Pak. J. Bot., 37(3): 673-684, 2005.

HIGH FREQUENCY REGENERATION FROM SCUTELLUM DERIVED CALLI OF BASMATI RICE cv. BASMATI 385 AND SUPER BASMATI

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Abstract

This study was conducted to obtain a high frequency regeneration from Basmati 385 and Super Basmati, which is a pre-requisite for transformation protocol. Seed was the explant source used in this study. Callus induction was obtained from N_6 media with 2 mg l^{-1} 2,4-D. Super Basmati exhibited high callus induction efficiency (93.3%) followed by Basmati 385 (90.2 %). Twenty one days old maintained calli when transferred on MS medium with different combinations of auxin-cytokinin for regeneration showed 90% frequency of plant regeneration for Super Basmati with NAA 1mg l^{-1} and BAP 2.5 mg l^{-1} and 83% for Basmati 385 on MS medium supplemented with NAA 1 mg l^{-1} and BAP 5 mg l^{-1}.

Introduction

Aside from *Arabidopsis* in dicot, rice (*Oryza sativa* L.) has been widely used as a plant material serving as a model system for plant genomics and *in vitro* studies in monocots. Due to its economic importance as a staple food for 2.7 billion people, rice has been a more attractive target for developing transgenic rice (Cho *et al*., 2004). Almost 90% of total rice is grown in Asia. Besides being an important food of people of Pakistan, rice has also gained a respectable position as a foreign exchange earning commodity (Salim *et al*., 2003).

In Pakistan, rice is cultivated over an area of 2225.2 million ha with a. production of 4478.5 million tons giving an average yield of 2013 kgs per hectare. Total area under basmati production is 1377.3 hectare in which 1316.8 hectare is for Punjab. Total production of basmati rice is 2175.5 million tons; Punjab contributes 2304.2 million tons with a 1673 kgs per hectare yield of basmati rice (Anon., 2002-2003).

Pakistani rice especially basmati rice is a major commercial variety grown in rice growing areas of Pakistan and is famous in the world for its particular aroma. Basmati rice, the best quality scented rice produced in Pakistan commands the international market have four times greater price than in the domestic market. Pakistan exports 7% of the total world market (Rashid *et al*., 2001). Despite all these, yield per unit area of rice in Pakistan is far below from the world average and low from many neighboring countries. The production of basmati rice is severely affected by various stresses, including fungal, bacterial and viral diseases which is one of the reasons for the low yields of rice. Hence, there is a need to improve these commercial cultivars by biotechnological applications.

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Whatever transformation system would be employed, efficient systems for embroygenic callus induction and shoot regeneration have been considered the basic matter in obtaining fertile transgenic rice. Successful callus induction from rice seeds has been reported by several researchers (Gonzalez, 2000., Navraj *et al*., 1999., Marassi 1996., Valdez *et al*., 1997., Xie *et al*., 1995). Among various tissues of rice, scutella-derived callus of mature seeds have been frequently employed as target materials for genetic transformation by *Agrobacterium* mediated and biolistics methods because of the manipulative effectiveness (Chen *et al*., 1998; Hashizume *et al*., 1999; Hiei *et al*., 1994; Rashid *et al*., 1996., Cho *et al*., 2004). Plant regeneration via somatic embryogenesis has many advantages in theoretical and applied tissue culture (Vasil *et al*., 1982). The application of this technique has made rapid progress in dicotyledons, but significantly low in graminae (Yamada, 1977). Cereals in general, are difficult to manipulate *in vitro* and regeneration is drastically reduced in successive subcultures. Regeneration of plants was achieved from callus of various origins eg., regenerated plants from seed callus (Tamura, 1968), and from embryo callus (Bajaj & Bidani, 1980). Most of the reported work has been done on "Japonica" or "Javanica" varieties and still there is a great deal of work to be done on optimization of media for plant regeneration in "Basmati" varieties, especially those that are most commonly cultivated in Pakistan. Although there are a few reports available on callus induction, regeneration and transgenic plant production in other Basmati cultivars like Basmati 6129, Basmati 370 and Basmati 385 (Rashid *et al*., 1996). Rashid *et al*., (2001 and 2004) reported a low regeneration frequency in Super Basmati, but still there is a strong need for more studies on developing high frequency regeneration from these commercially grown varieties

Enthused by the success in *Agrobacterium* mediated transformation, a study was initiated at the Agricultural Biotechnology Programme, National Agricultural Research Center, Islamabad, Pakistan to develop a high frequency callus induction and regeneration system from scutellum derived calli of two rice varieties viz., Basmati-385 and Super basmati for genetic manipulation.

