In vitro plant development of *Eleusine coracana* via indirect organogenesis and somatic embryogenesis using mature seeds as explants

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A complete plant regeneration system has been developed using mature seeds as explants. Mature embryos were used for induction of callus and regeneration of finger millet GE-3885 genotype. Highest callus induction and proliferation was found with MS + 1.5 mg/l NAA and MS+0.5 mg/l NAA respectively. Somatic embryos were obtained in MS + 0.5 mg/l 2,4-D. Histological analysis of somatic embryos revealed globular-shaped structure. Plantlets attained good length of shoots and roots on MS + 1.5 mg/l BA and MS + 1.5 mg/l IBA respectively, and were acclimatized under glasshouse conditions after proliferation of roots in hydroponics system.

Keywords: Embryogenesis, finger millet, indirect organogenesis, mature seeds, somatic millets.

MILLETS are the world's sixth most important food crops among cereals and are grown in Asian and African countries. There are two types of millets – major and minor. The minor millets have the ability to grow in infertile soils under severe drought and harsh conditions and are usually grassy plants having short, slender culms and small grains. Finger millet (*Eleusine coracana* (L.) Gaertn.), also known as African millet, is one of the eight minor millets which has outstanding attributes as sustenance food crop grown in Africa and South Asia. For millions of people in tropical drylands regions, it is a primary food source and is grown in more than 50 mt globally[1]. In India, finger millet constitutes about 81% of the minor millets produced. It has nutritional qualities superior to that of rice and is on par with that of wheat[2]. Finger millet grains are grounded and supplemented as healthy food for infants and diabetic patients, as they are rich in calcium, iron, phosphorus and proteins[3]. With a focus on these nutritional properties of finger millet, its production can be enhanced in biotic and abiotic stress-prone areas by developing genetic resistance in the genotype against major biotic (leaf blast disease) and abiotic (drought and cold stress) which constraints in seed yield loss in finger millet. So far, not much attention has been given to genetic transformation studies of finger millet compared to other major cereals crops[4,5]. Though a couple of studies have been reported on callus induction, somatic embryogenesis, maturation of somatic embryos and plant regeneration, all these studies, directly or indirectly, focus upon demolishing and raising plants against abiotic and biotic stresses or have used those varieties which are resistant to these stresses[6–10]. Also, protocols for systemizing callus induction, somatic embryogenesis and plant regeneration in finger millet varieties which are high in protein content are not yet reported. Thus understanding the pathways for nitrogen uptake and assimilation in this genotype could help in producing nitrogen use efficient crops.

High-frequency plant regeneration system along with efficient transformation protocol is desirable for genetic improvement of a species[11]. As monocotyledonous plants are recalcitrant in nature, their *in vitro* plant regeneration is less efficient. If a suitable protocol for plant regeneration is available, genetic transformation can be carried out efficiently throughout the year[12].

In the present study, phytohormone concentration and combination for developing highly regenerative embryogenic and non-embryogenic calluses are standardized. Also, complete plant regeneration from these calluses is studied.

Materials and methods

Plant materials

The mature seeds of *Eleusine coracana* (GE-3885, high protein content) were obtained from the Department of Molecular Biology and Genetic Engineering, GB Pant University of Agriculture and Technology, Pantnagar, Uttarakhand, India and used as explants.

Callus induction

Pretreatment of 20 min in sterile water was given to remove husk. The dehusked seeds were subjected to surface

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disinfestation with a few drops of Tween 20 for 3 min, followed by three washes with double-distilled water. Seeds were subsequently treated with 0.8% sodium hypochloride for 8 min, followed by three washes, each of 2 min duration with double distilled water. Then the seeds were rinsed with 70% ethyl alcohol for 2 min with continuous stirring and final washing was done two times with sterile distilled water. Sterilized seeds were aseptically inoculated on MS medium[13] supplemented with 3% (w/v) sucrose, 0.8% (w/v) agar (pH 5.8) and various combinations of different plant growth regulators for induction of callus from seeds under photoperiod of 16 h light at 27° ± 2°C. An experiment was carried out for identification of the optimal plant growth regulator, its concentration and combination for callus induction (Table 1).

