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Influence of Organic Derivatives on Direct Regeneration of finger millet genotype CO 9

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***Abstract*-** Finger millet (*Eleusine coracana* (L.) Gaertn.) is a commercially important food crop extensively cultivated in the arid and semi-arid tropical regions of India and East Africa. The current study was designed to develop an efficient protocol for micropropagation by the influence of additives to enhance the number of shoots per explant from Shoot Apical Meristems (SAMs) of finger millet genotype CO 9. The highest shoot regeneration frequency (95.89%) with an average of 46.52 shoots per explant and 10.86 cm shoot length per explant was achieved when SAMs were cultured in Shoot Regeneration Medium (SRM) containing Murashige and Skoog's (MS) medium supplemented with 3.0 mg/L 6-Benzyl Amino Purine (BAP), 2.0 mg/L Kinetin and 5% coconut water, 300 mg/L proline and 400 mg/L casein enzymatic hydrolysate, and 3 mg/L glycine. Sub-culturing the SAMs in SRM at 2 weeks interval for 8 weeks resulted in an increase in the number of shoots per explant. The highest rooting frequency (100%) with an average root length of 7.32 cm was obtained on full-strength MS medium supplemented with 0.25 mg/L IAA. and successfully acclimated in the field, subsequently developed into fertile plants. Thus, the procedure described is a rapid and consistent method useful for efficient large-scale propagation and genetic transformation in finger millet.

***Keywords*-** Eleusine coracana, shoot apical meristems, Shoot Regeneration Medium, Random amplified polymorphic DNA

I. INTRODUCTION

Finger millet (*Eleusine coracana* [L.] Gaertn.), a small grain crop, cultivated in more than 4 million ha worldwide is the staple diet for underdeveloped populations of Africa [1]. Millets are coarse-grained hardy crops of the Poaceae (Gramineae) family that include 10 genera and 14 species. Millets are the first domesticated crops of the tropics owing to their rapid growth, minimal harvesting period, and tolerance to abiotic stress like drought and high temperatures [2-3]. Compared to other cereal crops, Finger millet rich in amino acids (cysteine, tyrosine, methionine, and tryptophan) and minerals (calcium, phosphorus, and iron) [4]. These granules are vastly used in the food industry for the preparation of cakes and bread. There is a high demand for nutrient-rich finger millet is on the rise in recent years. In order to meet the worldwide dietary demand, there is an immediate necessity to improve existing crops with new approaches and technologies for abiotic and biotic stress tolerance [5]. One of these methods involves the generation of transgenic crops with desired traits. In the case of millets, little genetic improvement has been done so far, specifically using wide- or cross-hybridization among the closely related species [6]. Advances in regeneration and genetic transformation have been reported in cereal crops (rice, wheat, maize, oats, and barley), very less attention given so for improvement of millets [7]. There are various explants used for *in vitro* regeneration in finger millets *like* mesocotyl, leaf base segments, root and immature inflorescence [8-11], mature seeds [12-16], shoot tips [17-18]. Regeneration of whole plants from callus is a time-consuming process as redifferentiation of shoots requires multiple sub-culturing cycles. During the prolonged culture, most of the calli lose their regeneration potential [19-21]. In addition, the use of callus tissue for transformation makes it difficult to identify competent cells for plant regeneration [22-23]. From the literatures that there is no universal recipe for efficient culture and induction of direct shoot regeneration depends on the nature of the plant organ from which the explant was derived, and is highly dependent on plant genotype. Direct regeneration is an alternative approach to establish whole plants using shoot apical meristems (SAMs) present at the apical dome. SAMs possess superior redevelopment ability and thus stimulate multiple shoots clumps within a short duration of time [23-24]. Direct shoot regeneration is achieved by SAMs in monocots like rice [23- 24] wheat [25] maize [26-27] sorghum [28] pearl millet [29] oats, and barley [30-31].