Materials and Methods

Mature seeds of Basmati rice varieties Basmati 385 and Super Basmati were sterilized by protocol as described by Rashid *et al*., (1996). Treated seeds of Basmati 385 and Super Basmati were dehusked. Seeds were surface sterilized by 70% ethanol for one minute followed by 50% Clorox (*Sodium hypochlorite*) for 20 min. Seeds were continuously shaked during treatment with Clorox and subsequently rinsed with autoclaved distilled water for 3-4 times after a regular interval of 5 min.

The sterilized seeds were aseptically inoculated on N_6 medium (Chu *et al*., 1975) with 30 g l^{-1} sugar, 2, 4-D 2 mg l^{-1} and 6 g l^{-1} of agar which was used as solidifying agent .The pH of the medium was adjusted to 5.8 before autoclaving at 121°C for 20 min. These inoculated seeds were placed in the growth room for three weeks for callus induction. Only embryogenic calli (white to light yellowish in color, compact and friable) were selected and non-embryogenic calli (mucilaginous and smooth) were discarded.

Three weeks old scutellum derived calli were transferred on to maintenance medium which contained N_6 salts and vitamins supplemented with 2 mg l^{-1} of 2, 4-D, 30 g l^{-1} sugar and agar 6 g l^{-1} as a solidifying agent. Calli were placed on maintenance medium for about two weeks.

Media used for regeneration contained basic MS salts and vitamins (Murashige & Skoog, 1962), 3% sucrose and 0.4% gelrite. Regeneration media contained different combinations of BAP and NAA as growth regulators. Maintained calli were placed on regeneration media and were placed in the growth room (light and dark period at 23 ± 2°C with 16/8h photoperiod with light intensity of 16 m.mol.m.s). After shoot regeneration, plantlets were transferred to MS media without growth regulators for root initiation. In order to determine regeneration frequency, 40-60 cultures were raised for each treatment and each experiment was performed thrice. Subculture period was maintained at 2-3 weeks intervals. Observations were taken every week on the basis of % age of regeneration for the development of shoot/culture and shoot development.

Statistical analysis

All resulting scales of total callus browning, callus differentiation, production of green tissue or spots and plantlet formation among the two rice cultivars with 9 treatments along with three replicates on the MS medium were statistically analyzed using Analysis of Variance technique with a computer program MSTAT-C. Treatment means were compared using Duncan's Multiple Range Test (DMRT).

Establishment of plantlets in the glass house

Well-rooted plantlets were washed with running tap water to remove agar from the surface of roots. They remained suspended in water in test tubes for a few days before transfer to pots in the glass house. The soil mixture used for hardening was ordinary soil taken from rice fields and a high percentage 70-90% of humidity was maintained in the glass house for the first few days. Under these conditions it was to recover almost hundred percent plantlets.

Results and Discussion

The choice of suitable explant source as starting material for transgenic plant production is one of the most important factor. Although some reports used immature derived calli (Dong *et al.*, 1996), but others found that cali initiated from scutellum of mature seeds were excellent starting material for transformation of rice by *Agrobacterium* due to their compactness (Hiei *et al.*, 1994., Rashid *et al.*, 1996., Toki *et al.*, 1997., Lee *et al*., 1999, Rashid *et al*., 2001, 2003, 2004., Cho *et al*., 2004). In the present study calli derived from mature seeds of Basmati rice cv. 385 and Super basmati were compact.