Proliferation of callus

Callus was subcultured after 21 days on proliferation medium which contained lower concentration of auxin, i.e. 0.5 mg/l and 1.0 mg/l NAA. Callus was also subcultured on basal medium for comparing the response of different concentrations of auxin on callus proliferation (Table 2).

Induction, proliferation and maturation of somatic embryogenesis

For somatic embryogenesis, 21-day-old, well-proliferated, yellowish-white, compact calluses were used. Callus which was subcultured for proliferation on MS medium was used as control to determine the percentage of somatic embryogenic callus formation in finger millet. Cultures were maintained in the dark at 25° ± 2°C for seven weeks. Every two weeks of incubation, embryogenic cultures were routinely transfered on fresh medium with the same composition. The percentage of somatic embryogenesis and average number of globular-shaped embryos formed on MS medium containing 2,4-dichlorophenoxyacetic acid (2,4-D) in combination with benzyl adenine (BA) were determined after seven weeks of incubation in the dark. Influence of 2,4-D along with BA on the formation of different stages of somatic embryos was analysed after seven weeks of incubation. Then these stages were examined under a light microscope at magnifications of 4×, and 10×, and photographed.

Histological analysis of somatic embryos

The proliferation of somatic embryos and maturation stage were fixed in formaldehyde acetic acid ethanol (FAA) for 24 h in accordance with the method of Boissot *et al.*[14]. An aspirator set-up was used to remove air entrapped in the tissues. Tissue was then dehydrated in tertiary butyl alcohol series (10, 20, 35, 50 and 75 ml) for different time intervals (60, 60, 45, 45 and 30 min respectively). After dehydration, the embryogenic calluses were cleared and dealcohalized in xylol and absolute alcohol series. The callus tissue was infiltrated with paraffin (55–58°C) and sections were cut using rotary microtome (Ao Spencer, 820 Microtome, Pennsylvania, USA) to thickness of 6 µm. The cuttings were placed onto slides and fixed with Haupt's adhesive. Staining and mounting were done using safranin and diphenylxylene respectively.

Plant regeneration from somatic embryos

Well-developed somatic embryogenic calluses were transferred in MS basal, half MS medium, MS + 1.5% sucrose (w/v), MS + 1.5% sucrose + half nitrate and MS + half nitrate. The callus cultures were incubated in the light at 25° ± 2°C for four weeks of incubation. Then the percentage of plantlets produced by each embryogenic callus was determined.

Table 1. Callus response observed after three weeks of mature embryo culture in different media. Data were recorded from 10 replicates

Combination of phytohormones (mg/l)	Colour	Texture
2.0 2,4-D	Cream	Compact
2.5 2,4-D	Brownish	Friable
3.0 2,4-D	White	Compact
3.5 2,4-D	Brownish	Friable
0.5 2,4-D + 0.5 BAP	Cream	Compact
2.5 2,4-D + 0.5 BAP	Brownish	Compact
3.5 2,4-D + 0.5 BAP	Greenish	Compact
3.0 2,4-D + 1.0 Kinetin .	Brownish	Friable
3.0 NAA + 1.0 Kinetin	White	Friable
0.5 NAA	White	Friable
1.0 NAA	White	Friable
1.5 NAA	White	Compact
2.0 NAA	White	Friable
2.5NAA	White	Compact
3.0 NAA	Brownish	Friable
3.5 NAA	Greenish	Friable

Table 2. Media composition for callus proliferation and morphological analysis of the callus observed after 21 days of proliferation

Concentration of phytohormone (mg/l)	Callus proliferation (%)	Colour
MS basal medium	25	Yellow
0.5 2,4-D	75	Green
0.5 NAA	100	Greenish-white
1.0 NAA	50	White

Plant regeneration from callus

Well-proliferated calluses were subcultured on MS medium supplemented with different concentrations of BA alone (0.5, 1.0 and 1.5 mg/l) and in combination with NAA (0.5 NAA + 1.5 BA, 0.5 mg/l NAA + 2.0 mg/l BA and 0.5 mg/l NAA + 3.0 mg/l BA). The cultures were incubated at 27° ± 2°C under photoperiod of 16 h with cool white fluorescent light. Until excision of the shoots, the media were changed every 15 days. In this experiment best plant growth regulator combination was identified for plant regeneration.