Reports are also available on use of amino acids in media, plays an important role to improve callus induction, shoot and root formation in various plants; viz: wheat [32-33] pearl millet [34] maize [35], sorghum [36], and naga chilli [37-38]. Micropropagated plantlets are commonly subjected to genetic fidelity analysis using molecular markers to confirm their clonal identity to the mother plant [39]. Random amplified polymorphic DNA (RAPD) is an efficient marker system that makes use of arbitrary primers to cover the entire regions of the genome and has been routinely used to identify intra- and interspecies genetic variation in crops [40]. In the Present study, we report an efficient direct plant regeneration protocol for Indian finger millet genotypes CO 9 using amino acids and organic derivates. The genetic identity of the micropropagated plantlets from the genotype CO 9 was confirmed using RAPD markers.

II. MATERIALS AND METHODS

Plant Material and Surface Sterilization

Seeds of finger millet genotype CO9, obtained from the Tamil Nadu Agriculture University, Coimbatore, India. The seeds were de-husked in running tap water for 5 min and surface-disinfected using 0.1% (w/v) mercuric chloride for 5 min followed by 70% (v/v) ethanol for 25 s. The seeds were then rinsed five times with autoclaved, double-distilled water, blot dried on sterile filter paper.

Culture Conditions

Surface sterilized seeds were blot dried using autoclaved filter paper in a dry petri dish. The seeds were transferred to Petri plate containing MS medium [41] amended with sucrose 3% (*w/v*) and solidified with 0.8% agar. The pH of the medium was changed to 5.8 ± 0.02 using 1N NaOH or HCl, before the inclusion of agar and autoclaved at 121°c /15 lbs pressure for 20 mins. The seeds were cultured at 25 ± 2°c with a photoperiod of 16 hours light and 8 hours dark for 8 days.

Effect of cytokinins

Three days old *in vitro* germinated seedlings with Shoot Apical Meristem (SAM) 4 – 6 mm in size were used as explants. SAM explants were then transferred to Shoot Growth Medium (SGM) containing MS basal salts and vitamins supplemented with 6 – Benzylaminopurine (BAP) ,Kinetin (KIN) (0.5, 1.0, 2.0, 3.0 and 4.0 mg/L) individually and combination of BAP 3.0 mg/l and KIN (0.5, 1.0, 2.0, 3.0 and 4.0 mg/L) concentrations for *in vitro* propagation. Cultures were nurtured in glass bottles (15 cm height × 10 cm diameter) and incubated at 25 ± 2°c in 16/8h (light/dark) with a photosynthetic photon flux density of 50 µ mol m^{-2} S^{-1} supplied with cool white fluorescent lamps (Philips, Chennai, India). The shoot regeneration frequency was observed (number of explants with shoots/shoots per explants × 100) after 8 weeks, the total number of shoots per explant and average shoot length, were recorded. Shoot explants were subcultured at the 2nd week in the same medium. The experiment was repeated thrice with each treatment using four replicates.

Effect of cytokinins and coconut water

Shoot apical meristem were cultured on MS medium containing 3% (w/v) sucrose with 0.8% (w/v) agar and supplemented with 5% (w/v) coconut water and different concentrations and combinations of plant growth regulators, including BAP 0.5-4.0 mg/L, KIN 0.5-4.0 mg/L and combination of BAP 3.0 mg/L and KIN 0.5-4.0 mg/L .

Effect of amino acid and organic derivative

The best PGR identified (3.0 mg/L BAP and 2.0mg/L KIN) from the above experiment were further examined to maximize multiple shoot regeneration. The influence of some of the essential amino acids and organic derivatives such as proline (Pro; 50, 100, 200, 300, 400, 500 mg/L), glycine (Gly; 0.5, 1.0, 2.0, 3.0, 4.0, and 5.0 mg/L) casein enzymatic hydrolysate (CEH; 200, 300, 400, 500, 750, or 1000 mg/L), and 5% coconut water was systematically observed to improve shoot induction and proliferation. The cultures were incubated at 25±2°C for 16/8-h light/ dark conditions. The percentages of responding explants, mean number of shoots induced in each explant, and shoot lengths were calculated after 8weeks incubation in light.

