

Efficient protocols for *in vitro* regeneration of *Pennisetum glaucum* (L) Br.

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A system was developed for *in vitro* regeneration of *Pennisetum glaucum* through organogenesis and somatic embryogenesis. Mature embryo and leaf base explants of *Pennisetum glaucum* (L) Br. cv HH B60 (Poaceae) were cultured on Murashige and Skoog agar medium supplemented with 11.3 μM of 2,4-D for callus induction. Embryogenic calli were induced within eight weeks. Percentage of callus induction and somatic embryogenesis was significantly higher in mature embryo than leaf base explants. Maximum shoot regeneration was obtained via organogenesis on MS medium supplemented with 4.43 μM of BAP and 4.64 μM of kinetin from the calli of both the explants. The frequency of plant regeneration through somatic embryogenesis was comparatively lower than organogenesis. Regeneration frequency was higher in mature embryo explants than leaf base explants. The shoots regenerated via organogenesis were elongated and rooted efficiently on MS medium supplemented with IBA (0.49 μM). The rooted plantlets were hardened and transferred to soil.

Keywords: Bajra, Leaf base, Mature embryo, Pearl millet, Poaceae, Regeneration

Pearl millet (*Pennisetum glaucum*) is an important staple food grain in India and Western Africa and is widely cultivated in semi-arid regions especially under low soil fertility and harsh climatic conditions. The grains are rich in protein (12.3%), fat (5.3%), ash (2.4%), essential amino acid leucine and other minerals¹. Because of high nutritive value of its proteins, pearl millet is recommended for diabetic and cardiac patients². Furthermore, it is an important forage crop for ruminants³. Pearl millet is highly susceptible to downy mildew disease caused by the fungus *Sclerospora graminicola* (Sacc.) Schroet., which can cause up to 70% reduction in yield⁴. Insect pests also inflict severe damage in field and during storage. Therefore, improvement of pearl millet crop will have a great effect on socio-economic status of the people in rural areas⁵, where it is widely cultivated.

Recently, Kothari *et al.*⁶ have clearly outlined the role of biotechnology in the genetic improvement of millet crops. A reproducible regeneration procedure is essential in studies involving gene transfer for the genetic improvement of this crop plant⁷. Although plant regeneration through somatic embryogenesis and organogenesis have been reported in *Pennisetum*

*americanum*⁸⁻¹⁰ and *P. glaucum*.¹¹⁻¹⁸ from different explants, it has not been reported from mature embryo and leaf base explants. Here we report a highly reproducible plant regeneration protocol which is based on organogenesis and indirect somatic embryogenesis from mature embryo and leaf base explants.

Materials and Methods

Seeds of pearl millet, *Pennisetum glaucum* (L) Br. cv HH B60 were obtained from National Seeds Corporation Limited, New Delhi, India. Seeds were surface sterilized in 70% (v/v) ethanol for 30 sec, washed in sterile distilled water and then soaked in 0.1% (w/v) mercuric chloride solution for 5 min and thoroughly washed five times with sterile distilled water. Some of the seeds were cultured on MS basal medium for leaf base explants. Surface sterilized seeds and four-day-old *in vitro*-derived leaf base explants were cultured on MS medium¹⁹ supplemented with different concentrations of 2,4-dichlorophenoxyacetic acid (2,4-D; 2.25-18.0 μM) for callus induction. Calli were subcultured on MS basal medium for somatic embryogenesis. For regeneration, the callus was transferred to MS medium supplemented with different concentrations of cytokinins, 6-benzylaminopurine (BAP; 2.21-8.86 μM) and kinetin (2.32-9.29 μM) individually and in combination. The embryo-like structures from leaf