Root induction

Elongated shoots (6–8 cm long) were transferred to MS medium supplemented with IBA. For root induction, four different concentrations of IBA were used (0.5, 1.0, 1.5 and 2.0 mg/l). The cultures were incubated at 27° ± 2°C under 16 h photoperiod with cool white fluorescent light (60–70 µm M^{-2} s^{-1}). Until induction of roots, the media were changed every 15 days. Mean number of roots and average root length were recorded after two weeks of incubation.

Elongation of roots and acclimatization

Rooted plants were carefully removed from rooting media and cleaned with deionized water to remove excess media present on the surface of the roots. Isolated rooted plantlets were transferred in Hoagland nutrient solution for 15 days at 27° ± 1°C. Then they were transferred to pots containing autoclaved soil and maintained in natural conditions for further development.

Results and discussion

Callus induction and proliferation

For callus induction and embryogenic callus fomation, two different types of auxin (2,4-D and NAA) were used individually and also in combination with two different types of cytokinin (BA and kinetin). Mature seeds of finger millet were cultured on MS medium supplemented with different concentrations and combinations of phytohormones for three weeks of incubation. The mean percentage of callus induction ranged from 63 to 97.61 based on the type and concentration of phytohormone in the medium (Table 1 and Figure 1). MS medium supplemented with 1.5 mg/l NAA produced highest percentage (97.6%) of callus induction compared to those containing different concentrations of NAA (1.0 mg/l – 92.0%; 2.0 mg/l – 88.6% and 2.5 mg/l – 86.0%). Best results for callus induction were found using NAA, but optimum response of embryogenic callus was obtained using 2,4-D alone and also in combination with kinetin. When only 2,4-D was used, the highest callusing of 89.5% was obtained for 3.0 mg/l concentration, while only 77.6% of callus induction was obtained on using 3.0 mg/l 2,4-D + 1.0 mg/l kinetin. These results are in agreement with the previous studies on finger millet[2,9] and kodo millet[10]. Auxins play an important role in callus induction in finger millet and cereals. Induction of callus in millets or other cereals is frequently accomplished by supplementing 2,4-D in MS medium[12,15]. When compared to 2,4-D, NAA was found to be better for callus induction. Both 2,4-D and kinetin have also been used for callus induction and somatic embryogenesis. For callus proliferation a lower concentration of auxin was used and 0.5 mg/l NAA was optimized for the same (Table 2; Figure 2 *a* and *b*).

Induction of somatic embryogenesis

Auxin is the most important phytohormone affecting plant cell division, morphology and differentiation at the beginning of somatic embryogenesis[16]. Proliferated callus cultures were selectively transferred onto MS medium containing different concentrations of 2,4-D and also onto MS medium containing 2,4-D in combination with BA (induction medium). After four weeks of incubation in induction medium, the callus was transferred to somatic embryos proliferation medium (hormone-free medium) for the next four weeks. Among different concentrations and combinations used (induction medium), the maximum amount of somatic embryogenesis was seen in

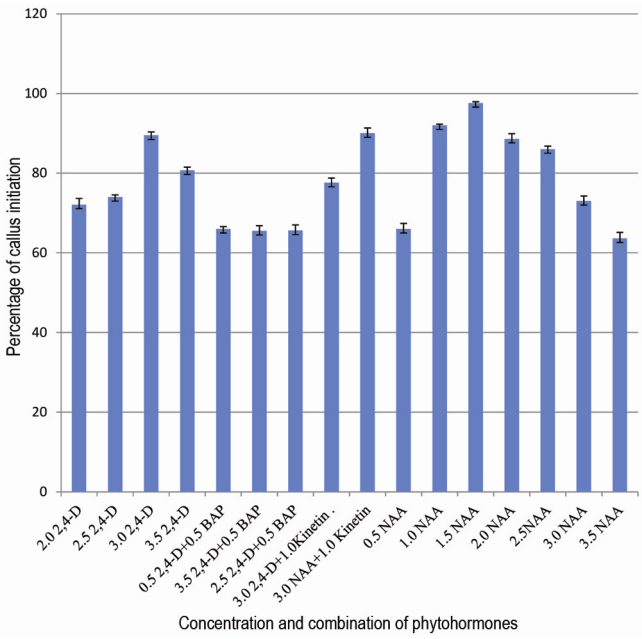

Figure 1. Callus response observed after three weeks of mature embryo culture in different media.