Rooting Medium

In vitro grown shoots (3 – 6 cm long, 8 weeks old) were isolated individually from the multiple shoot clumps. The separated shoots were transferred to MS medium amended with different concentration of (0.1, 0.25, 0.5 and 1.0 mg/L) Indole Acetic Acid (IAA), (0.1, 0.25, 0.5 and 1.0 mg/L) Indole Butyric acid (IBA) and (0.1, 0.25, 0.5 and 1.0 mg/L) 1-Naphthalene Acetic Acid (NAA) for rooting.

Acclimatization

Two weeks old, rooted shoots were detached from agar medium and they were thoroughly washed under running tap water and transferred to the soil mixture. Root induction frequency (number of shoots with root / total number of shoots × 100) and mean root length (average length of three longest roots) were measured and recorded. This experiment was repeated thrice to evaluate the efficacy. Plantlets (6 -7 cm in length) were then acclimatized to a plastic pot containing sterilized cow dung manure and red soil (1:1 ratio, *v/v*) and they were watered at regular intervals.

RAPD Analysis

Genomic DNA of the haphazardly selected mother plant and micropropagated plantlets were isolated from fresh leaves using CTAB method [42]. RAPD reactions were performed as described by [43]. RAPD study of both mother plant and micro propagated plantlets was performed using RAPD primers (Eurofins Genomics, Bangalore, India). PCR

amplification was done using proflex thermocycler (Applied Biosystems, Singapore). The PCR amplified products were electrophoresed on 1.2% agarose gel in 1X TAE buffer at 75V for 1h. The results were photographed using a gel documentation system (GELSTAN 1012 Economy Imaging System, Chennai, India). Lab Image ID Software, version 3.3.0 (Kapelan Bio-Imaging solutions, Leipzig, Germany) was used for the determination of amplicon size by visual comparison with 1 Kb ladder (250 – 10000 bp).

Statistical Analysis

All the above experiments were repeated thrice with four independent replicates for each treatment. The study of variance appropriate for the design was carried out to spot the implication of difference among the treatment means. IBM SPSS Statistics version 20 Duncan's multiple range test at $P < 0.05$ was used to compare the treatment means.

III. RESULT AND DISCUSSION

Effect of cytokinins on shoot regenerations

Shoot apical meristems (SAMs) excised from 3-d-old *in vitro* germinated seedlings of CO 9 (Fig. 3a) were evaluated for multiple shoot induction in Shoot regeneration medium (SRM). The initial response of shoot regeneration was observed within 3 d and multiple shoot buds emerged from the bottom of the explants after 7 d, without any basal callus growth (Fig. 3b). Multiple shoot induction increased spontaneously after 2 wk. Among the PGRs tested, 3.0 mg/L BAP showed 88.03% shoot regeneration frequency, 25.5±0.33 of shoots per explant and 5.5±0.15 cm of shoot length (fig 1). KIN also showed a positive influence on shoot regeneration frequency of 73.67% and 14.7±0.31 of shoots per explant (fig 1) when MS medium supplemented with 2.0 mg/L KIN which is less compare to BAP. However, MS medium amended with the combination of 3.0 mg/L BAP and 2.0 mg/L KIN show highest regeneration frequency (90.56%), multiple shoot tip per explant (32.5±0.26) and multiple shoot clumps (5.8±0.17cm) (fig 1). BAP and KIN commonly induced multiple clumps in millets and cereals [44, 45, and 46]. In pearl millet and wheat, higher shoot proliferation was observed in medium containing BAP greater than 3.0 mg/L [25].

Effect of cytokinins and coconut water on shoot regenerations

Synergistic effect of cytokinins and coconut water on multiple shoot induction were tested (fig 2). Medium supplemented with (0.5-4.0 mg/L) BAP, (0.5-4.0 mg/L) KIN individually and combination of (3.0 mg/L) BAP with (0.5-4 mg/L) KIN along with 5 % coconut water, were tried for shoot induction. Addition of coconut water (5 %(*w/v*)) increased the shoot number as well as shoot length (fig 2). The most significantly shoot proliferation frequency was attained in MS medium added with 3.0 mg/L BAP, 2.0 mg/L Kin along with 5 % coconut water produced 94.36 %. And 37.5 ±0.2 shoots per explant (fig 2). A similar result was reported by [45] in *Sorghum bicolor*. However, the increase in the concentration of BAP or KIN in the medium resulted in decrease in shoot number and shoot length. The shoot length varied significantly in all combination of shoot regeneration medium (SRM) (fig 2).

