Similarly, the Green plantlet differentiation frequency (GPDF) was calculated on the number of green plantlet differentiation as percentage of the number of transferred calli. Analysis of variance was performed using NCSS Statistical Software (2019).

Results and Discussion

Callus initiation

Sterilized dehusked seeds of 12 genotypes were inoculated on MS media supplemented with growth regulators, 2,4-D and Kinetin to estimate the differential response of genotypes towards tissue culture. After placing the seeds on MS media, seeds started germinating in a week's time and scutellum region of seeds swelled. Embryonic calli, light yellow and granular in appearance, started emerging from the swollen junction between radicle and mesocotyl in all the 14 genotypes. Callus induction was established after two weeks period. The genotypes depicted varied response towards callus induction and regeneration potential as evident from results based analysis of variance (Table 1). Highly significant mean squares were noted for parameters which define tissue culture response over rice genotypes. Differential genotype response for callus induction has been reported by Diawuoh *et al.,* (2016).

Success intissue culture mainly depends upon the source of explant, genotype and medium Growth regulator 2,4-D is the most suitable Auxin for callus induction.

Different concentrations of 2,4-D havebeen assessed by different workers to find the optimal working concentration that gives best callus induction. A study conducted by Vennapusa *et al.,* (2015) showed that highest callus frequency was obtained on LS media containing 2.5 mg/L 2,4- D. Diawuoh *et al.,* (2016) also worked with dehusked rice and reported that 2,4-D is the best auxin for callus induction.

The highest callus induction was observed in rice germplasm line GS-88, aromatic landrace Kamad and fine grained aromatic variety, Pusa Sugandh 3.

Table.1 Analysis of variance for traits related to tissue culture response across a set of temperate rice genotypes

Source of Variation	d.f.	Mean squares		Probability
		Callus Induction (%)	Regeneration (%)	
Genotypes	11	17.58	21.37	0.000005
Error	12	0.05	0.09	

Table.2 Callus induction and regeneration response of genotypes

Genotypes	Characteristic features	Callus Induction (%)	Regeneration (%)
Madew-2	Japonica red rice	71.4	30
SKUA-420	Non- aromatic basmati	93.3	70
GS-19	Japonica aromatic rice	84.6	90
Jehlum	Indica variety for high altitudes (1600 msl)	25.0	5
Zeera Rice	Short grained indica	68.0	50
Tangdar Zag	Japonica red rice	80.0	30
MB 4-40	Pyramided MushkBudji	64.3	95
K-332	Japonica variety for high altitudes (2000 msl)	94.4	100
Pusa Sugandh 3	Fine grained aromatic	100.0	80
Kohsar	Japonica variety for high altitudes (2000 msl)	20.0	20
Kamad	Short grained aromatic landrace	100.0	100
GS-88	Japonica	100.0	95

Fig.1 Percent callus induction and plant regeneration of 12 genotypes in MS media