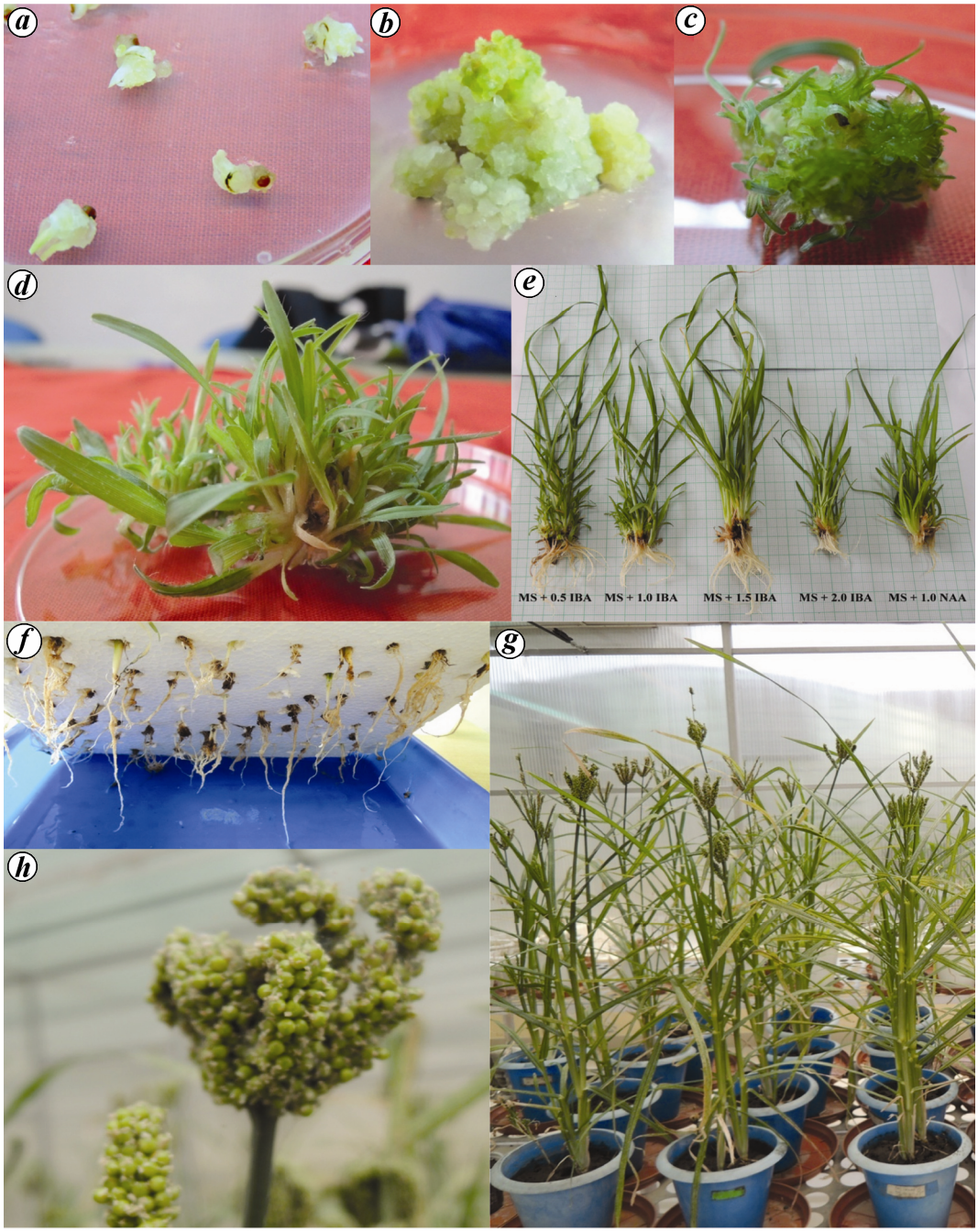

Figure 2. ***a***, Callus induction from mature seeds of *Eleusine coracana* on MS medium + 1.5 mg/l NAA. ***b***, Callus proliferation on MS medium + 0.5 mg/l NAA. ***c***, Plant regeneration from callus proliferated for 21 days on MS medium + 1.5 mg/l BA. ***d***, Shoot proliferation from regenerated embryogenic calluses. ***e***, Root induction from well-shooted plants from embryogenic calluses on MS medium supplemented with different concentrations of phytohormones. ***f***, Root proliferation in hydroponics system after seven days in Hoagland solution. ***g*** and ***h***, Regenerated and well-rooted plantlets transplanted in the glasshouse showing normal morphological characters and good seed setting at maturity stage.

0.5 mg/l of 2,4-D, along with 1 mg/l of 2,4-D after eight weeks of incubation. Lowest percentage of somatic embryogenesis was found in MS medium supplemented with 3.0 mg/l 2,4-D + 0.5 mg/l BA (Figure 3).

Localized group of cells was produced by embryogenic calluses, differentiated into embryoids on the peripheral region of the callus and later differentiated into white, globular embryoids after 28 days of incubation (Figure 4 *a* and *c*). This process continued in subsequent subculturing in MS medium supplemented with 5×10^{-3} mg/l abscisic acid resulting in the production of post-globular to heart-shaped embryos. On maturing, these somatic embryos were differentiated into cylindrical shape or elongated club-shaped and initiation of cotyledon, as well

Table 3. Effect of different media on regeneration of somatic embryos

MS medium	Shoot initiation (%; mean ± SE)	No. of shoots per embryogenic callus (mean ± SE)
MS basal	95.2 ± 2.68	8.0 ± 0.53
Half MS medium	66.6 ± 1.66	3.9 ± 0.21