Sterilization is the major factor, which affects the tissue culture. Clorox (Sodium hypo chlorite) was used as a surface sterilization agent, played very important role in the germination of seeds. Seeds of both varieties, Basmati-385 and Super basmati treated with different concentrations of Clorox for 20 minutes for sterilization showed that 100% Clorox when applied for 20 minutes gave 97-98% sterilization but no healthy callus was formed and it affected the growth of callus, Whereas 50% Clorox gave 78.5% sterilization in Super Basmati and 70% sterilization in Basmati 385 with the formation of large, granular, healthy callus. With the increased concentration of Clorox, the growth of callus decreased because at high Clorox concentration there are more chances of the tissue to be destroyed. Clorox 50% was found more suitable for sterilization without browning and inhibition of germination. Our results are nearly similar to Li *et al*., (1992) who reported that sterilization of seeds with 45%(v/v) Clorox for 30 minutes were effective (Table 1).

Table 1. Effect of Clorox on sterilization in rice seeds for 20 minutes for callus Induction.

Clorox	Super Basmati		Basmati 385	
	Frequency %	Growth	Frequency%	Growth
100%	98	-	97	-
80%	95	+	88.7	+
70%	89	+	77	+
60%	80	+	75.0	+
50%	78.5	+ + + +	70	+ + +
40%	50	+ + +	34	+ +

- = No growth, + = Poor, + + = Average, + + + = Good, + + + + = Excellent

Selected utilization of basal medium, growth regulators, carbon sources, and amino acids are important considerations for improving efficiencies of callus induction and shoot regeneration. Especially, shoot regeneration from the callus, the final stage for producing transformants. The N_6 basal media supplemented with 2-6 mgl^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D) for embryogenic callus induction and 0.5-10 mgl^{-1}6-benzylaminopurine (BAP) combined with 0.02-1 mgl^{-1} ∝-naphtalenacetic acid (NAA) for shoot regeneration have been selectively used depending on rice cultivars (Jiang *et al.*, 2000; Cho *et al*., 2004).

For obtaining transgenic plants of rice with scutella derived embryogenic callus (Fig. 1A-C), N_6 media (Hiei *et a*l., 1994; Rashid *et al*., 1996) have been selectively used depending on genotype of rice. A number of reports have emphasized the importance of the type of calli for regeneration efficiency in many monocot plants such as maize (Tomes & Smith, 1995) and rice (Lee *et al*., 2002). In the present study N_6 media was used for callus induction and callus growth. Sterilized seeds were placed on these media for three weeks. Both varieties showed callus induction and growth with 2 mg l^{-1} 2, 4-D. Embryogenic callus was selected on the basis of their physical properties as described by Cho *et el*., 2004. Morphology of the embryogenic callus was slick, yellowish-compact type with globular shape, soft and friable. Both the varieties were good for callus induction. However, Super basmati has more potential for callogenesis. The significant difference between two varieties for callogenesis under the same nutritional condition indicate that callus induction efficiency is genotype specific. These findings are in accordance with the reports of Abassi *et al*., (2000) and Khanna & Raina (1998), Nasreen & Mohammad (2000). The callus of Super basmati was found more embryogenic than Basmati-385. Callus induction and growth frequency was observed as 93.3% in Super Basmati and 90.2 % in Basmati 385. Our results are contradictory to Rashid *et al.,* (2001) who reported that callus induction frequency of Super Basmati on N_6 medium was 47.7%. Comparing callus induction and growth frequency of two varieties on N_6 media proved that Super Basmati was good as compared to Basmati 385 for callus induction (Table 2). Further these calli were placed on N_6 media with 2 mg l^{-1} 2, 4-D for growth and proliferation. After two weeks, it was observed that 76% calli showed growth and increased 2-3 times in size (Table 3, Fig. 1-D)

On regeneration media, embryogenic callus tended to show positive responses related with regeneration for green tissue formation or green spotting and shoot regeneration or plantlet formation and a negative response for callus browning (Cho *et el*., 2004). Additionally, the types and concentrations of cytokinins combined with NAA have been considered to be important factors on shoot regeneration of both indica (Xue & Earle, 1995) and Japonica rice (Lee *et al*., 2002). Supplementation of higher level of

