Comparison of Regeneration Efficiency of Three Different Genotypes of Basmati Rice under *in Vitro* Condition

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Abstract
Seeds of three basmati rice genotypes namely Pusa Sugandha, Pusa1 and Pusa 1121 were assessed for callus induction and plant regeneration on different concentrations and combinations of plant growth regulators in MS medium. A two stage sterilization treatment was found to be the most effective leading to maximum number of uncontaminated explants in three rice genotypes. The number of shoots was observed to be more in MS media supplemented with BAP and the shoot length was more in MS media supplemented with Kn. An optimum concentration of 1.0mg/l of 2,4-D was found to be effective for callus induction but Pusa Sugandha had more potential towards callus formation than Pusa 1 and Pusa 1121. However, highest percentage of shoot regeneration from callus was observed in Pusa Sugandha (90.0%) followed by Pusa 1 (80.0%) and Pusa 1121 (50.0%) in MS media supplemented with 0.5 mg/l of 2,4-D, 1.0 mg/l of Kn and 0.5 mg/l of BAP.

Key words: *Oryza sativa*, basmati, *in vitro*, callus, growth regulators, genotypes

INTRODUCTION

Rice (*Oryza sativa* L.) is the main staple food for more than half of the world population and has also become a model monocot system for genetic and functional genomics studies. The Asian cultivated rice provides 35% of calorie intake worldwide **[1].** In recent years, considerable efforts are being directed toward the improvement of important agronomic traits of rice through biotechnological techniques. A number of factors such as the genotype of the donor plant, the physiological status of the explant, the

composition and concentration of the culture medium play an important role for rice callus induction and regeneration **[2].** Among these, auxins and cytokinins are mostly employed in plant tissue culture systems to regulate cell division and differentiation in the explants. Aromatic rice such as basmati is well known for its super grain quality. It is an important export commodity of India due to its aroma and excellent cooking qualities. Its production is limited by being susceptible to number of insects and pests. Thus there is a need to improve the existing germplasm of Basmati cultivars by introducing variability into plants **[3]**. Thus callus production and its regeneration are the prime steps for the manipulation of a crop by biotechnological means and their regeneration ability highly depends upon genotypes, carbohydrate sources, plant growth regulators, culture medium and culture conditions [**4].** Present study was, therefore, carried out to study the effect of growth regulators on developing a high frequency callus induction and regeneration system in three different genotypes of basmati rice i.e. Pusa 1, Pusa 1121 and Pusa Sugandha.

MATERIALS AND METHODS:

Plant Material and establishment of cultures:

The plant material used in present study were the rice seeds of Pusa 1, Pusa 1121 and Pusa Sugandha which were brought from SKUAST Jammu and were used for all the experiments. The seeds of all three varieties: Pusa 1, Pusa 1121 and Pusa Sugandha were dehusked manually. The dehusked seeds were washed with detergent Tween 20, rinsed thrice with tap water and finally with distilled water. The seeds then were treated aseptically in laminar flow with 70% alcohol for 30 sec and with mercuric chloride (0.1%, 0.2%, 0.4%, 0.7% and 1.0%) for 3-4 mins. The seeds were established on basal MS nutritive media with 3% sucrose and 0.8% agar for their initial establishment and to monitor the percentage of contamination.

Salt Stress:

Proliferation efficiency of different basmati genotypes was studied after a pre-treatment of seeds to a salt concentration of 100 mM, 200 mM, 300mM, 400mM and 500mM NaCl for 7 days and then inoculating the seeds on basal MS media.