MS + 1/2 sucrose	89.2 ± 0.37	4.2 ± 0.08
MS + 1/2 nitrate	84.1 ± 0.79	4.6 ± 0.16
MS + 1/2 sucrose + 1/2 nitrate	93.3 ± 3.33	5.8 ± 0.13

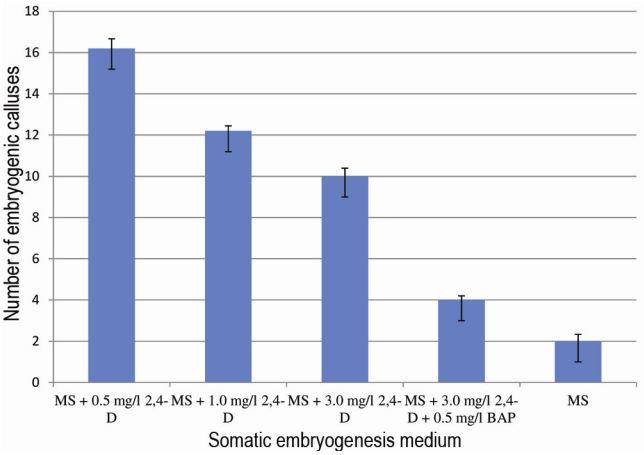

Figure 3. Effect of different concentrations of phytohormones on induction of somatic embryos. Experiments were performed with three replicates (for each treatment 20 calluses were inoculated).

as signs of initiation of scutellum occurred after 45 days of incubation. Green coleoptile was visible and shoot completely differentiated with one or two leaf plumules (Figure 4 *e*).

Histological analysis of somatic embryos

Microscopic examination confirmed the presence of globular and heart stages of somatic embryos which were freely attached on the callus and contained different layers with well-developed suspensor cells. Histological observations showed that somatic embryos originated from the more external cells of the embryogenic calluses, in agreement with previous observations in sugarcane[17], guinea grass[18] and oil palm[19]. Histological analysis of embryogenic globular and heart-shaped tissues confirmed that they had proliferated to form a mass of cells, is identified as embryogenic mass. Compact tissues were found in the central portion which was equivalent to the nodular callus and the outward loose portion corresponded to friable callus. These observations confirmed by a study in maize[20], indicated that the formation of prominent air spaces is due to the death and differentiation of vacuolated cells in the cell aggregates. Cross-section analysis revealed the asynchronous embryo development with compact cell mass and dense cytoplasm as characteristics. Embryos at globular and heart stage of growth could be noticed in the histological observations (Figure 4 *b*, *d* and *f*).