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base and mature embryo explants were transferred to MS basal medium without growth regulators for regeneration. The shootlets (2-3 cm height) regenerated through organogenesis were transferred to the MS medium containing various concentrations of auxins, indole butyric acid, (IBA) indole acetic acid (IAA) or α -naphthalene acetic acid (NAA), for root induction. All the media were gelled with 0.7% agar (w/v) (Hi-media, Mumbai, India) after adjusting the pH to 5.7 and were autoclaved at 1.4 kg/cm^2 for 15 min. Cultures were incubated at $27^\circ \pm 1^\circ \text{C}$ under cool white-fluorescent light providing a quantum flux density of $30 \mu\text{mol m}^{-2}\text{s}^{-1}$ with the photoperiod of 16 h light and 8 h dark. The fully developed plantlets were removed from the culture tubes, thoroughly rinsed with running tap water and transferred to paper cups containing a mixture of sterilized vermiculite and sand (1:1). The cups were maintained in the culture room for two weeks under controlled temperature ($27^\circ \pm 1^\circ \text{C}$) and 16 h photoperiod and 80-90% relative humidity for hardening. Plants were initially irrigated with 1/4 strength Hoagland solution²⁰ on alternate days followed by tap water after one week. Thereafter, the acclimatized plants were transferred to field and the percentage of survival of plantlets was recorded. The experiment was repeated at least three times with twenty replicates per treatment.

Results and Discussion

Mature embryo and leaf base explants cultured on MS medium supplemented with $4.50 \mu\text{M}$ or more 2,4-D, produced callus. Mature embryos gave rise to white, soft and friable callus (Fig. 1A, 1B) whereas leaf base explants formed compact, shiny, yellowish and nodular embryogenic calli (Fig. 1C). With both the explants, the highest frequency of callus initiation was recorded on MS medium supplemented with $11.3 \mu\text{M}$ 2,4-D (Table 1). Callus induction from the mature

embryo was higher (75.0%), than leaf base explants (42.0%). The primary callus was subcultured on the callus induction medium for maintenance and regeneration of shoots occurred from the embryogenic calli of both the explants at high frequency on MS medium containing BAP ($4.43 \mu\text{M}$) and kinetin ($4.64 \mu\text{M}$). Though the differentiation of plants is