Shoot Proliferation and Rooting:

The explants that formed shoots after salt stress treatment were inoculated into fresh Murashige and Skoog medium **[5]** with different concentrations and combinations of Benzyl amino purine (BAP) (0.5mg/l, 1.0mg/l and 1.5mg/l) and Kinetin (Kn)

(0.5mg/l, 1.0mg/l and 1.5mg/l) separately as well as in combinations. Data was recorded after 12 days of culturing. Every possible care was taken to prevent any further contamination. Rootable shoots were excised from lavishly multiplying shoot clusters of all three basmati varieties (Pusa 1, Pusa 1121 and Pusa Sugandha) and were then transferred singly to culture tubes. Rooting medium was MS supplemented with different concentrations of IAA (Indole-3-acetic acid) (0mg/l, 0.2mg/l, 0.4mg/l, 0.6mg/l and 1.0mg/l). The percentage of root induction was recorded after 12 days of culture. After the roots were well developed, the rooted plants were taken out of culture tubes, washed gently to remove agar and then transferred to the pots with a mixture of sand and soil in the ratio of 2:1. The plantlets in the pots were covered with jars to maintain the humidity. After 2 weeks, the jars were removed and the established plants were then transferred to soil in the field conditions and their survival rate was observed.

Callus induction and Shoot regeneration:

Dehulled seeds of Pusa 1, Pusa 1121 and Pusa Sugandha were surface sterilized with 0.7% mercuric chloride for 3-4 mins and cultured on MS media supplemented with 0.5 mg/l and 1.0 mg/l concentrations of 2,4-D (2,4-dichlorophenoxy acetic acid). The cultures were kept at 25 ± 3°C in diffused light for initial 12 days and then were transferred to 2000 lux intensity for 16 hour photoperiod. Seeds were screened regularly for contamination and callus induction. Healthy callus (Granular, light yellow in color) was subcultured on fresh callus proliferating media for another two weeks. The callus induction frequency was recorded considering that each callus piece originated from a single seed. After 15 days the callus formed was transferred to fresh MS media containing 2,4-D, kinetin and BAP. Regenerated plantlets were counted based on the number of callus-producing plantlets.

Data collection and analysis:

The average number and length of shoots per explants and the total number of shoots produced were calculated for the different treatments. To measure the number and length, the shoots and roots were removed from the cultures. The statistical analysis was based on mean values and standard error

RESULTS AND DISCUSSION

Shoot establishment:

A single sterilant for different time periods was not applicable for the surface sterilization of seeds (explant) in Pusa 1, Pusa 1121 and Pusa Sugandha, as it could not control the contamination completely. A two stage sterilization treatment which included the combination of 70% alcohol (for 30 sec) and $HgCl_2$ (0.7% for 3-4 minutes) was found to most effective leading to maximum uncontaminated explants (Table I).

Table I. Effect of different sterilants on surface sterilization of explants

S.No.	Treatments	Time duration	Percentage survival of uncontaminated explants		
			Pusa 1	Pusa 1121	Pusa Sugandha
1	70% ethanol	25-30 sec	0.1	0.1	0.2
2	$HgCl_2$ (0.1%)	4 min	32.6	32.2	36.4
3	$HgCl_2$(0.2%)	4 min	37.5	35.2	38.5
4	$HgCl_2$ (0.4%)	4 min	42.4	40.3	35.2
5	$HgCl_2$(0.7%)	4 min	81.4	72.6	81.4
6	$HgCl_2$(1.0%)	4 min	69.5	62.4	76.0
7	70% ethanol+ 0.7% $HgCl_2$	30 sec +4 min	82.3	80.8	89.9

Salt stress:

After 7 days of inoculation, it was observed that the all the explants were established in the MS medium without any salt concentration and as the salt concentration was increased in the media there was a reduction in the survival rate of the explants, clearly

stating that the increased salinity adversely affect the plant growth. However the number of explants survived in Pusa 1 and Pusa Sugandha were more as compared to Pusa 1121 at 100 mM salt concentration. None of the explants survived in all the three basmati genotypes when MS media was supplemented with 400mM and 500 mM salt concentration (Table II). Our results were at par with those obtained by Rajakumar in 2013.

