RESEARCH ARTICLE

Role of activated charcoal and amino acids in developing an efficient regeneration system for foxtail millet (*Setaria italica* (L.) Beauv.) using leaf base segments

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Abstract An efficacious, reproducible direct in vitro regeneration system has been developed from leaf base segments (LBs) of six high yielding genotypes of foxtail millet (*Setaria italica* (L.) Beauv.). LBs excised from 4-day-old seedling were inoculated on Murashige and Skoog (MS) medium supplemented with different types and concentrations of cytokinins. The shoots induced per explant significantly increased with the supplementation of BAP to auxin containing medium. The results showed that a maximum shoot induction, 58.8% was obtained on MS medium incorporated with 8.9 μM BAP and 2.7 μM NAA in ‘CO5’ genotype. Further, the highest frequency of multiple shoots was produced on MS(I) medium containing 8.9 μM BAP, 2.7 μM NAA, 700 mg L^{-1} proline, 0.5 mg L^{-1} cysteine, 2.0 mg L^{-1} glycine and 150 mg L^{-1} arginine. MS(I) medium additionally fortified with 5.0 g L^{-1} activated charcoal (AC) was found to achieve the best precocious plant regeneration. Elongated shoots were rooted on half-strength MS medium amended with 2.9 μM IAA and achieved maximum root number (8.7) within 10 days. Rooted plantlets were acclimated in soil with 92% survival rate. Molecular marker analysis of in vitro regenerated and field grown plants revealed no somaclonal

variations. Briefly, amino acids and activated charcoal could significantly enhance the foxtail millet direct multiple shoot proliferation and plant regeneration. Here we report, a short-term, genotype independent, direct plant regeneration protocol for future genetic transformation studies in foxtail millet genotypes.

Keywords Additives · Direct multiple shoots · Molecular markers · Plant growth regulators · Plant regeneration

Introduction

Foxtail millet (*Setaria italica* (L.) Beauv.) belongs to the family Poaceae that includes 125 species widely distributed all over the world, specifically in arid and semi-arid regions of Asia and Africa and has a long history of cultivation (Sato et al. 2013; Pan et al. 2015). In the global millet production, foxtail millet ranks second after pearl millet (FAOSTAT 2005; Lata et al. 2013). It is widely adapted to abiotic stress and adverse conditions than any other crops like sorghum and maize (Panaud 2006; Pan et al. 2015). Foxtail millet is one of the important millet while it contains higher level of fiber and lipid content, it is also been used as medicine to cure diarrhea (Kim et al. 2009). Foxtail millet is well known as a model crop which shares a significant genome synteny with several millets and cereals, such as pearl millet, common millet, maize and sorghum, and it is also closely related to biofuel grasses (Ceasar et al. 2014; Satish et al. 2016a). Moreover, the availability of genome sequence facilitated its use as an excellent model crop with a small genome size of ~ 515 Mb that helped in studies related to tolerance of stresses like salinity and dehydration, plant architecture, and photosynthesis.

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Direct plant regeneration is a valuable approach to reduce the culture duration and to minimize the somaclonal variation extensively (Burner and Grisham 1995; Satish et al. 2015). The efficient and reproducible in vitro plant regeneration is a first step for successful biotechnological application in crop improvement to achieve the requirement of food in the shortest period (Parmar et al. 2012; Sivanesan et al. 2015; Aydin et al. 2016). However, the effective utilization of plant tissue culture relies on the interaction between culture media, explants, plant species and environmental growth conditions (Chutipaijit and Sutjaritvorakul 2018). The previously published reports include in vitro regeneration of foxtail millet from various types of explants viz., somatic embryos (Wang et al. 2011; Satish et al. 2016a), mature seed derived callus (Rao et al. 1988), mature seeds and immature glumes (Reddy and Vaidyanath 1990), shoot apices and young inflorescence (Osuna-Avila et al. 1995) and mesocotyl (Rout et al. 1998). All these studies have described the de novo plant regeneration from organogenesis of foxtail millet genotypes. Till date and to the best of *our knowledge*, there is no study to emphasize direct regeneration of foxtail millet devoid of an intervening callus phase. Leaf base segment (LBs) are an excellent source for direct in vitro regeneration due to its advantages over somatic embryos and other explants. LBs have been successfully reported for direct multiple shoot regeneration in many food crops viz., wheat (Yu et al. 2012), kodo millet (Rashid 2003), oats (Gless et al. 1998) and rice (Ramesh et al. 2009). However, there is necessity of a simple and rapid direct multiple shoot regeneration in foxtail millet genotypes which will be applicable for further genetic engineering studies.

In plant tissue culture, optimization of nutrient media plays a crucial role in plant regeneration. The exogenous application of amino acids in the medium will enhance the regeneration frequency of crop plants (Grewal et al. 2006; Sivakumar et al. 2010; Satish et al. 2016a). The improvement of cell growth and development in plant tissue culture is often achieved by activated charcoal (AC). AC having ample surface volume with a network of fine pores gives a unique adsorption capacity, which will reduce the oxidation property of the phenolic compounds, also extensively decreasing the toxic metabolites and accumulation of brown exudates (Pan and van Staden 1998; Thomas 2008; Chutipaijit and Sutjaritvorakul 2018). In previous studies, AC has been used widely to ameliorate the callus induction and plant regeneration in aromatic rice (Chutipaijit and Sutjaritvorakul 2018), for development of somatic embryogenesis in pearl millet (Lambe et al. 1999), and for improving the efficiency of anther culture in maize (Buter et al. 1993). The substance present in AC naturally involves in a number of stimulatory activities such as promoting growth, adsorption of vitamins, cytokinins,

auxins and metal ions in the culture medium which improve the embryogenesis, in vitro rooting, multiple shoot proliferation, shoot elongation and regeneration efficiency in plants (Dumas and Monteuiis 1995; Nhut et al. 2001; Thomas 2008; Manchanda and Gosal 2012). Moreover, in foxtail millet genotypes, there is no detailed report on the influence of AC on in vitro direct plant regeneration system.