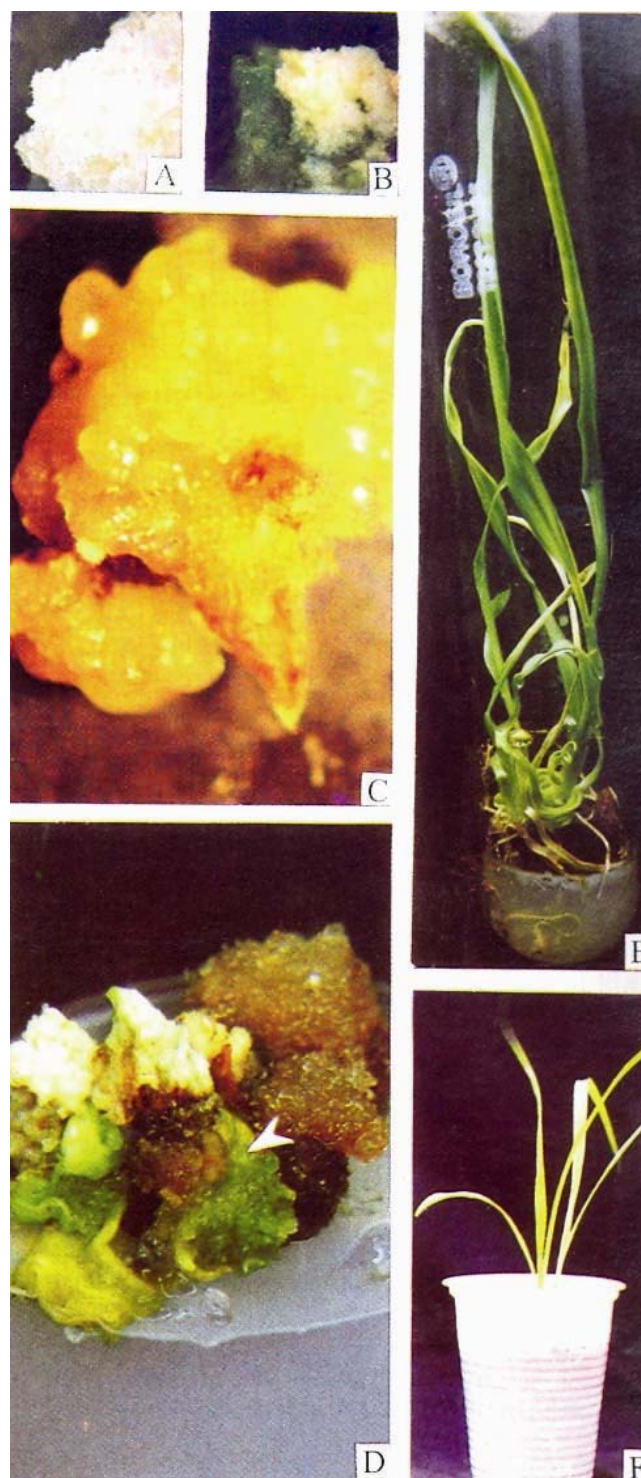


Fig. 1 — (A) Callus derived from mature embryo on MS medium containing $11.3 \mu\text{M}$ 2,4-D after 21 days of culture; (B) Mature embryo-derived callus showing shiny embryoid on MS basal medium devoid of growth regulators; (C) Embryogenic calli derived from leaf base explants on MS medium containing $11.3 \mu\text{M}$ 2,4-D after 21 days of culture (Scale bars indicate 0.5 cm); (D) Mature embryo-derived calli showing regeneration of shoots and whitish leathery embryoid structures on MS medium containing ($4.43 \mu\text{M}$ BAP) and kinetin ($4.64 \mu\text{M}$) after 21 days of culture (Scale bars indicate 0.5 cm); (E) Regenerated shoots showing elongation and root induction on MS medium supplemented with IBA ($0.49 \mu\text{M}$) and (F) *In vitro* regenerated plantlet of *P. glaucum* transferred into a plastic cup

reported to be mediated by the relative amount of auxins and cytokinins²¹, in this study the frequency of plant regeneration was enhanced by addition of cytokinins in the medium without auxins, as reported in *Sorghum dimidiatum*²². Although BAP or kinetin could induce shoot regeneration their combined application enhanced shoot regeneration (Table 2). Although good amount of callus was obtained from the leaf base explants, the frequency of shoot regeneration was found to be lower than that obtained from mature embryo derived callus (Table 2). Similarly, calli from mature embryo gave more number of shoots per callus than leaf-derived calli. The regeneration frequency in both the explants was maintained up to seven months. However, regeneration frequency was found to decline after fourth subculture and the mean number of plants was 3.3 per callus culture of mature embryo and 2.01 plants per callus culture of leaf base explants.

Further, the primary calli of both the explants were cultured on MS basal medium devoid of growth regulators for somatic embryogenesis. The calli formed from mature embryo produced two types of somatic embryoids. Calli cultured on MS basal medium induced globular, translucent and slimy somatic embryoids (Fig. 1B). In contrast, the calli

Table 1 — Effect of 2,4-D on callus induction (%) from mature embryo and leaf base explants on MS medium after four weeks

2,4-D Conc. (μ M)	of culture [Values are $\pm$ SD] Callus induction (%)	
	Mature embryo	Leaf base
	Mean $\pm$ sd	Mean $\pm$ sd
2.25	00 $\pm$ 00 ^a	00 $\pm$ 00 ^a
4.50	28.0 $\pm$ 2.25 ^b	14.5 $\pm$ 0.75 ^c
6.75	52.5 $\pm$ 1.75 ^d	27.0 $\pm$ 1.95 ^e
9.00	67.5 $\pm$ 2.35 ^f	35.3 $\pm$ 1.08 ^g
11.30	75.0 $\pm$ 1.58 ^h	42.0 $\pm$ 1.48 ⁱ
13.50	62.5 $\pm$ 2.20 ^j	29.5 $\pm$ 2.24 ^e
15.75	51.0 $\pm$ 2.75 ^k	21.5 $\pm$ 2.15 ^l
18.00	22.4 $\pm$ 1.65 ^l	19.0 $\pm$ 1.05 ^l

Data were recorded after three weeks. Data were analysed by ANOVA test and means were separated by LSD; means followed by identical alphabets were not statistically different within the column and rows. ($P < 0.05$).

Table 2 — Morphogenetic responses of the calli of mature embryo and leaf base explants on MS medium supplemented with different concentrations of BAP and kinetin.