Influence of amino acids and organic derivatives on shoot proliferation

The addition of supplements in the media has been found to exhibit significant improvement in shoot tip initiation, proliferation, and maturation. In this study, we evaluated additives such as CEH, 5 % coconut water, Gly, and Pro on improved proliferation, elongation, and maturation. The SAMs of CO 9 responded very well to amino acids supplement in a dose-dependent manner. The induction, proliferation, and elongation frequency of shooting improved quickly when amino acid levels were systematically increased in the culture medium. A higher shoot frequency (95.89%), shoot number per explant (46.52), and shoot length (10.8 cm) were obtained for CO 9 genotype (Table 1), when the MS medium containing BAP 3.0 mg/L, KIN 2.0 mg/L was supplemented with 300 mg /L Pro, 400 mg/L CEH, 3.0 mg/L Gly and 5 % coconut water (fig 3). In responds to this unique combination of PGRs and amino acid supplement's, the multiple shoots remained green and continued to proliferate and elongate. When the concentration of supplements were increased beyond this, there was no further improvement in regeneration, but rather a decline in induction rate, shoot number, and shoot length. Pro, CEH and 5% coconut water have been successfully employed for improving regeneration in rice and sorghum via high-frequency embryogenesis [47]. CEH is primarily act as an organic nitrogen source shows beneficially impact on multiple shoots development [48]. Multiple shoot proliferation and plant regeneration in cereals depends upon on numerous factors such as age of the explant, explant size, genotype and region are most important [49-51]. In our study, SAMs from 3-d-old seedlings were found to be optimal, along with amino acid resulting in an excellent regeneration response.

Rooting and Acclimatization

Eight-week-old shoots from shoot regeneration medium were rooted in MS medium amended with IAA, IBA, and NAA. Roots initiated spontaneously after 1 wk in MS medium devoid of PGRs. However, the inclusion of IAA (0.1 – 1.0 mg/L) positively influenced the rooting frequency and root length. The best root growth response in terms of frequency of root induction (100%), mean number of roots per shoot (9.05±0.10) and mean root length (7.32 cm) was observed when 0.25 mg/l IAA was incorporated into MS medium (Table 2; Fig. 3f.g.h). Our results were consistent with [25] in wheat.

RAPD analysis

Finger millet plantlets propagated under *in vitro* conditions were subjected to clonal fidelity analysis using RAPD markers. A total of 71 monomorphic bands was generated by ten RAPD primers. The number of bands ranged from 5 (OPA7 and OPA10) to 9 (OPA1) with 7.1 bands per primer on average (Table 3). The amplified products ranged in size from 250 to 1990 bp (Fig. 4). The identical banding pattern between micropropagated plants and the mother plant revealed no epigenetic variation induced under *in vitro* conditions.

IV. CONCLUSION

The method demonstrates an effective and low costs protocol that helps in the rapid multiplication, large-scale production in finger millets. Influence of additives on enhanced shoot multiplication was established by the use shoot tip explants. Shoot tip proliferation, without any intervening callus phase, supposedly leads to the production of genetically stable, true-to-type plantlets. Reproducible bands were obtained from RAPD marker confirmed the genetic integrity of regenerated plantlets. Hence, this method could be used as a potential alternative *en masse* propagation and further for genetic transformation.