Plant regeneration from somatic embryos

The percentage of somatic embryogenic callus producing rooted shoots was determined by subculturing somatic embryogenic calluses with nodular structures containing coleoptiles, root, and shoot poles derived from each treatment onto regeneration medium consisting of MS basal medium supplemented with half MS medium, MS + 1/2 sucrose, MS + 1/2 nitrate and MS containing 1/2 sucrose + 1/2 nitrate. Germination of a somatic embryo was apparent by the emergence of a coleoptile and coleorhiza simultaneously within a week of transfer. Somatic embryos were regenerated at the highest rate (95.2%) with 8 shoots per embryogenic callus when cultured on MS basal medium. MS medium containing 1/2 sucrose + 1/2 nitrate also produced the significant response (93.3%) with formation of 5.8 leaves per embryogenic callus. MS medium containing 1/2 sucrose and 1/2 nitrate produced average percentage of shoots (89.2 and 84.1 respectively) with an average of 4.2–4.6 shoots per embryogenic callus. Half MS medium was less effective than the other four combinations in induction of shoot regeneration (Table 3). Multiple shootings were also observed when shoot regenerated embryogenic callus was subcultured on the same media.

Shoot formation and plant regeneration

The induction of shoots in cereals and millets is commonly achieved by BA or thidiazuron[9,21]. In order to achieve direct adventitious shoot regeneration in finger millet, proliferated callus was subcultured on MS medium containing various concentrations of BA and also in combination with NAA (Table 4). Among different combinations and concentrations of phytohormones used, the highest percentage of callus regeneration (76) was obtained when MS medium was supplemented with 1.5 mg/l BA, where mean numbers of 37 viable shoots per callus of 7 cm length were observed. When NAA was used in combination with BA, the highest percentage (62.05) of callus regeneration was observed in MS medium