BAP (μ M)	Kinetin (μ M)	Mature embryo callus		Leaf base callus	
		% regeneration	Shoots/callus (mean $\pm$ SD)	% regeneration	Shoots/callus (mean $\pm$ SD)
2.21	-	0	0	0	0
4.43	-	30.7	4.7 $\pm$ 1.23 ^a	42.7	3.8 $\pm$ 0.04 ^a
6.64	-	19.4	3.3 $\pm$ 0.41 ^{ab}	54.7	1.2 $\pm$ 0.54 ^b
8.86	-	0	0	0	0
-	2.32	0	0	0	0
-	4.64	55.1	3.8 $\pm$ 0.04 ^{ab}	25.4	2.3 $\pm$ 0.05 ^{ab}
-	6.96	40.2	2.2 $\pm$ 1.24 ^{bc}	17.4	2.6 $\pm$ 0.31 ^{ab}
-	9.29	12.5	1.6 $\pm$ 0.42 ^c	8.4	1.4 $\pm$ 0.21 ^{bc}
2.21	2.32	68.7	10.2 $\pm$ 1.14 ^{df}	39.5	5.4 $\pm$ 0.18 ^e
4.43	4.64	70.1	12.2 $\pm$ 2.54 ^d	60.4	6.6 $\pm$ 1.42 ^e
6.64	6.96	64.5	9.4 $\pm$ 0.25 ^f	37.8	4.7 $\pm$ 1.23 ^e
8.86	9.29	0	0	0	0

Data were recorded after four weeks. Data were analysed by ANOVA test and means were separated by LSD; means followed by identical alphabets were not statistically different within the columns and rows. ($P < 0.05$).

Table 3 — Effect of auxin on root induction in regenerated shoots of *Pennisetum glaucum* on MS medium

Auxin (μM)	Shoots from mature embryo callus		Shoots from leaf base callus	
	Rooted shoots (%)	Number of roots/shoot (means $\pm$ SD)	Rooted shoots (%)	Number of roots/shoot means $\pm$ SD)
IBA				
0.04	53	10.1 ± 1.14^a	51	8.4 ± 1.31^a
0.49	72	18.4 ± 2.06^b	64	15.4 ± 2.21^c
2.46	49	8.2 ± 0.71^{ah}	48	11.5 ± 1.12^d
4.92	37	5.1 ± 0.5^e	35	7.5 ± 0.5^{fa}
IAA				
0.05	19	2.4 ± 0.16^g	9	$2.8 \pm 0.03+g$
0.57	28	5.6 ± 0.19^e	11	4.5 ± 1.04^e
2.85	31	6.7 ± 1.12^{eh}	28	6.1 ± 1.01^{ef}
5.7	40	8.5 ± 1.43^{ha}	35	7.2 ± 1.40^{ha}
NAA				
0.05	9	2.1 ± 0.24^g	9	3.2 ± 0.12^g
0.53	15	2.3 ± 0.07^g	11	4.1 ± 0.09^e
2.86	27	5.5 ± 0.74^e	27	5.9 ± 0.21^e
5.37	35	8.8 ± 1.0^i	30	6.5 ± 0.5^j

Data were recorded after four weeks. Data were analyzed by ANOVA and means were separated by LSD; means followed by identical alphabets were not statistically different within the columns and rows. ($P < 0.05$).

cultured for regeneration on MS medium containing BAP and kinetin induced white, leathery embryo-like structures unexpectedly (Fig. 1D), which did not give rise to plantlets after one month culture. Moreover, the embryogenic calli of leaf base explants induced yellowish, shiny, globular somatic embryoids (Fig. 1C) on MS basal medium. The embryo-like structures derived from both mature embryo and leaf base explants were removed separately and subcultured on MS basal medium devoid of growth regulators for regeneration and they were readily converted into plantlets. However, differences were observed in terms of frequency of somatic embryogenesis and mean number of plantlets between mature embryo and leaf base explants. It was observed that 42.5% of mature embryo-derived calli formed somatic embryos and produced an average of 7.2 plants per culture, whereas in the case of leaf base explants the percentage of somatic embryogenesis

was 15.5% and 4 plants were obtained per culture. These observations indicate that phenotype of the somatic embryoids induced from these explants and the efficiency of plant regeneration may be dependent on plant growth regulators used and the type of explants.

MS medium with $0.49 \mu\text{M}$ of IBA was found to be the best for root induction of shoots. (Table 3, Fig. 1E). However, the frequency of root formation varied with different concentrations of various auxins (Table 3). Maximum rooting frequency of shoot was recorded on MS medium supplemented with IBA ($0.49 \mu\text{M}$). Sixty-five phenotypically normal plants were transferred to plastic cups and out of these forty-three plants survived and resumed growth. The fully developed plantlets were established in the soil and they showed 65% survival (Fig. 1F). The regenerated plants showed normal morphology under *in vivo* conditions.

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