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Table 1 Effect of BAP, Organic Derivatives and Amino acid supplements on multiple shoot proliferation in finger millet genotype CO9 after 8 wks of incubation in the light

PGRs mg/L		Amino acids mg/L		Organic derivatives		Explants with Multiple shoot induction (%)	Mean no. of shoots induced per explant	Mean length of shoots (cm)
BAP	KIN	pro	Gly	CHE	%Coconut water			
3	2	50	0.5	100	5	60.35	19.65 ±0.2^{f}	6.82 ±0.1^{j}
3	2	100	1	200	5	67.46	23.45 ±0.2^{e}	7.24 ±0.4^{hi}
3	2	200	2	300	5	81.61	30.91 ±0.3^{b}	9.11 ±0.6^{g}
3	2	300	3	400	5	95.89	46.52 ±0.3^{a}	10.86 ±0.2^{g}
3	2	400	4	500	5	79.74	29.31 ±0.5^{c}	8.60 ±0.6^{h}
3	2	500	5	600	5	64.52	24.48 ±0.4^{d}	7.21 ±0.5^{hi}

Table 2 Effect of Auxins on Root formation of finger millet genotype CO9

Auxins mg/L	Percentage of Rooting (%)	No. of Roots per Shoot	Root Length (cm)
IAA			
0.1	81.83	4.44 ± 0.13^{e}	3.58 ± 0.08^{f}
0.25	100	9.05 ± 0.10^{a}	7.32 ± 0.06^{b}
0.5	98.26	8.61 ± 0.03^{b}	4.443± 0.7^{e}
1.0	92.59	5.45 ± 0.06^{d}	2.34 ± 0.07^{g}
IBA			
0.1	60.96	6.56 ± 0.10^{c}	3.90 ± 0.05^{f}
0.25	66.61	8.22 ± 0.10^{b}	4.27 ± 0.07^{e}
0.5	76.19	6.37 ± 0.09^{c}	3.20 ± 0.08^{f}
1.0	69.43	4.71 ± 0.07^{e}	3.00 ± 0.11^{f}
NAA			
0.1	64.62	3.40 ± 0.07^{f}	1.94 ± 0.07^{h}
0.25	61.21	3.70 ± 0.11^{f}	2.45 ± 0.01^{d}
0.5	77.41	5.46 ± 0.08^{d}	2.51 ± 0.10^{g}
1.0	72.72	4.59 ± 0.07^{e}	2.18 ± 0.04^{g}

Table 3 List of RAPD primer sequences with the number and size of amplified fragments generated in genotype CO 9 of finger millet mother plant and micropropagated plantlets.

Genotype		CO 9	
Primer code	**Primer sequence (5′–3′)**	**No. of scorable bands**	**Range of band sizes (bp)**
OPA 1	CAGGCCCTTC	9	460-1500
OPA 2	TGCCGAGCTG	8	430-1450
OPA 3	AGTCAGCCAC	7	530-1600
OPA 4	AATCGGGCTG	7	640-1500
OPA 5	AGGGGTCTTG	8	300-1500
OPA 6	GGTCCCTGAC	8	490-1990
OPA 7	GAAACGGGTG	5	520-1900
OPA 8	GTGACGTAGG	8	250-1480
OPA 9	GGGTAACGCC	6	360-1490
OPA1 0	GTGATCGCAG	5	330-1350
		71	250-1990