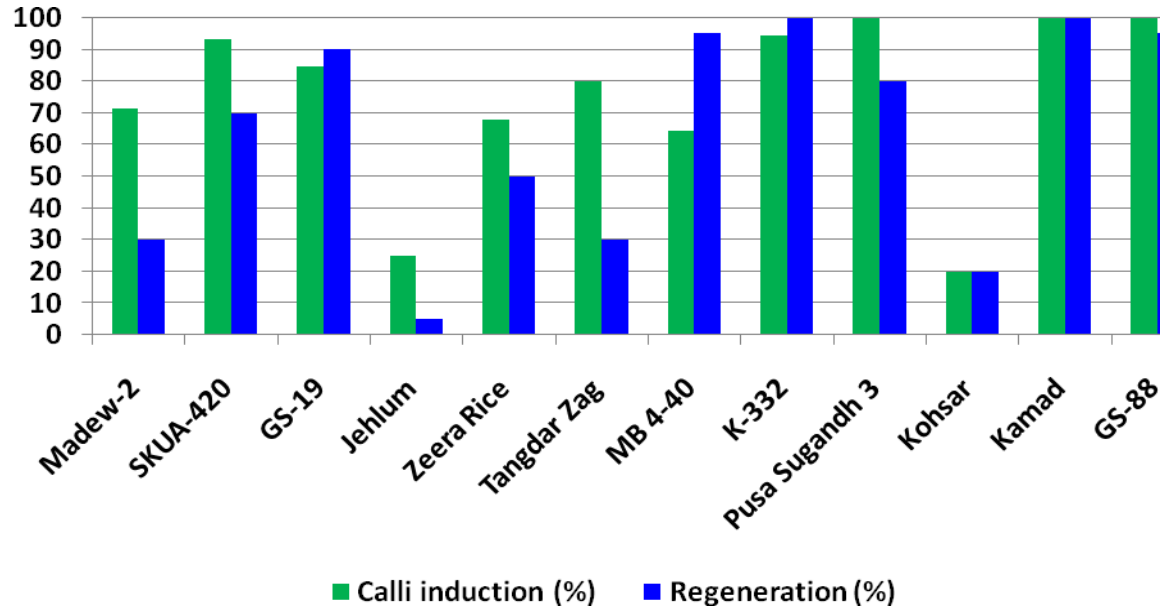
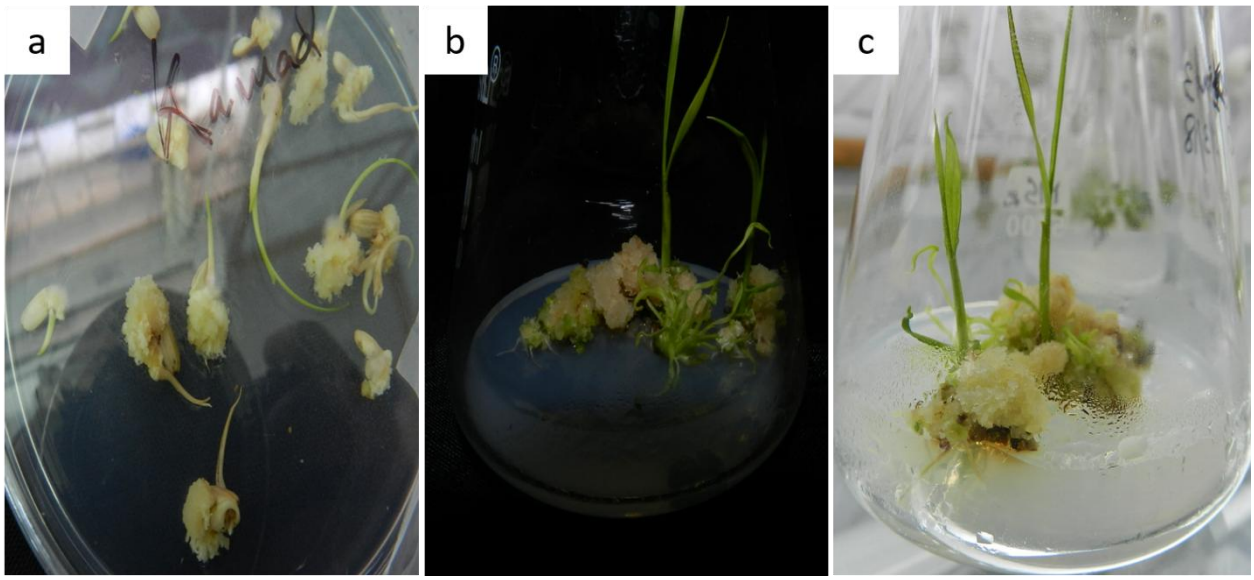


Fig.2 Seed explant tissue culture of rice genotypes



a: Calli induction; b & c: green plant regeneration

Callus induction potential varied across genotypes, K-332 (94.4%), SK-420 (93.3%), GS-19 (84.6%), Tangdar Zag (80%), Madew-2 (71%), Zeera rice (68%), MB 4-40 (64.3%), Jehlum (25%) and was lowest for Kohsar (20%) (Table 2 and Figure 1).

Shoot regeneration

Among the various genotypes, the enhanced regeneration potential was observed in K-332 and Kamad followed by MB 4-40. Jehlum and Kohsar had lowest regeneration potential (Figure 1 and 2). Variable regeneration potential was observed by Biswas *et al.*, (2007) among the genotypes inoculated on regeneration media which is in line with the present study and emphasizes that regeneration is genetically controlled trait and may show differential behaviour.

Acclimatization

Around 95% of the *in-vitro* developed plants survived the hardening process both in green house as well as the net house conditions. Regenerants were transferred to field where they exhibited normal growth and development and produced viable seeds.

Overall, the genotypes K-332, GS-88 and Kamad performed better as regards callus induction and green plant regeneration followed by Pusa Sugandh 3, MB 4-40 and GS-19. These genotypes can easily be utilized in transformation studies due to their excellent calli induction and green plant regeneration rates. Moreover, the study helped to validate the given media composition on set of germplasm adapted to temperate high altitude climate.

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