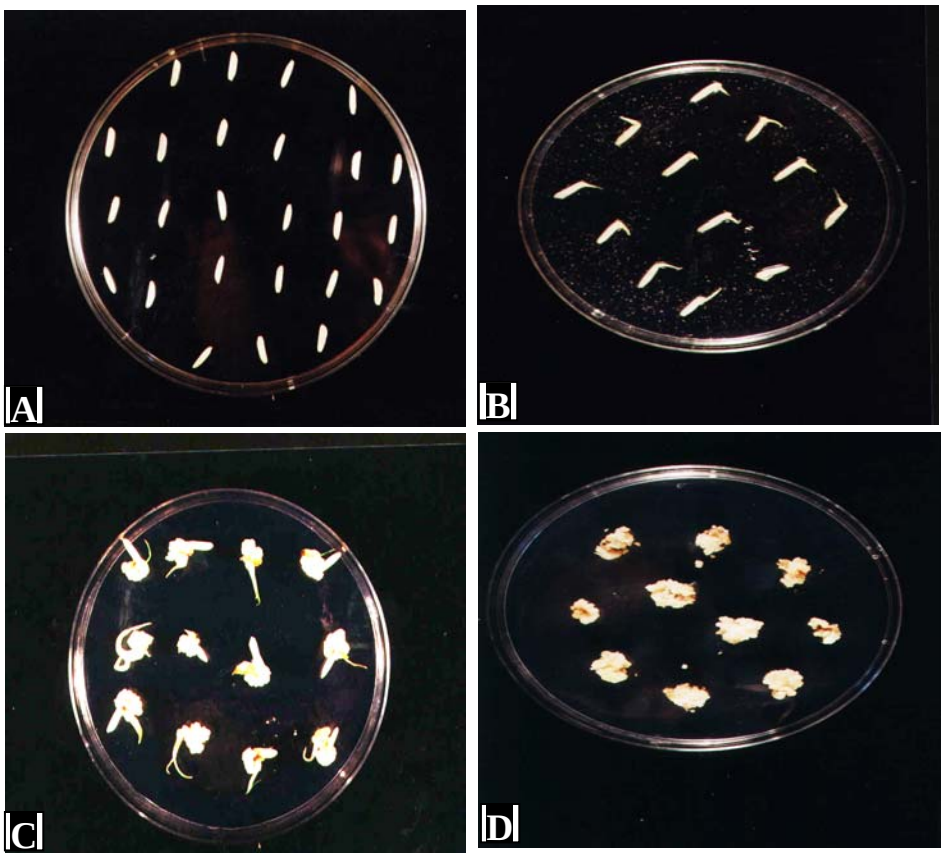

Fig. 1. Callus induction from seed as a explant source

A. Seeds of rice cv. Bas 385 on N_6 media
B. Arising of calli from scutellum
C. Three week old scutellum derived calli
D. Calli on maintenance media.

BAP was more effective on regeneration of rice (Lee *et al*., 2002, Xue & Earle, 1995). In the present study, maintained calli were transferred to regeneration medium containing basic MS salts and vitamins with different concentrations of NAA and BAP i.e. NAA 1 mg l^{-1} and BAP .5,1, 1.25,2, 2.5 and 5 mg l^{-} Generally, the callus having high regeneration potential formed green tissue or green spots on the surface of callus with fast growth for 4-7 days of cultivation (Fig. 2-B) and, consequently, 0.2-1.0 cm size shoot emerged at 10-14 days (Fig.2-C). The callus regenerated on all the concentrations of growth hormones, but the highest frequency 90% was observed on NAA 1 mg l^{-1} and BAP 2.5 mg l^{-1} in Super Basmati (Fig. 2- E & F) and 83 % in Basmati 385 in combination of NAA 1 mg l^{-1} and BAP 5 mg l^{-1}.
(Fig. 2-D).

Table 2. Comparison of callus induction, callus growth frequency of Basmati rice varieties i.e., Basmati 385 and Super basmati on N_6 medium supplemented with N_6 salts and vitamins, 30g/l sucrose, 2mg/l 2,4-D and 4g/l gelrite.

# of Exp.	# of seeds	Callus induction		Callus growth	Percentage of callus growth		
		Bas 385	Super Bas.	Bas. 385	Super Bas.	Bas. 385	Super Bas.
1	240	218	199	203	153	84%	63.7%
2	240	215	222	210	216	87.5%	90%
3	240	233	208	225	183	90.8%	76.2%
4	240	189	150	167	123	69.5%	51.2%
5	240	236	232	196	224	81.6%	93.3%
Average	**240**	**218.2**	**202.2**	**198.8**	**179.8**	**82%**	**74%**

Table 3. Maintenance of embryogenic calli of Basmati 385 on N_6 media supplemented with 2 mg l^{-1} 2, 4-D.