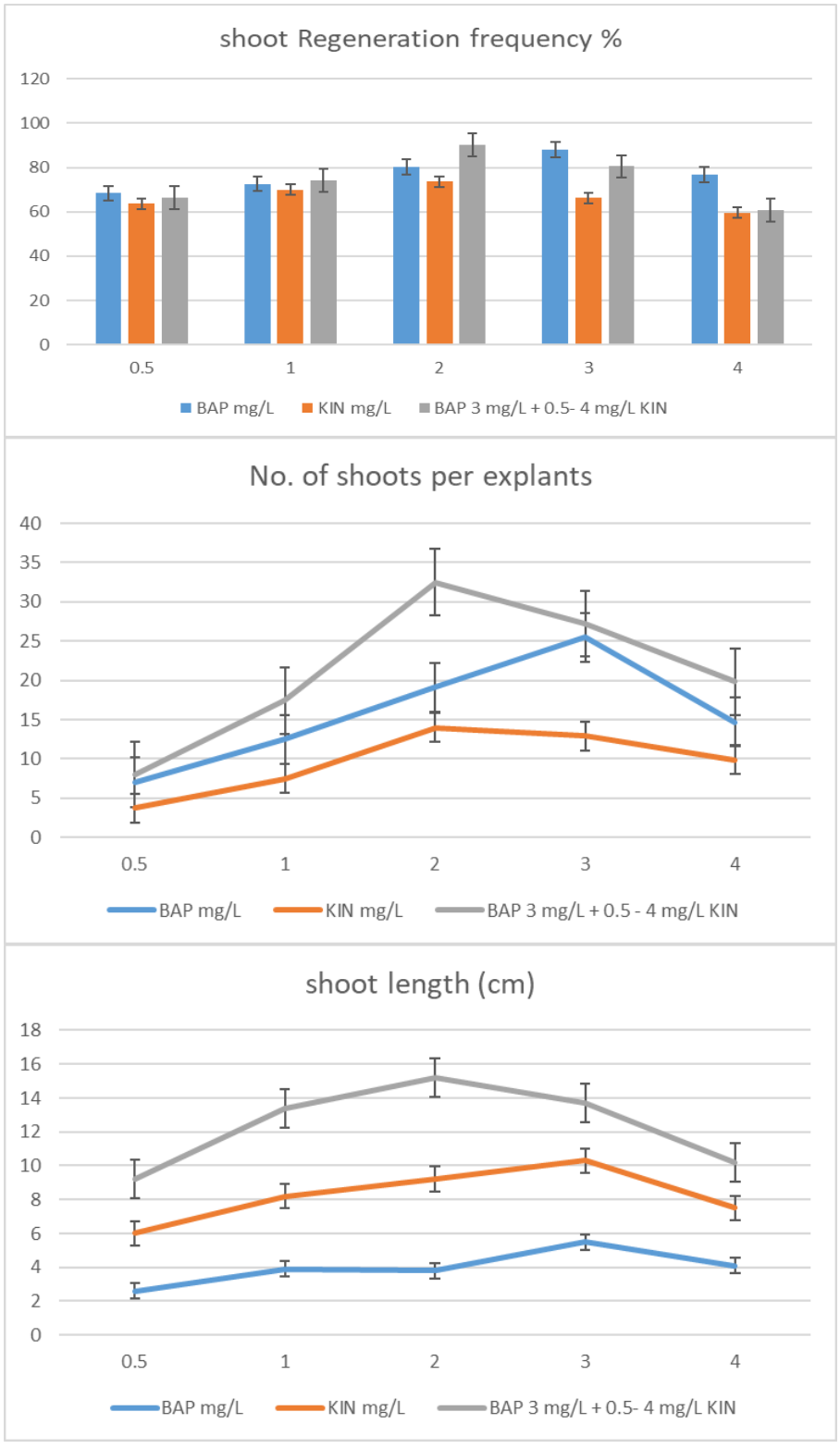

Figure 1 Effect of Cytokinins in Direct Regeneration of finger Millet genotype CO 9. Values represent mean ± SE of individual treatments obtained from three repeated experiments. Each treatment consisted of four replicates with ten explants per replicate

Figure 2 Effect of Cytokinins along with 5% coconut water in Direct Regeneration of finger Millet genotype CO 9 Values represent mean ± SE of individual treatments obtained from three repeated experiments. Each treatment consisted of four replicates with ten explants per replicate