Shoot multiplication:

The explants that survived in all the three basmati genotypes viz. Pusa 1, Pusa 1121 and Pusa Sugandha after salt stress were transferred to MS media supplemented with BAP and Kinetin. Their effect on shoot multiplication was studied as plant growth regulators are the most important factors for successful plant regeneration. Hill and schaller **[6]** reported that cytokinins play a crucial role as promoters of cell division and act in the induction and development of meristematic centers leading to the formation of organs mainly shoots. The maximum number of shoots established in MS media supplemented with (1.0 mg/l) BAP and (1.0 mg/l) Kinetin were 93.33% in Pusa Sugandha, 80.0% in Pusa1, whereas it was only 66.6% in Pusa1121 (Table III, Fig I)). However when the MS media was supplemented with different concentrations (0.5mg/l, 1.0mg/l and 1.5mg/l) of either BAP or kinetin then the shoot length was longer in MS supplemented with 1.5mg/l Kn than in MS supplemented with 1.5mg/l BAP but the number of shoots obtained in MS supplemented with BAP (1.5mg/l) were more (multiple shoot regeneration) than in MS supplemented with Kn (1.5mg/l) (Table IV, Fig II).

The results were at par with the earlier studies where cytokinins induced multiple shoot formation and in increased shoot length **[7].** Verma *et al.* **[8]** obtained 95% of shoot regeneration in MS media supplemented with a combination with BAP and GA3 in cotyledonary node explants of gaur.

Table II: Effect of different salt concentrations on in vitro growth of explants

S.No.	Salt concentration	Time period	Percentage survival rate of explants		
			Pusa 1	Pusa 1121	Pusa Sugandha
1	Control	7 days	100.0	100.0	100.0
2	100 mM	7 days	92.0	80.1	95.2
3	200 mM	7 days	60.8	42.4	72.8
4	300 mM	7 days	42.7	30.2	50.8
5	400 mM	7 days	0	0	0
6	500 mM	7 days	0	0	0

Table III: Effect of different concentrations of BAP and Kinetin on shoot regeneration in Basmati genotypes after 12 days of inoculation

MS Media + growth regulators (mg/l)	Basmati genotypes	No. of explants inoculated	Percentage shoot regeneration	Average No. of shoots per explant Mean±S.E
MS+BAP(1.0)+ Kn(1.0)	Pusa Sugandha	15	93.3	3.11±0.43
	Pusa 1	15	80.0	2.98±0.89
	Pusa 1121	15	66.6	2.12±0.65
MS+BAP(1.0)+ Kn(0.5)	Pusa Sugandha	15	80.0	2.98±0.89
	Pusa 1	15	66.6	2.12±0.65
	Pusa 1121	15	53.3	2.12±0.65
MS+BAP(0.5)+ Kn(0.5)	Pusa Sugandha	15	66.6	2.72±0.72
	Pusa 1	15	66.6	2.12±0.65
	Pusa 1121	15	53.3	2.12±0.65
MS+BAP(0.5)+ Kn(1.0)	Pusa Sugandha	15	53.3	2.12±0.65

	Pusa 1	15	46.6	1.56±0.34
	Pusa 1121	15	33.3	1.78±0.447
MS+BAP(1.5)+ Kn(1.5)	Pusa Sugandha	15	60.0	1.99±0.41
	Pusa 1	15	60.0	1.99±0.32
	Pusa 1121	15	46.6	1.98±0.89

Fig I: Effect of BAP and Kinetin on shoot multiplication (a) Pusa Sugandha (b) Pusa 1 (c) Pusa 1121

Table IV: Effect of different concentrations of BAP and Kinetin on shoot multiplication in Pusa 1, Pusa 1121 and Pusa Sugandha