# of calli	Contamination		Browning		Growth and proliferation		Percentage of Growth	
	Bas. 385	Sup. Bas	Bas. 385	Sup. Bas	Bas 385	Sup. Bas	Bas. 385	Sup. Bas.
25	5	4	3	5	12	21	60	70
30	4	5	6	6	20	24	66	68.5
40	5	7	10	4	25	29	62.5	72.5
40	6	4	5	7	30	30	75	75
Average	**5**	**5**	**6**	**5**	**21**	**26**	**65.8**	**71.5**

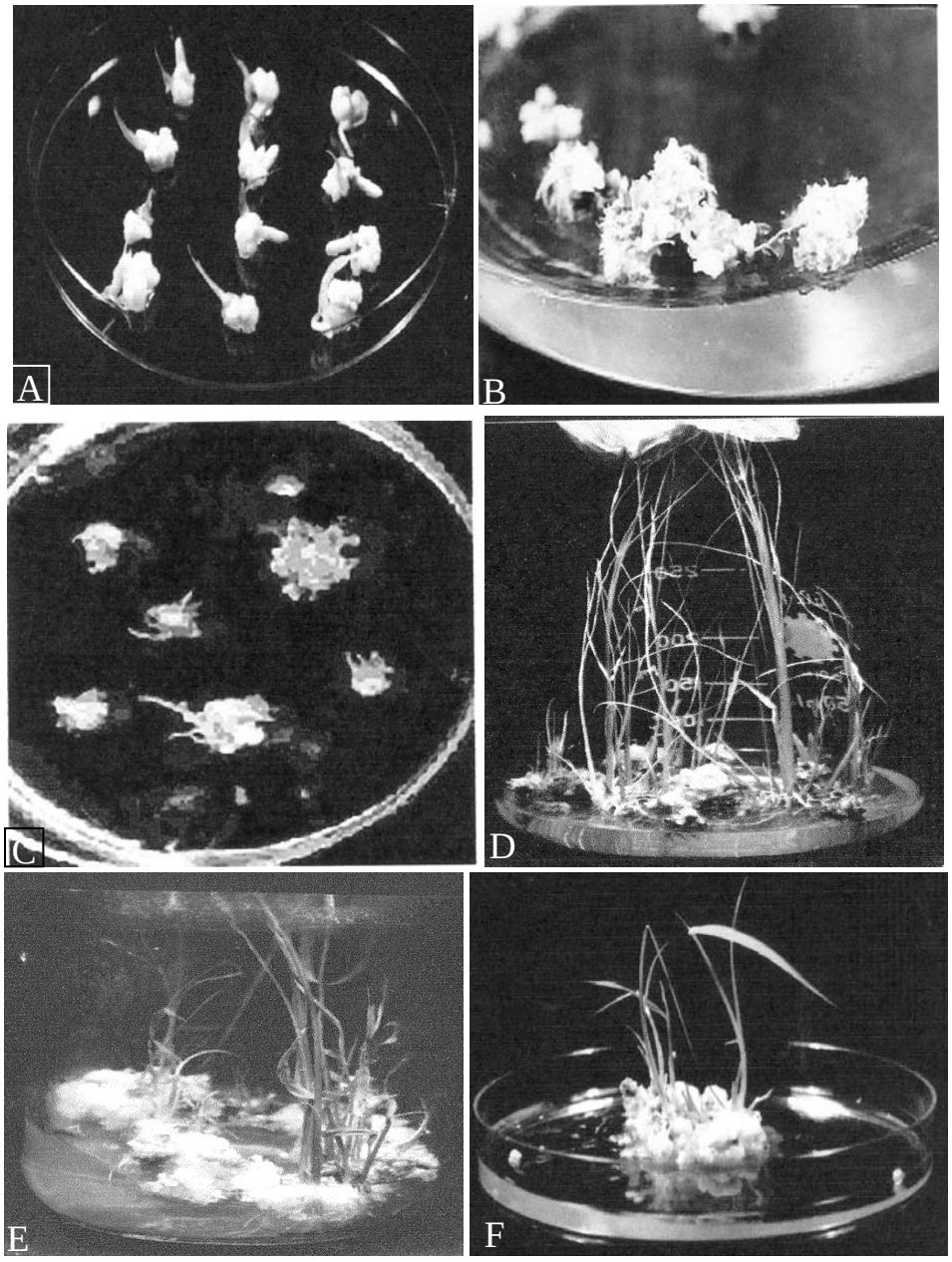

Fig. 2. Regeneration from scutellum derived calli.
A. Three week old scutellum derived calli
B. Calli become green on regeneration media
C. Calli showing further growth on regeneration medium
D. Calli showing regeneration on MS media with NAA I mg^{-l} and BAP 5 mg^{-l} in Bas. 385
E & F. Calli showing regeneration on MS media with NAA I mg^{-l} and BAP 2.5 mg^{-l} in Super Basmati

Our results are different from the reports of Rashid *et al.,* (2001) in which regeneration frequency was 45.3%. Brar *et al.,* (1985) also reported that the presence of a cytokinin either BAP or Kinetin is essential in promoting plant regeneration from cultured cells (Table 4). Both NAA and BAP was not required for shoot initiation (Azria *et al*., 2000). Pons *et al., (*2000) reported that BA yielded more shoots than kinetin in all varieties while in case of using auxin NAA and IAA, it depends on the varieties. Analysis of variance indicates that there are significant differences of regeneration efficiencies of two rice cultivars observed between the supplementation of BAP. As carbon source and osmotic regulator, sucrose has been most acceptable for *in vitro* culture of rice. However maltose (Zhang, 1995) and sorbitol (Rashid *et al*., 1996) were substituted in the media for callus induction and shoot regeneration. Even though sorbitol is a major factor for embrogenic callus formation in the monocot plant such as maize (Swedlund & Locy, 1993). Cho *et al*., (2004) reported that growth of callus is stimulated and multiple shoots of regenerated plants could be obtained in higher frequency by supplementation of sorbitol in combination with sucrose or maltose under 5 mgl^{1-}kinetin. The supplementation of sorbitol and prolin in the medium was attributed to be more effective to obtain regenerated plants of rice rather than utilization