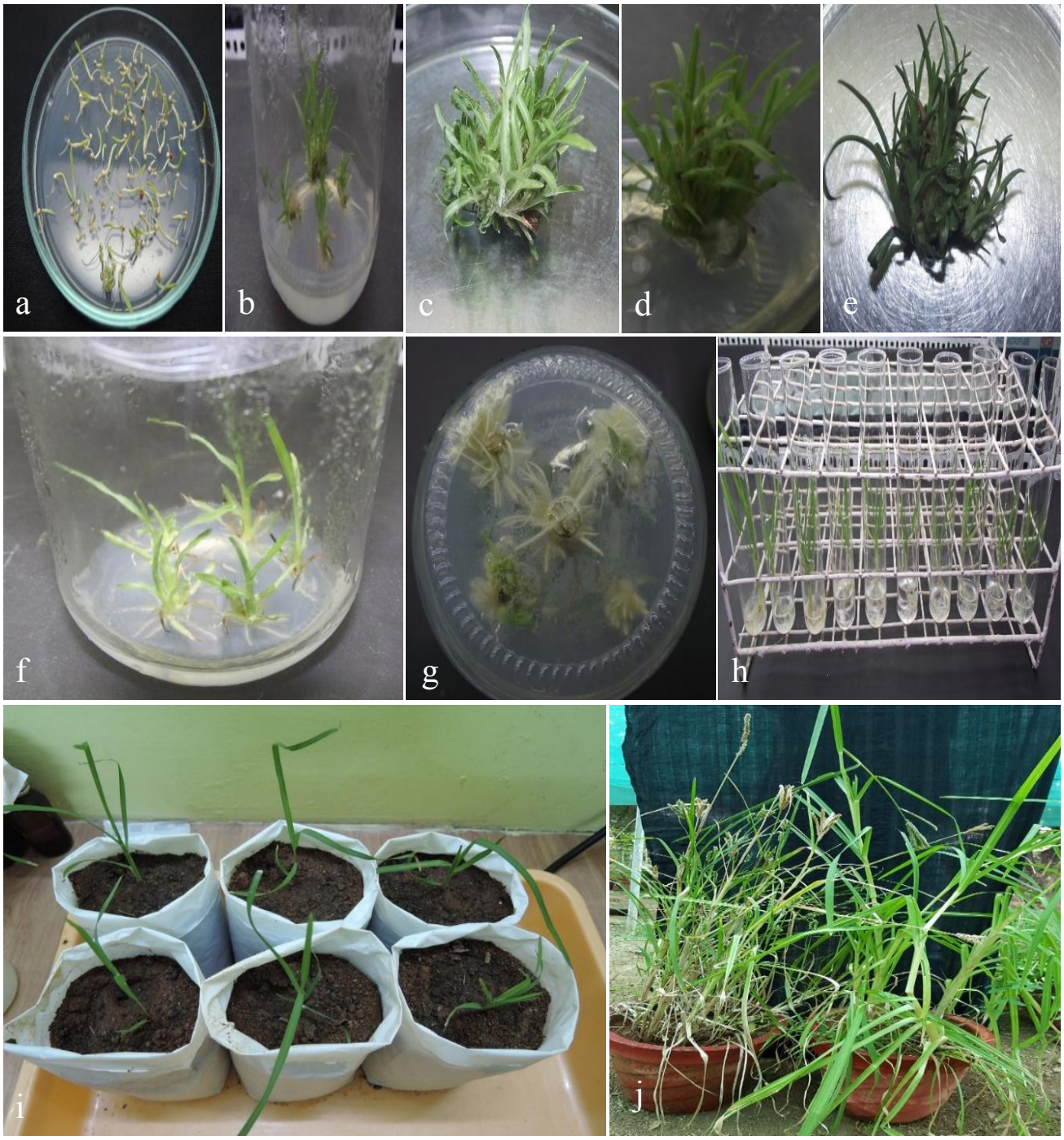

Figure 3 Direct regeneration from shoot apical meristems of *E. coracana.* (a) Initiation of shoot apical meristems (3–4 mm) from mature seeds germinated on MS medium after 3 d; (b) Induction of multiple shoots in shoot regeneration medium(SRM) containing MS basal salts and vitamins supplemented with with 3 mg/L 6-Benzyl Amino Purine (BAP), 2 mg/L μM Kinetin and 5% coconut water in combination with 300 mg/L proline, 400 mg/L casein enzymatic hydrolysate, and 3 mg/L glycine.; (c) Proliferation of shoots in SRM after 4 wk; (d) Multiple shoot regeneration in SRM after 6 wk; (e) Elongated shoots of CO 9 in SRM after 8 wk; (f,g) Rooting in MS medium supplemented with 0.25 mg/l IAA after 1 wk; (h) Rooting in liquid MS for 1 wk (i) Acclimatized plantlets in grow bags containing sterile red soil and manure (1:1, *v/v*); (j) Seed set of micropropagated plants grown in the field.