The molecular marker technique is a powerful and valuable tool used for assessing the genetic fidelity of the in vitro regenerated and seedling derived plants (Gupta and Roy 2002). Random amplified polymorphic DNA (RAPD) and inter simple sequence repeats (ISSR) markers are simple, quick, inexpensive and highly reproducible method (Al Khateeb et al. 2013; Satish et al. 2016c). RAPD analysis uses single arbitrary decamer primers to determine the genetic variation of in vitro regenerated plants (Salvi et al. 2001). ISSR method was proven to be helpful for the analysis of closely isogenic lines of the Poaceae family (Al-Turki and Basahi 2015). Similarly, combined use of various molecular marker techniques has been utilized for the analysis of genetic diversity in various crop plants (Gupta et al. 2010; Rajendran et al. 2016, Satish et al. 2016c). In tissue culture plants, it has been confirmed that RAPD and ISSR markers are more frequently used and are suitable technique for the detection of somaclonal variations (Ramakrishnan et al. 2014). In this study RAPD and ISSR analysis were employed to evaluate the genetic fidelity among the in vitro regenerants and seed derived field grown foxtail millet plants.

The conventional organogenesis in foxtail millet depends on multi phases with continuous subculturing posing more limitations to achieve entire plant regeneration system (Rao et al. 1988; Satish et al. 2016a). Hence, the present study aimed to develop a highly efficient plant regeneration method without intermediate callus phase in foxtail millet genotypes using in vitro derived LBs. To our knowledge, this is the first study discussed on the influence of AC and amino acids on successful in vitro direct plant regeneration from the LBs in these important Indian foxtail millet genotypes, followed by genetic fidelity analysis. Perhaps, this short-term regeneration protocol will aid in future for genetic transformation, induction of mutants and genomic studies of this important C4 model species.

Materials and methods

Plant material

Mature seeds of six high yielding and widely cultivated Indian foxtail millet genotypes were used in this study. The genotypes 'CO5', 'CO7' and 'TNAU-43' were procured

from Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu, India and the other three genotypes ‘H-1’, ‘K-221-1’ and ‘RS-118’ were procured from Main Agricultural Research Station, University of Agricultural Sciences, Dharwad, India. Seed material was stored in air tight container at room temperature (RT) in the dark until the time of use.

Explant preparation

Seeds of all the six foxtail millet genotypes were manually de-husked using a palm husker (MRC 707; Mercer Co. Haryana, India). De-husked seeds were rinsed in sterile distilled water for 5 min, then with 70% (v/v) ethanol solution for 1 min, washed twice with sterile distilled water, followed by 0.1% (w/v) mercuric chloride (HgCl₂) for 5 min (Satish et al. 2016a). Finally, seeds were washed with sterile distilled water for five times to remove any trace of HgCl₂. Surface-sterilized seeds were inoculated on MS basal medium (Murashige and Skoog 1962) enriched with 3% sucrose without any plant growth regulators (PGRs) supplementation. The pH of all the media used in this study was adjusted to 5.75 ± 0.2 , added solidifying agent agar–agar type-1 (0.8%) and CleriGelTM (0.2%) (Hi-Media, Mumbai, India), and the medium was autoclaved at 121 °C for 20 min. Sterilized media were poured into Petri dishes (50 × 12 mm) (Borosil, India) each 20 mL and cooled to RT and used. Surface sterilized seeds from all the genotypes were cultured on MS basal medium and incubated for 4 days in the dark at 26 ± 2 °C. Four-day-old in vitro derived seedlings were collected and aseptically cut to obtain LBs using sterile scalpel blade no. 22 (Swann-Morton®, England) and used as explant source for in vitro direct regeneration. Experiments were performed to analyse the optimal PGR concentration and types on shoot regeneration of foxtail millet genotypes.

Shoot induction

MS medium supplemented with thidiazuron (TDZ) (0.5, 0.9, 1.4 or 1.8 µM), kinetin (Kin) (4.6, 9.3, 13.9 or 18.5 µM), 6-benzylaminopurine (BAP) (4.4, 8.9, 13.3 or 17.7 µM) or zeatin (4.6, 9.1, 13.7 or 18.2 µM) (Hi-Media, Mumbai, India), were individually tested for shoot induction using 4-day-old in vitro seedling derived LBs from all six foxtail millet genotypes. Further, with the best responding cytokinin, three different types of auxins such as indole-3-butyric acid (IBA) (1.2, 2.5, 3.6 or 4.9 µM), 1-naphthaleneacetic acid (NAA) (1.3, 2.7, 4.0 or 5.4 µM) or indole-3-acetic acid (IAA) (1.4, 2.9, 4.3 or 5.7 µM) (Hi-Media, Mumbai, India), individually at different concentration was used to enhance the shoot induction frequency. LBs cultured on MS medium without any PGR has been

used as control. All the cultures were incubated in a growth chamber (Sanyo versatile environmental test chamber, MLR 351H, Japan) by providing 16/8-h light/dark photoperiod under cool white fluorescent lamp with 150 µmol photons m⁻² s⁻¹ and 60% relative humidity (RH) for 10 days at 26 ± 2 °C. Percentage of explants survival, initial shoots produced in each LBs explant, shoot induction frequency and average number of shoots induced per explant was calculated after 10 days of incubation in light. These experiments were performed to optimize the best PGR concentration and type for direct shoot bud initiation from all the six foxtail millet genotypes studied.