MS+ Growth regulators (mg/l)	No. of explants inoculated	Average no. of shoots per explant (Mean±S.E)			% shoot regeneration		
		Pusa 1	Pusa 1121	Pusa Sugandha	Pusa 1	Pusa 1121	Pusa Sugandha
MS + 0.5 BAP	15	7±1.41	0	6±0.41	46.6	0	40.0
MS+ 1.0 BAP	15	8±0.70	0	8±1.23	53.3	0	53.3
MS+ 1.5 BAP	15	7±2.07	0	11±0.22	80.0	0	73.3
MS+ 0.5 Kn	15	6±1.11	4±1.41	5±0.32	40.0	26.6	33.3
MS+ 1.0 Kn	15	9±0.32	4±0.21	7±0.87	60.0	26.6	46.6
MS+ 1.5 Kn	15	8±0.32	6±0.77	13±1.21	80.0	40.0	86.6

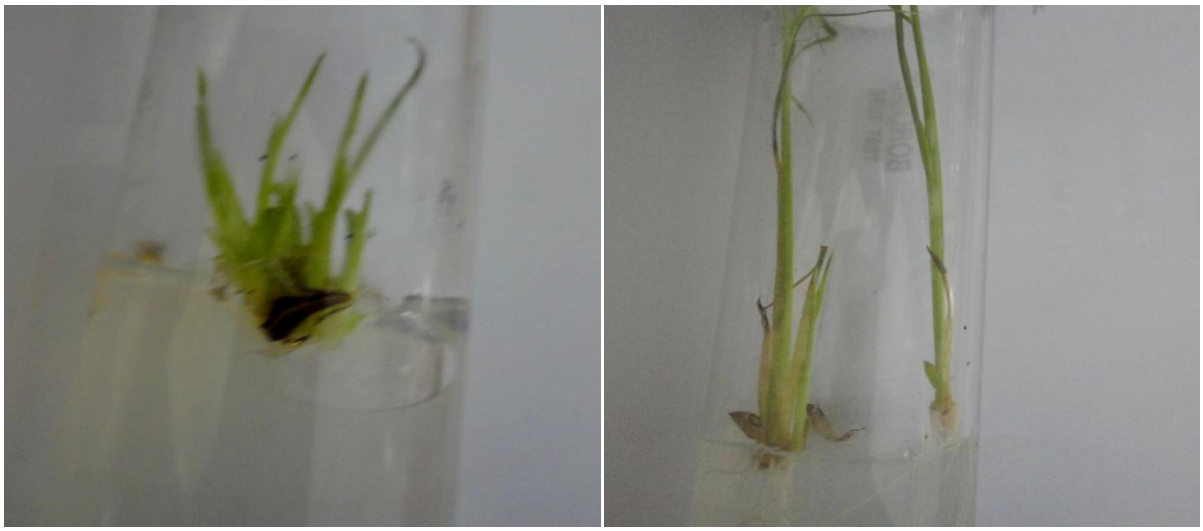

Fig II: Effect of (a) 1.5mg/l BAP and (b) 1.5mg/l Kn on shoot regeneration in Pusa Sugandha

***In vitro Rooting and Hardening**:*

The shoots obtained in Pusa 1, Pusa 1121 and Pusa Sugandha were transferred to MS medium containing different concentrations of IAA. Root initiation occurred after 10-15 days of inoculation and well developed root system was attained in 3 weeks in all the three genotypes. The maximum percentage of root regeneration was 97.83% in Pusa Sugandha, 93.48% in Pusa 1 and 78.23% in Pusa 1121 on MS medium containing 1.0 mg/l IAA whereas basal MS media has 78%, 32.40% and 33.24% roots in Pusa Sugandha, Pusa 1 and Pusa 1121 respectively (Table V).

Table V: Effect of different concentrations of IAA on root initiation in Pusa 1, Pusa Sugandha and Pusa 1121.