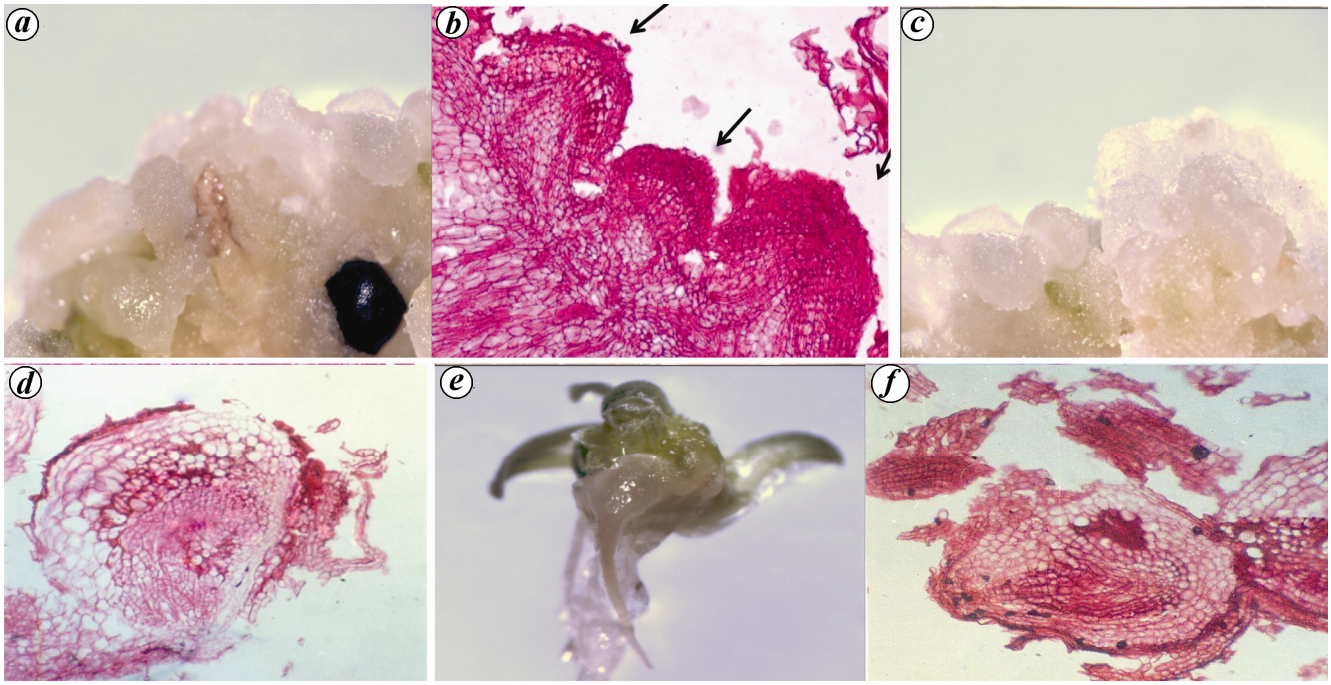

Figure 4. ***a***, ***c***, Embryogenic callus showing oval-shaped embryos with distinct globular shapes. ***b***, ***d*** and ***f***, Histological analysis of somatic embryogenesis in finger millet showing embryogenic cells with globular-shaped embryo. ***e***, Emergence of coleoptile and coleorhizae from somatic embryos.

Table 4. Effect of MS medium supplemented with different concentrations of BAP and NAA on morphological parameters of regenerated calluses. Data were recorded after 21 days of inoculation

Combination of phytohormones (mg/l)	Callus regeneration (%)	Average no. of shoots per callus	Average length of regenerated shoots (cm)	Average no. of leaves per plant
0.5 BAP	44.6 ± 1.33	14.3 ± 1.33	2.7 ± 0.21	2.4 ± 0.14
1.0 BAP	59.5 ± 1.33	20.6 ± 1.33	6.6 ± 0.41	4.6 ± 0.66
1.5 BAP	76.3 ± 1.57	37.0 ± 1.75	7.0 ± 0.32	4.0 ± 0.57
0.5 NAA + 1.5 BAP	62.05 ± 1.45	35.0 ± 1.85	5.0 ± 0.57	1.8 ± 0.63
0.5 NAA + 2.0 BAP	41.2 ± 1.57	26.3 ± 1.13	5.9 ± 0.33	4.9 ± 0.23
0.5 NAA + 3.0 BAP	42.03 ± 1.54	Very small	1.9 ± 0.33	1.0 ± 0.42

supplemented with 0.5 mg/l NAA + 1.5 mg/l BA, in which average number of shoots, shoot length and number of leaves per plant were 35, 5 and 1.8 cm respectively (Figure 5). BA is one of the active phytohormones frequently used for tissue culture of monocotyledonous plants[22]. We found that BA alone and in combination with NAA resulted in the formation of consistent multiple shoot clumps within 14 days, that continued further proliferation and maturation (Figure 2 *c* and *d*).

Root induction

Immediate rooting occurred when the elongated shoots were subcultured on MS medium containing various concentrations of IBA (Table 5). The best root growth response in terms of root induction, mean root length (5 cm) and number of roots per plant (13) was observed when 1.5 mg/l of IBA was incorporated into MS medium (Figure 2 *e*). Spontaneously, roots were developed after two weeks even in the absence of IBA in the medium. These results are in agreement with those of Ahmad *et al.*[23] in wheat. Sharma *et al.*[24] observed good rooting response in barley on MS medium containing 4.9×10^{-3} mg/l IBA.