Effect of amino acids on multiple shoot induction

Shoots induced on MS medium containing optimized concentration of BAP and NAA were further used to study the influence of various types and concentrations of essential amino acids such as proline (Pro; 250, 400, 550, 700, 850 or 1000 mg L⁻¹), cysteine (Cys; 0.2, 0.3, 0.4, 0.5, 0.6 or 0.7 mg L⁻¹), glycine (Gly; 0.5, 1.0, 1.5, 2.0, 2.5 or 3.0 mg L⁻¹) and arginine (Arg; 25, 50, 100, 150, 200 or 250 mg L⁻¹) to enhance the development of multiple shoot induction and proliferation. MS medium supplemented with PGRs and different concentration of amino acids was specified as MS(I) medium for further experiments. Shoots cultured on MS medium containing PGRs and without amino acids served as a custom control. The percentage of multiple shoot induction and number of shoots induced from each explant was calculated after 12 days of incubation in light.

Effect of activated charcoal on shoot elongation and maturation

Multiple shoot induced on MS(I) medium were subcultured on to MS(I) elongation medium amended with various concentrations of AC (2.5, 5.0, 7.5 or 10 g L⁻¹). MS(I) medium containing PGRs and amino acids devoid of AC served as a control to compare the multiplication and regeneration ability of the LBs. The percentage of multiple shoot induction, elongation and mean length of shoots produced per explant were calculated after 12 days of incubation in light.

Root induction

Elongated shoots (~ 12 cm long) were transferred to half-strength MS medium with reduced concentration of sucrose 15% as carbon source and various concentrations of IAA (1.4, 2.9, 4.3 or 5.7 µM), individually. Half-strength MS medium devoid of IAA was used as a control to test the rooting efficiency in all six foxtail millet genotypes

studied. The frequency of shoots producing roots, mean number of roots and average length of roots was recorded after 10 days of incubation in light.

Hardening and acclimatization

The rooted plants were gently removed from the culture tubes and washed thoroughly with distilled water to remove any adherent on the surface of the roots and then transferred to the Yoshida solution (Yoshida et al. 1976). Plantlets were shifted to growth chamber for 4 days to maintain high humidity and thereafter transferred to soil pots containing sterile red soil, peat and SoilriteTM (Keltech, Bengaluru, India) at 1:1:1 ratio (w/w/w) and kept in a greenhouse at natural conditions for further growth, flowering and seed setting. Plantlets were watered for every 2 days and grown to maturity.

Genetic fidelity analysis

Initially, genomic DNA of foxtail millet plant was amplified using 20 RAPD and 15 ISSR primers (Sigma Aldrich, USA) (Table S1 and S2). For molecular analysis, only 5 RAPD and 5 ISSR primers were employed to check the genetic fidelity of in vitro regenerated and seed derived field grown foxtail millet plants. The detail of the primers used in this study is given in Table S1 and S2. Genomic DNA was isolated using 100 mg fresh leaves of randomly selected in vitro regenerated and field grown plants using CTAB method (Doyle and Doyle 1990). Both RAPD and ISSR reactions were carried out in 12.5 μ L volumes which contained the following components, template DNA (30 ng/ μ L), 200 mM of each dNTP mix, 1.5 mM MgCl₂, 0.4 μ M of RAPD or ISSR primer, 1X *Taq* buffer and 1.0 unit of the *Taq* DNA polymerase (MBI, Fermentas, Germany). For RAPD primers, the amplification reactions were carried out using a Thermal Cycler (AB Applied Biosystems, USA), which was programmed to include pre-denaturation at 94 °C for 5 min, followed by 40 cycles of denaturation at 94 °C for 1 min, annealing at 37 °C for 1 min and extension at 72 °C for 2 min next to a final cycle with an additional 7 min at 72 °C for extension. For ISSR primers reaction, setup and PCR program was similar where the annealing temperature was at 55 °C for 1 min. For both the primers, reaction was repeated thrice to confirm the constant reproducibility of the results. The amplified products were electrophoresed in 8% (w/v) polyacrylamide gel and stained with 2 μ L ethidium bromide (10 mg mL⁻¹ stock). The banding pattern was documented using a gel documentation system (Molecular Imager[®] GEL DocTM XR + system, Bio-Rad, USA).

Data collection and statistical analysis

All the experiments were done in a completely randomized design with 3 replications for all the six foxtail millet genotypes. For multiple shoot induction, 75 mature LBs from each genotype were used in each experiment. For that, 75 explants were cultured for each concentration with three replicates per experiment. The frequency of multiple shoot induction, mean number of shoots induced per explant, percentage of shoot elongation and shoot length were calculated after 12 days of culture. Statistics on the percentage of shoots producing roots, mean number of roots induced and average length of roots were recorded after 10 days of incubation in light. The survival percentage was documented after 40 days of transfer in pots. The data were analysed using one-way analysis of variance (ANOVA), the significance of variations within means was carried out at $p \leq 0.05$ using Duncan's multiple range test with Statistical Package for Social Science, version 17.0 for Windows, SPSS Inc. (SPSS Inc. 2003).