Medium (mg/l)	Pusa1		Pusa1121		Pusa Sugandha	
	% rooting	Type of roots	% rooting	Type of roots	% rooting	Type of roots
MS	32.4	Thin, fragile roots with fewer root hair	33.2	Thin, fragile roots with fewer root hair	78.0	Small, thread like, with lesser root hair
MS + 0.2 IAA	73.6	Thin, fragile roots with few root hair	48.5	Thin, fragile roots with few root hair	87.5	Small, thread lik, with less root hair
MS + 0.4 IAA	81.0	Thin long roots with many root hair	67.2	Thin, long roots with few root hair	90.2	Thin, long roots with many root hair
MS + 0.6 IAA	82.4	Long roots with fewer root hair	69.4	Thin, long roots with few root hair	93.8	Long roots with many root hair
MS + 1.0 IAA	93.4	Long-well developed roots with numerous root hair	78.2	Long-well developed roots with numerous root hair	97.8	Long-well developed roots with numerous root hair

Callus Induction:

Though 2, 4-D is the most suitable auxin for callus induction in rice tissue culture, yet it depends on the explant source and rice genotypes. In the present study, the effectiveness of different concentrations of 2, 4-D alone was evaluated for callus induction from dehusked seed of Pusa 1, Pusa 1121 and Pusa Sugandha. The initiation of callus occurred after15

days of inoculation of dehusked seeds in all three genotypes of basmati rice viz. Pusa 1, Pusa 1121 and Pusa Sugandha (Table VI, Fig III). After three weeks of inoculation the MS medium supplemented with 1.0mg/l of 2, 4-D produced only 50% of the callus in Pusa 1121 whereas high frequency of callus induction occurred in Pusa Sugandha followed by Pusa 1. However, Mahajan *et al.* **[9]** reported an optimum concentration of 2.0mg/l of 2,4-D effective for callus induction in Ranbir basmati and Basmati 370. Though difference was found between callus of all three varieties but it was observed that the Pusa Sugandha had more potential towards callus formation than Pusa 1 and Pusa 1121. The observed difference was not only in callus induction frequency but also in the quality of callus produced. Callus produced by Pusa Sugandha was more embryogenic. Significant difference between all three varieties for callogenesis under the same nutritional condition indicated that the callus induction quality is genotype dependent. The strong influence of genotypes on callus induction and plant regeneration was also observed in different plants **[10, 11].**

Table VI: Genotypic response of basmati rice to callus induction in MS media supplemented with 1.0 mg/l 2, 4-D.

Genotypes	No. of seeds inoculated	No. of calli regenerated	Percent callus induction (%)	Appearance
Pusa1	30	21	70	Creamy white, granular and small in size
Pusa 1121	30	15	50	Creamy white, compact and small in size
Pusa Sugandha	30	27	90	Creamy white, granular and large in size

Fig III: Callus induction in (a) Pusa Sugandha (b) Pusa 1 (c) Pusa 1121 after 3 weeks of seed inoculation

Shoot Regeneration and Multiplication:

The differential application of growth regulators such as BAP and Kn in the culture medium results in the induction of callus and subsequent differentiation and organogenesis. The plant regeneration ability of plated calli generally depends on the variety and the callus inducing media. Pusa Sugandha regenerated maximum number of plants. The current study showed that the best combination of plant growth regulator that caused plant regeneration from callus was 2,4-D (0.5 mg/l), Kn (1.0 mg/l) and BAP (0.5 mg/l) in all three genotypes of basmati rice i.e Pusa 1, Pusa 1121 and Pusa Sugandha. However, highest percentage of shoot regeneration was observed in Pusa Sugandha (90.0%) followed by Pusa 1 (80.0%) and Pusa 1121 (50.0%) (Table VII, Fig IV). No shoot regeneration was observed in Pusa 1121 when the MS media was supplemented with 2,4- D (0.5mg/l) and 1.0 mg/l Kn whereas shoot regeneration was observed in Pusa Sugandha and Pusa 1 (Table VII). Duangsee and Bunnag **[12]** observed the effect of nutrient composition and plant growth regulators on callus induction and plant regeneration in the glutinous rice cultivar Khunvang. They studied that the increasing casein hydrolysate and proline concentrations did not show a significant effect on callus growth but, proline concentration of 900 mg/ L yielded 85.67% of callus growth.