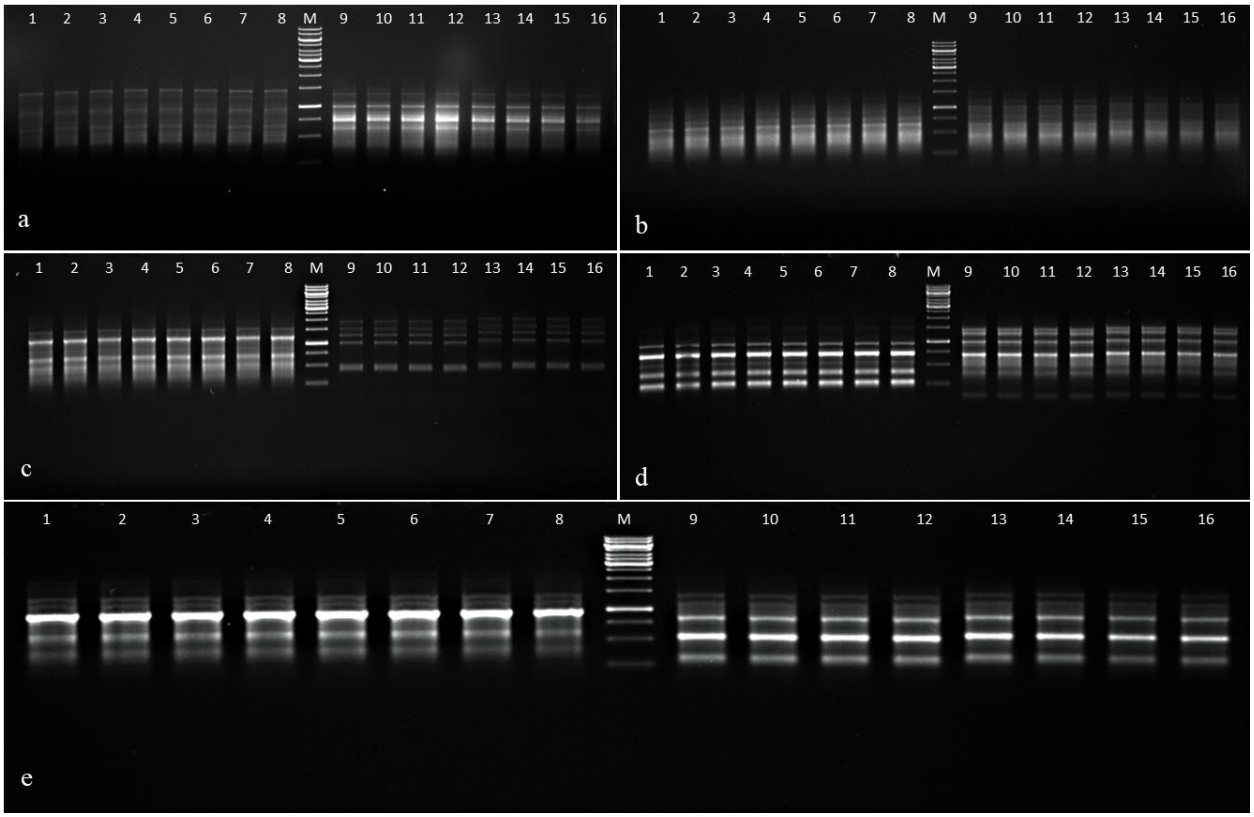

Figure 4 Assessment of genetic fidelity of the mother plant and micropropagated plantlets of *Eleusine coracana* using RAPD markers. (a)RAPD primers 1 (left) and 2 (right); (b) RAPD primers 3 (left) and 4 (right); (c) RAPD primers 5 (left) and 6 (right) (d) RAPD primers 7 (left) and 8 (right); (e) RAPD primers 9 (left) and 10 (right) The same seven *ex vitro* plants are represented in the same order in each of the 10 gel halves. In each panel, lanes 1 and 9 are mother plants; lanes 2–8 and 10–16 are *ex vitro* plants; lane M is 1 Kb DNA marker.

Author's Profile

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