Results

Effect of PGRs on shoot induction and multiple shoot initiation

Surface sterilized foxtail millet seeds were germinated on MS basal medium in dark and 4-d-old seedlings derived LBs were used as explants (Fig. 1a). The cut ends of foxtail millet LBs explants showed shoot initiation within 2-3 days of incubation in light and most of the LBs initiated green shoots at high rates without callus intervention based on the various types and concentrations of PGRs used. MS medium amended with BAP 8.9 μ M produced shoot initiation after 10 days of incubation in light (Table 1). Among all the genotypes tested, the highest frequency 96.6% of the explant survival rate was found in 'CO5' genotype where in 81.2%, 87.5%, 91.7%, 79.6% and 68.5% of survival rate was observed for 'TNAU-43', 'CO7', 'K-221-1', 'H.1' and 'RS-118', respectively (Table 1). All the LBs on MS medium containing BAP 8.9 μ M at the initial stage were green (Fig. 1a). The highest shoot induction frequency 35.1% was noted in 'CO5' genotype where in 'TNAU-43', 'CO7', 'K-221-1', 'H.1' and 'RS-118' genotypes produced 20.6, 25.5, 29.6, 17.9 and 11.6% of shoot induction, respectively (Table 2) and there was no shoot initiation observed in the control. However, BAP showed best explant survival rate with maximum shoot initiation percentage when compared to other cytokinins TDZ, Kin and zeatin in all the six foxtail millet genotypes studied. At higher concentration of BAP (8.9 μ M or above), the shoot induction frequency

Fig. 1 Direct regeneration from in vitro derived leaf base segments of foxtail millet ‘CO5’ genotype. **a** Four-day-old leaf base segments from ‘CO5’ genotype inoculated on MS medium containing 8.9 μ M BAP. **b** Primary shoot initiation at the cut end of the LBs on MS medium containing 8.9 μ M BAP along with 2.7 μ M NAA after 10 days of incubation in light. **c** Multiple shoots initiated from LBs after 10 days of incubation on MS medium containing 8.9 μ M BAP along with 2.7 μ M NAA. **d** Induction of multiple shoots in MS(I) medium supplemented with amino acids 700 mg L^{-1} Pro, 0.5 mg L^{-1} Cys, 2.0 mg L^{-1} Gly and 150 mg L^{-1} Arg after 12 days of incubation in light. **e**, **f** Multiple and elongated shoot of ‘CO5’ genotype foxtail millet on MS(I) medium with 5 g L^{-1} AC after 12 days of incubation in light. **g** In vitro rooting in half-strength MS medium containing 2.9 μ M IAA after 10 days of incubation in light. **h** Acclimatized and hardened 55-day-old in vitro regenerated foxtail millet plantlets of genotype ‘CO5’ in plastic pots containing 1:1:1 (w/w/w) ratio of red soil, sterile soilrite™ and peat with seed panicles

decreased significantly in all foxtail millet genotypes tested.

An increased efficiency of shoot induction was noticed on MS medium fortified with lower concentrations and types of auxins (NAA, IAA or IBA) tested individually along with BAP (8.9 μ M) (Fig. 2). Combination of BAP 8.9 μ M and NAA 2.7 μ M considerably increased the shoot induction frequency in ‘TNAU-43’ (35.4%), ‘CO7’

(41.3%), ‘CO5’ (58.8%), ‘K-221-1’ (50.4%), ‘H.1’ (31.4%) and ‘RS-118’ (29.7%) (Fig. 2a), and the average number of shoots in ‘K-221-1’ (6.9), ‘CO7’ (6.1), ‘CO5’ (8.4), ‘TNAU-43’ (5.2), ‘H.1’ (4.7) and ‘RS-118’ (3.0), respectively after 10 days of incubation in light (Fig. 2d). Next to NAA 2.7 μ M, IAA 2.9 μ M and IBA 2.5 μ M showed the reduced frequency of shoot induction in combination of BAP (8.9 μ M) (Fig. 2e, f) and no shoot

Table 1 Effect of different plant growth regulators on explants survival from LBs of six foxtail millet genotypes after 10 days of incubation in light