The interaction between different growth regulators and callus induction and shoot multiplication in three different basmati genotypes was observed. Pusa Sugandha responded better than two other basmati genotypes i.e. Pusa 1 and Pusa 1121 which shows that the ability of plant regeneration from seed

derived callus of rice is not influenced by physiological factors but also seems to be genetically controlled **[13]**. Same observations have also been reported earlier **[14].** Though rice cultivars respond differently to culture techniques and the genotype is a critical factor in tissue culture yet different histological processes leading to callus induction and organ differentiation, needs to be fully understood.

Table VII: Effect of different concentration of 2,4-D, BAP and Kn on plantlet regeneration from callus after 12 days

Combinations (mg/l)	Basmati genotypes	Total seeds inoculated	No. of calli regenerated	Plantlet regeneration	% regeneration	Type of shoots
Basal MS	Pusa Sugandha	15	5	1	20.0	Callus proliferation without shoot regeneration
	Pusa 1	15	5	2	40.0	Weak, fragile proliferation without shoot regeneration
	Pusa 1121	15	0	0	0	No plant regeneration
MS + 2,4-D (0.5mg/l)	Pusa Sugandha	15	8	3	37.5	2 long shoots with numerous short shoots
	Pusa 1	15	8	5	62.5	Numerous short shoots regeneration
	Pusa 1121	15	4	2	50.0	Callus proliferation without shoot regeneration
MS+2,4D (0.5mg/l)+ Kn (1mg/l)	Pusa Sugandha	15	12	9	75.0	4 long shoots with numerous short shoots
	Pusa 1	15	9	7	77.7	2 long shoots with numerous short shoots
	Pusa 1121	15	5	3	60.0	Callus proliferation without shoot regeneration
MS+2,4D (0.5mg/l)+ Kn (1mg/l) + BAP (0.5mg/l)	Pusa Sugandha	15	14	13	92.8	8 long shoots with numerous short shoots
	Pusa 1	15	13	11	84.6	5 long shoots with numerous short shoots
	Pusa 1121	15	7	5	71.4	Callus proliferation with fewer shoot shoots

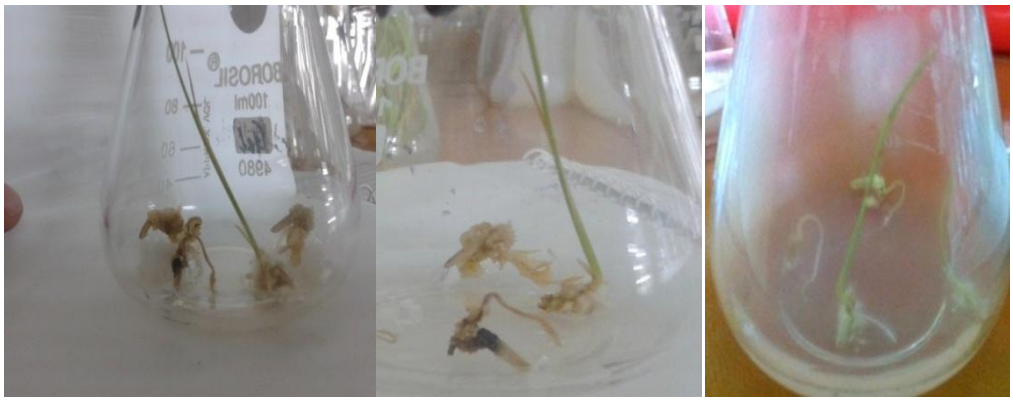

Fig IV: Effect of BAP, Kn and 2,4 D on shoot regeneration from callus in (a) Pusa Sugandha (b) Pusa 1 (c) Pusa 1121

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