of the medium containing sucrose or maltose alone. Yang *et al*., (1999) reported that proliferation and regeneration of rice callus were suppressed by sorbitol, in contrast controversy to that of Kishor & Reddy (1986) who reported that sorbitol has been used for restoring and enhancing the plant regeneration ability of rice callus. Swedlund & Lucy (1993) also reported that sorbitol, as osmoticum imparted beneficial effect on embryogenesis in cereal crops. In this study sorbitol supplementation in combination with sucrose critically stimulated the growth rate of rice calli. The green spots appeared after 3-4 days of transferring the rice calli on regeneration medium.

Sorbitol as the osmoticum was found not necessary for the induction and growth of rice callus. When seeds of both varieties were inoculated on the N_6 Media (CIM) supplemented with 2,4-D and sorbitol, there was no induction and growth of callus formation. Calli showed necrosis after one week of inoculation (Table 5). Our results are similar to the reports of Alkhayri *et al.,* (1996) who used MS medium with 2,4-D and Kinetin either with sucrose alone or combined with sorbitol. It was reported that medium without sorbitol enhanced callus growth and callus proliferation as compared to sucrose–sorbitol combination. However, the sucrose–sorbitol combination improved regeneration of rice cultures.

Twenty two plantlets of Super basmati and 18 of Basmati 385 have been established on ordinary soil contained in pots by maintaining a high percentage of humidity in the glass house at 25^{o}C and were assessed for their growth by using two parameters i.e., height and tiller. As compared to control, they showed less growth due to sensitivity and lack of development of efficient root system for water and nutrient absorption from the soil (Table 6). The somaclones were short in height as it was reported by Su *et al.*, (1992).

The present study showed that now it is possible to obtain high regeneration frequency of up to 90% from Super Basmati and 83% from Basmati 385, as compared to other varieties. This high regeneration frequency will lead to improve these varieties by genetic transformation technology.