Plant growth regulators (μM)	Percentage of explants survival (mean $\pm$ SE)					
	Foxtail millet genotypes					
	TNAU-43	CO7	CO5	K-221-1	H.1	RS-118
<i>TDZ</i>						
0.5	58.3 $\pm$ 0.4 ^l	67.7 $\pm$ 0.2 ^k	82.3 $\pm$ 0.3 ^l	77.3 $\pm$ 0.3 ^k	51.3 $\pm$ 0.4 ^j	47.2 $\pm$ 0.3 ^k
0.9	67.4 $\pm$ 0.2 ⁿ	74.3 $\pm$ 0.2 ⁿ	86.4 $\pm$ 0.2 ^m	82.4 $\pm$ 0.2 ^m	63.1 $\pm$ 0.3 ^l	55.2 $\pm$ 0.5 ^m
1.4	46.2 $\pm$ 0.5 ⁱ	53.3 $\pm$ 0.2 ^h	73.5 $\pm$ 0.2 ⁱ	62.5 $\pm$ 0.3 ^h	78.7 $\pm$ 0.6 ^o	35.5 $\pm$ 0.4 ^g
1.8	39.3 $\pm$ 0.4 ^f	41.6 $\pm$ 0.2 ^d	69.4 $\pm$ 0.1 ^g	60.3 $\pm$ 0.5 ^g	33.2 $\pm$ 0.4 ^e	29.4 $\pm$ 0.4 ^f
<i>Kin</i>						
4.6	34.3 $\pm$ 0.4 ^d	44.3 $\pm$ 0.3 ^e	58.2 $\pm$ 0.4 ^d	49.2 $\pm$ 0.4 ^d	37.3 $\pm$ 0.5 ^f	24.5 $\pm$ 0.2 ^d
9.3	40.2 $\pm$ 0.5 ^g	51.2 $\pm$ 0.4 ^g	60.3 $\pm$ 0.2 ^e	54.4 $\pm$ 0.3 ^e	41.6 $\pm$ 0.2 ^g	35.3 $\pm$ 0.2 ^g
13.9	25.3 $\pm$ 0.4 ^b	38.3 $\pm$ 0.5 ^c	49.5 $\pm$ 0.2 ^b	38.6 $\pm$ 0.2 ^b	21.4 $\pm$ 0.3 ^b	18.3 $\pm$ 0.5 ^b
18.5	21.3 $\pm$ 0.4 ^a	29.2 $\pm$ 0.4 ^a	37.2 $\pm$ 0.2 ^a	31.4 $\pm$ 0.2 ^a	16.2 $\pm$ 0.3 ^a	11.4 $\pm$ 0.5 ^a
<i>BAP</i>						
4.4	72.3 $\pm$ 0.3 ^o	78.7 $\pm$ 0.3 ^o	90.3 $\pm$ 0.5 ^o	88.2 $\pm$ 0.3 ^o	66.2 $\pm$ 0.4 ^m	59.3 $\pm$ 0.5 ⁿ
8.9	81.2 $\pm$ 0.5 ^p	87.5 $\pm$ 0.2 ^p	96.6 $\pm$ 0.4 ^p	91.7 $\pm$ 0.4 ^p	79.6 $\pm$ 0.2 ^o	68.5 $\pm$ 0.3 ^o
13.3	63.5 $\pm$ 0.4 ^m	70.5 $\pm$ 0.3 ^m	89.6 $\pm$ 0.5 ⁿ	85.3 $\pm$ 0.3 ⁿ	71.3 $\pm$ 0.5 ⁿ	50.6 $\pm$ 0.4 ^l
17.7	55.2 $\pm$ 0.3 ^k	69.2 $\pm$ 0.5 ^l	81.6 $\pm$ 0.3 ^k	79.7 $\pm$ 0.3 ^l	58.3 $\pm$ 0.5 ^k	45.4 $\pm$ 0.3 ^j
<i>Zeatin</i>						
4.6	44.5 $\pm$ 0.2 ^h	58.4 $\pm$ 0.3 ⁱ	71.5 $\pm$ 0.3 ^h	65.8 $\pm$ 0.3 ⁱ	42.7 $\pm$ 0.5 ^h	39.8 $\pm$ 0.3 ^h
9.1	52.1 $\pm$ 0.3 ^j	64.6 $\pm$ 0.2 ^j	77.1 $\pm$ 0.3 ^j	73.5 $\pm$ 0.2 ^j	48.5 $\pm$ 0.3 ⁱ	41.2 $\pm$ 0.4 ⁱ
13.7	36.7 $\pm$ 0.3 ^e	47.3 $\pm$ 0.5 ^f	62.4 $\pm$ 0.4 ^f	59.5 $\pm$ 0.3 ^f	31.0 $\pm$ 0.2 ^d	26.5 $\pm$ 0.3 ^e
18.2	28.3 $\pm$ 0.5 ^c	36.8 $\pm$ 0.3 ^b	55.6 $\pm$ 0.3 ^c	46.2 $\pm$ 0.5 ^c	29.2 $\pm$ 0.3 ^c	22.4 $\pm$ 0.3 ^c
MS alone	0.0	0.0	0.0	0.0	0.0	0.0

For each plant growth regulator type and concentration, 75 LBs per each genotype were placed in petri dishes with 20 ml media with three replicates. MS medium devoid of PGRs have been used as control

Means followed by the same letter in each column are not significantly different at $p \leq 0.05$ based on Duncan's multiple range test

induction was observed in the control experiment. Shoot initiation appeared from 'CO5' genotype at the cut end of the LBs (Fig. 1b) on MS medium containing 8.9 μM BAP along with 2.7 μM NAA. Concentration higher than the optimum level of NAA (2.7 μM) reduced the shoot induction by developing the earlier roots. Among the six Indian foxtail millet genotypes studied, 'CO5' showed superior responses for shoot induction than the other five genotypes with all three PGRs tested. MS medium amended with the optimum concentration of BAP 8.9 μM and NAA 2.7 μM were used for further investigations in this study.

Influence of amino acids on multiple shoot induction and proliferation

Even though LBs initiated shoot buds in the initial experiments, they did not consequently proliferate, mature or elongate. The supplementation of additives in the media

has been initiated to show significant development of adventitious shoot regeneration, proliferation and maturation in all foxtail millet genotypes. Shoots induced on MS medium supplemented with BAP (8.9 μM), NAA (2.7 μM) and different amino acids, showed a stimulatory effect on multiple shoot induction. In this optimized medium, cluster of multiple shoots was initiated within 12 days of incubation in light (Fig. 1c). In this study, maximum percentage (78.5) of multiple shoot induction was observed in 'CO5' genotype on MS(I) medium containing 8.9 μM BAP and 2.7 μM NAA, along with 700 mg L^{-1} Pro, 0.5 mg L^{-1} Cys, 2.0 mg L^{-1} Gly and 150 mg L^{-1} Arg, after 12 days of incubation in light (Table 3). In other genotypes viz., 'K-221-1', 'CO7', 'TNAU-43', 'H.1' and 'RS-118', multiple shoot induction frequency in the same medium were 71.5%, 68.5%, 61.7%, 55.9% and 52.2% respectively. The average number of shoots 29.5 was produced in 'CO5' genotype where in 'K-221-1' (20.4), 'CO7' (17.4), 'TNAU-43' (11.2), 'H.1' (9.9) and 'RS-118' (8.2)

Table 2 Effect of different cytokinins TDZ, Kin, BAP and zeatin on shoot induction from LBs of six foxtail millet genotypes after 10 days of incubation in light