Acclimatization and field transfer

The rooted shoots obtained from organogenesis and somatic embryogenesis were transferred to Hoagland nutrient solution for 15 days for root proliferation before being transferred to greenhouse conditions. Finally, the plants were transplanted into pots with 100% survival rate (Table 6). All *in vitro* regenerated plants grew well and showed similar phenotypic homogeneity such as

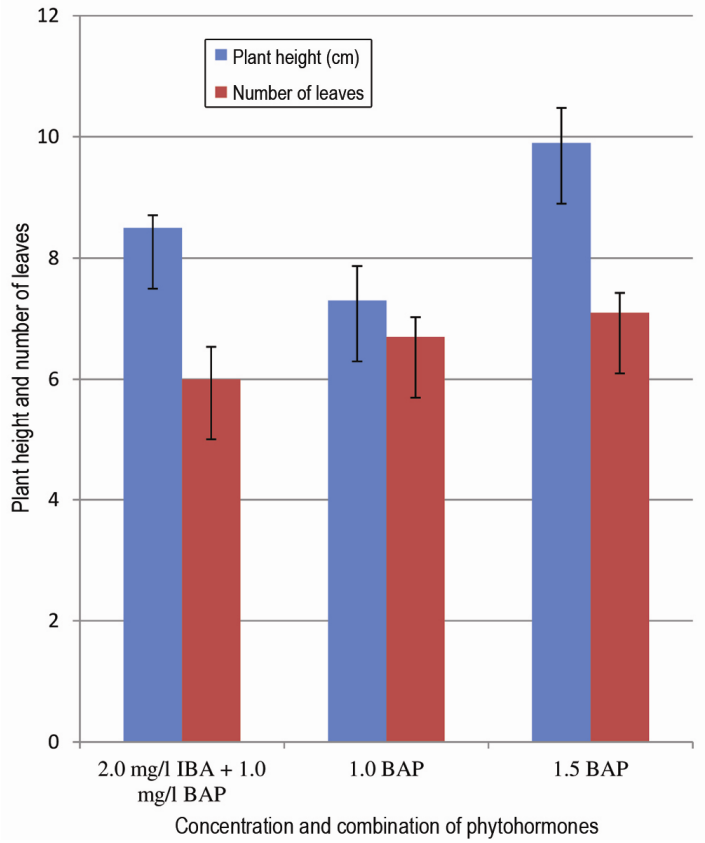

Figure 5. Shoot proliferation and analysis of morphological parameters in MS medium supplemented with different concentrations of BA.

Table 5. Root induction in proliferated shoots. Data were observed after 21 days of subculture

Concentration of phytohormone (mg/l)	Root length (cm)	No. of roots per plant
0.5 IBA	5.03 ± 2.52	9.12 ± 6.16
1.0 IBA	4.13 ± 0.15	10.81 ± 7.24
1.5 IBA	5.23 ± 0.15	13.06 ± 8.78
2.0 IBA	3 ± 0.60	6.65 ± 4.63
1.0 NAA	2 ± 0.23	4.25 ± 2.35

Table 6. Height of plants and number of leaves per plant at harvesting stage

Pot no.	Plant height (cm)	Number of leaves
1	70.33 ± 1.52	9.75 ± 1
2	64 ± 2	4.5 ± 1
3	74 ± 1	5.25 ± 2
4	65.33 ± 3.51	5 ± 1.52
5	77.33 ± 2.08	6.25 ± 1.52
6	76.33 ± 3.05	8.25 ± 1
7	77 ± 3	5.25 ± 1
8	70 ± 2	5.25 ± 2
9	72.33 ± 3.51	5.25 ± 2
10	64 ± 3	4.75 ± 2.51
11	60.33 ± 1.52	4.75 ± 1.52
12	71 ± 1	5.75 ± 1.52
13	63 ± 2	5.5 ± 2.08
14	65.66 ± 1.52	6.75 ± 2
15	64.66 ± 1.52	3.75 ± 2.64

flowering, seed-setting, and grain yield, as in the case of plants grown from seeds in natural environment (Figure 2 *f–h*).

Conclusion

In this study, high-frequency complete regeneration protocol using mature seeds as explants has been optimized. Different plant growth regulators were used, namely NAA, BA, 2,4-D and IBA. Regeneration of both somatic embryo and embryogenic callus was also optimized. Exposure of embryogenic callus to different concentrations of carbon and nitrogen led to the induction of somatic embryos, which was confirmed by histological analysis. Finger millet, due to its recalcitrant property, is not a good choice for research, especially in tissue

culture. Very few studies have been reported on transformation of finger millet, and only limited studies are available on reporter gene assessment and expression of marker gene in this crop.

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