Table 4. Comparison of regeneration efficiencies of rice calli of the two rice cv. Basmati 385 and Super basmati on MS media supplemented with 30g^{-1} Sucrose, 2 g^{-1} Casine hydrolysate, 30 g^{-1} Sorbitol with different treatments of NAA and BAP.

Number of Treatment	Callus browning		Callus differentiation		# of green spots		Plant formation		% frequency of plant formation	
	Bas. 385	Sup. Bas	Bas. 385	Sup. Bas	Bas. 385	Sup. Bas	Bas. 385	Sup. Bas	Bas. 385	Sup. Bas
NAA* 0mg^{-1} + BAP** 0mgl^{-1}	0.00 h	0.00 h	0.00f	0.00f	0.00e	0.00e	0.00f	0.00f	0.00	0.00
NAA 1mg^{-1} + BAP 0mgl^{-1}	16.6 a	14.00 ab	0.00f	0.00f	0.00e	0.00e	0.00f	0.00f	0.00	0.00
NAA 1mg^{-1} + BAP 0.5mgl^{-1}	3.33 e-h	6.6 de	5.00def	6.67cde	6.33bc	7.67bc	3.33c-f	2.00def	16.6	10
NAA 1mg^{-1} + BAP 1.0mgl^{-1}	5.00 d-g	5.00 d-g	7.33cde	7.33cde	9.67b	3.333cde	2.67 c-f	2.667c-f	13.3	13.3
NAA 1mg^{-1} + BAP 1.25mgl^{-1}	6.66 de	1.67fgh	11.6bc	9.33cd	7.67bc	9.67b	5.33cd	5.00cde	26.6	25
NAA 1mg^{-1} + BAP 2.0mgl^{-1}	11.6 bc	4.00 e-h	12.00bc	16.00ab	6.67bc	15.33a	5.00 cd	13.33b	25.0	66.65
NAA 1mg^{-1} + BAP 2.5mgl^{-1}	1.33 gh	3.33 e-h	80.6a	12.00bc	19.33a	9.667b	16.67 ab	6.667c	83.3	33.31
NAA 1mg^{-1} + BAP 5.0mgl^{-1}	3.00 e-h	0.00h	16.67 ab	19.00a	18.67a	19.33a	16.00ab	18.00a	80	90.0
NAA 2mg^{-1} + BAP 1.0mgl^{-1}	6.00 def	8.33cd	2.00ef	4.00def	1.67de	5.00bcd	1.00ef	3.33d-f	5	16.66
Mean= Coefficient of variance=	5.963	4.778	8.148	8.259	7.778	7.778	5.556	5.667		
	42.33%		34.87%		32.40%		38.65%			

Coefficient of variance (ANOVA) indicate that, here is no significant difference between two varieties tested for regeneration in our studies.
*∝-Naphtalenacetic acid (NAA), **6-benzylaminopurine (BAP)

Table 5. Comparison of effect of sorbitol on the induction of callus on N_6 media supplemented with 2,4-D 2mg l^{-1}, sucrose 3%, 0.6% agar and 0.3% sorbitol and without sorbitol.

Media with Sorbitol (0.3 %)								
No. of Seeds	**Basmati 385**				**Super Basmati**			
	CI	**CG**	**Browning**	**% freq.**	**CI**	**CG**	**Browning**	**% freq.**
192	150	40	42	20	142	25	48	17.6
Media without Sorbitol (0.3 %)								
192	185	180	3	93	175	170	11	88.5

CI = Callus Induction, CG = Callus Growth

Table 6. Glasshouse assessment of the somaclones of Basamti rice.

No. of plants	**Basmati 385**				**Super Basmati**			
	Control		**Somaclone**		**Control**		**Somaclone**	
	Height (cm)	**No. of tiller**	**Height (cm)**	**No. of tillers**	**Height (cm)**	**No. of tiller**	**Height (cm)**	**No. of tillers**
1	105.5	13	32.5	4	65	5	20	1
2	99.9	16	44.9	7	76.5	9	43	6
3	150	19	89.5	10	88.3	8	50.6	4
Average	**118.4**	**16**	**55.6**	**7**	**76.6**	**7**	**40.8**	**3.6**

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(Received for publication 20 July 2005)