Plant growth regulators (μM)	Percentage of shoot induction (mean $\pm$ SE)					
	Foxtail millet genotypes					
	TNAU-43	CO7	CO5	K-221-1	H.1	RS-118
<i>TDZ</i>						
0.5	7.3 $\pm$ 0.5 ^{fg}	13.8 $\pm$ 0.7 ^h	15.9 $\pm$ 1.0 ^f	18.8 $\pm$ 0.9 ⁱ	5.8 $\pm$ 0.6 ^{de}	3.8 $\pm$ 0.9 ^{def}
0.9	15.6 $\pm$ 0.4 ^j	18.9 $\pm$ 0.8 ^j	27.7 $\pm$ 0.3 ^k	22.8 $\pm$ 0.5 ^k	13.7 $\pm$ 1.1 ⁱ	9.1 $\pm$ 1.1 ^h
1.4	10.8 $\pm$ 0.6 ^h	9.1 $\pm$ 0.5 ^f	24.6 $\pm$ 0.7 ^j	12.8 $\pm$ 0.7 ^f	8.4 $\pm$ 0.3 ^g	6.5 $\pm$ 1.3 ^g
1.8	4.8 $\pm$ 0.9 ^{cd}	5.0 $\pm$ 0.9 ^c	11.7 $\pm$ 0.5 ^d	7.4 $\pm$ 1.4 ^{cd}	3.2 $\pm$ 0.4 ^c	2.7 $\pm$ 0.8 ^{cd}
<i>Kin</i>						
4.6	5.7 $\pm$ 1.1 ^{de}	6.6 $\pm$ 0.9 ^d	8.4 $\pm$ 0.5 ^b	9.5 $\pm$ 0.6 ^c	4.6 $\pm$ 0.5 ^d	3.6 $\pm$ 0.5 ^{cde}
9.3	8.2 $\pm$ 0.6 ^g	10.8 $\pm$ 1.1 ^j	17.7 $\pm$ 0.9 ^g	14.8 $\pm$ 0.7 ^g	7.5 $\pm$ 1.0 ^{fg}	4.8 $\pm$ 0.4 ^{ef}
13.9	3.3 $\pm$ 0.5 ^b	4.7 $\pm$ 0.7 ^c	10.5 $\pm$ 0.6 ^c	6.2 $\pm$ 0.5 ^{bc}	2.5 $\pm$ 0.5 ^{bc}	2.4 $\pm$ 0.5 ^{bc}
18.5	1.3 $\pm$ 0.6 ^a	3.0 $\pm$ 0.9 ^{ab}	5.1 $\pm$ 0.8 ^a	4.0 $\pm$ 1.1 ^a	1.3 $\pm$ 0.7 ^{ab}	0.9 $\pm$ 0.4 ^a
<i>BAP</i>						
4.4	16.2 $\pm$ 0.3 ^j	19.8 $\pm$ 0.7 ^j	22.3 $\pm$ 0.5 ⁱ	24.5 $\pm$ 0.5 ⁱ	14.5 $\pm$ 0.6 ⁱ	9.6 $\pm$ 0.7 ^h
8.9	20.6 $\pm$ 1.2 ^k	25.5 $\pm$ 0.9 ^k	35.1 $\pm$ 0.4 ^m	29.6 $\pm$ 0.8 ^m	17.9 $\pm$ 0.8 ^j	11.6 $\pm$ 0.4 ⁱ
13.3	8.4 $\pm$ 0.7 ^g	14.2 $\pm$ 0.3 ^h	29.5 $\pm$ 0.6 ⁱ	20.6 $\pm$ 0.5 ^j	6.0 $\pm$ 0.5 ^f	4.9 $\pm$ 0.5 ^{ef}
17.7	6.6 $\pm$ 0.5 ^{ef}	7.8 $\pm$ 0.5 ^{de}	18.1 $\pm$ 0.7 ^g	16.1 $\pm$ 0.4 ^h	5.5 $\pm$ 0.4 ^{de}	4.2 $\pm$ 0.7 ^{ef}
<i>Zeatin</i>						
4.6	7.6 $\pm$ 1.0 ^{fg}	8.4 $\pm$ 0.6 ^{ef}	16.5 $\pm$ 0.8 ^f	13.1 $\pm$ 0.7 ^f	6.4 $\pm$ 0.6 ^{ef}	5.1 $\pm$ 0.4 ^f
9.1	12.5 $\pm$ 0.3 ⁱ	15.6 $\pm$ 0.5 ⁱ	20.4 $\pm$ 0.5 ^h	17.3 $\pm$ 0.8 ^h	11.6 $\pm$ 0.5 ^h	7.1 $\pm$ 0.7 ^g
13.7	3.6 $\pm$ 0.7 ^{bc}	4.1 $\pm$ 0.4 ^{bc}	13.4 $\pm$ 0.6 ^e	8.5 $\pm$ 0.6 ^{de}	2.3 $\pm$ 1.0 ^{bc}	1.4 $\pm$ 0.5 ^{ab}
18.2	2.4 $\pm$ 0.5 ^{ab}	2.6 $\pm$ 0.5 ^a	9.7 $\pm$ 0.5 ^c	5.5 $\pm$ 0.5 ^b	1.0 $\pm$ 0.5 ^a	1.0 $\pm$ 0.6 ^a
MS alone	0.0	0.0	0.0	0.0	0.0	0.0

Data collected after 7 days of incubation in medium containing different concentrations of each plant growth regulator tested for shoot induction. 75 LBs segments per each genotype were placed in petri dishes with 20 ml media with three replicates. MS medium devoid of PGRs have been used as control

Means followed by the same letter in each column are not significantly different at $p \leq 0.05$ based on Duncan's multiple range test

genotypes produced multiple shoot induction, on MS(I) medium (Table 3). In MS medium devoid of amino acids, no multiple shoots were induced. In this specific combination of PGRs and amino acid supplements, multiple shoots remained green, and sustained to proliferate (Fig. 1d). Beyond these concentrations of additives supplemented in the medium, there was no further multiple shoot proliferation and instead relative declines were observed in induction rate and shoot number, respectively.

Effect of activated charcoal on multiple shoot induction and elongation

In order to achieve maximum shoot elongation, various concentrations of AC were tested on MS(I) medium containing PGRs and amino acids. The multiple shoots were separated individually and transfer to the MS(I) medium amended with various concentrations of AC. Increased

percentage of shoot elongation (97.2) was found in 'CO5' genotype on MS(I) medium containing 5.0 g L⁻¹ AC (Table 4). On the same medium, the other genotypes 'K-221-1', 'CO7', 'TNAU-43', 'H.1' and 'RS-118' showed 91.8, 88.3, 80.6, 73.6 and 68.4% of shoot elongation frequency, respectively. The multiple shoot induction frequency range from 98.2 to 21.2% in all six Indian foxtail millet genotypes (Table 4) and genotype 'CO5' (18.9) produced a superior response while 'RS-118' (6.6) showed less response in terms of the mean length of shoots. The shoot attained maximum elongation with thick, green, constantly developed shoots (Fig. 1e, f) and maturity after 12 days in the MS(I) medium incorporated with 5.0 g L⁻¹ AC in all the foxtail millet genotypes tested.

Fig. 2 Effect of BAP 8.9 μM in combination with different auxins on shoot induction from LBs of six foxtail millet genotypes after 10 days of incubation in light

Percentage of shoot induction

Fig. 2 continued

Average no. of shoot induction

Table 3 Influence of amino acids on multiple shoot induction of foxtail millet genotypes in MS(I) medium after 12 days of incubation in light

PGRs (μM)	Amino acids (mg L^{-1})					Percentage of multiple shoot induction (mean $\pm$ SE)					
	NAA	Pro	Cys	Gly	Arg	TNAU-43	CO7	CO5	K-221-1	H.1	RS-118
8.9	2.7	250	0.2	0.5	25	21.5 $\pm$ 0.3 ^a	26.9 $\pm$ 0.5 ^a	31.7 $\pm$ 0.4 ^a	29.4 $\pm$ 0.4 ^a	19.8 $\pm$ 0.7 ^a	14.4 $\pm$ 0.5 ^a
8.9	2.7	400	0.3	1.0	50	32.6 $\pm$ 0.8 ^c	37.9 $\pm$ 0.7 ^c	45.9 $\pm$ 0.8 ^c	41.9 $\pm$ 0.4 ^c	29.7 $\pm$ 0.8 ^c	21.8 $\pm$ 0.4 ^c
8.9	2.7	550	0.4	1.5	100	36.5 $\pm$ 0.4 ^d	46.7 $\pm$ 0.6 ^c	49.5 $\pm$ 0.7 ^d	51.7 $\pm$ 0.5 ^e	37.6 $\pm$ 0.5 ^d	33.9 $\pm$ 0.6 ^c
8.9	2.7	700	0.5	2.0	150	61.7 $\pm$ 0.7 ^f	68.5 $\pm$ 0.4 ^f	78.5 $\pm$ 0.5 ^f	71.5 $\pm$ 1.1 ^f	55.9 $\pm$ 0.8 ^e	52.2 $\pm$ 0.3 ^f
8.9	2.7	850	0.6	2.5	200	40.5 $\pm$ 0.4 ^c	40.1 $\pm$ 1.1 ^d	58.7 $\pm$ 0.6 ^c	43.8 $\pm$ 0.3 ^d	30.8 $\pm$ 0.6 ^c	27.7 $\pm$ 0.4 ^d
8.9	2.7	1000	0.7	3.0	250	27.7 $\pm$ 0.2 ^b	31.6 $\pm$ 0.2 ^b	38.5 $\pm$ 0.3 ^b	32.3 $\pm$ 0.5 ^b	21.8 $\pm$ 0.3 ^b	16.6 $\pm$ 0.3 ^b
Control											
8.9	2.7	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
<i>Total number of shoots induction per explant (mean $\pm$ SE)</i>											
8.9	2.7	250	0.2	0.5	25	1.2 $\pm$ 0.4 ^a	2.4 $\pm$ 0.7 ^a	10.5 $\pm$ 0.4 ^b	7.6 $\pm$ 0.2 ^a	4.7 $\pm$ 0.5 ^c	3.2 $\pm$ 0.6 ^b
8.9	2.7	400	0.3	1.0	50	6.2 $\pm$ 0.1 ^d	8.0 $\pm$ 0.4 ^d	13.1 $\pm$ 0.8 ^d	11.5 $\pm$ 0.4 ^b	5.8 $\pm$ 0.3 ^d	5.1 $\pm$ 0.4 ^d
8.9	2.7	550	0.4	1.5	100	9.7 $\pm$ 0.2 ^e	13.0 $\pm$ 0.4 ^c	16.6 $\pm$ 0.5 ^e	13.8 $\pm$ 0.2 ^c	8.6 $\pm$ 0.2 ^e	6.5 $\pm$ 0.6 ^c
8.9	2.7	700	0.5	2.0	150	11.2 $\pm$ 0.4 ^f	17.4 $\pm$ 0.3 ^f	29.5 $\pm$ 0.3 ^f	20.4 $\pm$ 0.5 ^d	9.9 $\pm$ 0.3 ^f	8.2 $\pm$ 0.3 ^f
8.9	2.7	850	0.6	2.5	200	4.1 $\pm$ 0.2 ^c	6.4 $\pm$ 0.3 ^c	12.2 $\pm$ 0.3 ^c	7.2 $\pm$ 0.6 ^a	3.3 $\pm$ 0.3 ^a	4.9 $\pm$ 0.8 ^c
8.9	2.7	1000	0.7	3.0	250	2.4 $\pm$ 0.5 ^b	3.6 $\pm$ 0.3 ^b	8.2 $\pm$ 0.4 ^a	7.4 $\pm$ 0.5 ^a	4.1 $\pm$ 0.4 ^b	2.9 $\pm$ 0.9 ^a
Control											
8.9	2.7	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

The percentage of multiple shoot induction and mean number of shoots per explant were calculated after 12 days of incubation in light. 75 LBs with three replicates of the six foxtail millet genotypes were tested in MS(I) medium containing 8.9 μM BAP, 2.7 μM NAA, 700 mg L^{-1} pro, 0.5 mg L^{-1} cys, 2 mg L^{-1} gly and 150 mg L^{-1} arg. Data shown in this table are the mean of 3 replicates $\pm$ standard error

Means followed by the same letter in each column are not significantly different at $p \leq 0.05$ based on Duncan's multiple range test

In vitro root induction

To promote the rooting, shoots approximately 12 cm were excised and transferred onto a half-strength MS medium containing IAA 2.9 μM . All the six foxtail millet genotypes in the present study showed the best rooting response on half-strength MS medium containing 2.9 μM IAA. The number of roots and its length varied among the six genotypes after 10 days of incubation in light. The highest rooting frequency 91.5% with mean number of roots 8.6 per shoot and mean root length 6.9 cm was observed in 'CO5' genotype when 2.9 μM IAA was incorporated on half-strength MS medium (Fig. 3a–c). The lowest rooting frequency 63.2% was noticed in 'RS-118' genotype. Half-strength MS medium containing 2.9 μM IAA produced thinner, longer and profuse roots (Fig. 1g) while in control plants, fine and short roots were noticed after 10 days incubation in light.

Hardening

The rooted plantlets after 10 days of culture under light were carefully transferred to the Yoshida solution (Yoshida et al. 1976) and maintained in growth chamber for 4 days.

Plantlets readily shifted to plastic cups showed 92% survival rate 20 days after transplanting. Regenerated shoots grew to mature plants were free of any morphogenetic difference upon field transfer (Fig. 1h). Schematic representation for the different steps involved in direct multiple shoot proliferation and plant regeneration protocol in foxtail millet is provided in Fig. 4.

Genetic fidelity analysis

RAPD and ISSR markers amplified different regions of the genome, allowing better analysis of genetic stability. The genomic DNA isolated from the in vitro and field grown plants were amplified with 20 RAPD and 15 ISSR primers. Five RAPD primers produced well resolved, reproducible amplification pattern with monomorphic bands and size of the amplicons ranged from 200 bp in OPC 13–1250 bp in OPA 10. In ISSR analysis, a total of five primers produced well resolved bands with the size range of 100 bp (ISSR A1) to 2000 bp (ISSR A3). Amplification profiles obtained from in vitro grown plants and that of the field grown plants were comparably similar in all respects. All the primers shown monomorphism of the amplified DNA bands confirming the genetic homogeneity of the in vitro

Table 4 Influence of activated charcoal on regeneration of six foxtail millet genotypes in MS(I) medium after 12 days of incubation in light

Activated charcoal (g L ⁻¹)	Amino acids (mg L ⁻¹)				Percentage of multiple shoot proliferation (mean ± SE)					
	Pro	Cys	Gly	Arg	TNAU-43	CO7	CO5	K-221-1	H.1	RS-118
Control										
0.0	700	0.5	2.0	150	41.7 ± 0.9 ^b	46.9 ± 0.8 ^b	62.2 ± 0.4 ^b	56.3 ± 0.4 ^b	37.6 ± 0.4 ^b	32.2 ± 0.8 ^b
2.5	700	0.5	2.0	150	55.5 ± 0.4 ^d	58.7 ± 0.4 ^c	70.6 ± 0.7 ^d	61.5 ± 0.5 ^c	44.5 ± 0.7 ^c	51.3 ± 0.7 ^d
5	700	0.5	2.0	150	83.5 ± 0.7 ^e	91.8 ± 0.6 ^e	98.2 ± 0.3 ^e	94.8 ± 0.2 ^e	78.1 ± 0.4 ^e	70.6 ± 0.9 ^e
7	700	0.5	2.0	150	50.3 ± 0.5 ^c	61.3 ± 0.2 ^d	67.7 ± 0.4 ^c	68.7 ± 0.5 ^d	57.5 ± 0.5 ^d	34.2 ± 0.6 ^c
10	700	0.5	2.0	150	31.7 ± 0.6 ^a	39.7 ± 0.6 ^a	52.2 ± 0.4 ^a	48.1 ± 0.2 ^a	28.7 ± 0.8 ^a	21.2 ± 0.7 ^a
<i>Percentage of plant elongation (mean ± SE)</i>										
Control										
0.0	700	0.5	2.0	150	42.1 ± 0.2 ^a	52.2 ± 0.9 ^a	58.8 ± 0.4 ^a	56.6 ± 0.9 ^a	39.2 ± 1.0 ^a	25.2 ± 0.7 ^a
2.5	700	0.5	2.0	150	63.6 ± 0.8 ^d	67.7 ± 0.8 ^d	75.8 ± 1.1 ^d	70.4 ± 0.6 ^c	61.7 ± 0.5 ^d	53.8 ± 0.8 ^d
5	700	0.5	2.0	150	80.6 ± 0.7 ^e	88.3 ± 0.6 ^e	97.2 ± 0.2 ^e	91.8 ± 0.8 ^d	73.6 ± 0.7 ^e	68.4 ± 0.6 ^e
7	700	0.5	2.0	150	57.1 ± 1.5 ^c	61.1 ± 0.7 ^c	71.9 ± 0.8 ^c	69.6 ± 0.5 ^c	48.8 ± 0.2 ^c	39.6 ± 0.4 ^c
10	700	0.5	2.0	150	51.8 ± 0.3 ^b	57.7 ± 1.0 ^b	68.6 ± 0.7 ^b	63.1 ± 0.4 ^b	41.5 ± 0.4 ^b	31.1 ± 0.6 ^b
<i>Mean length of shoot (cm) (mean ± SE)</i>										
Control										
0.0	700	0.5	2.0	150	6.5 ± 0.8 ^b	7.4 ± 0.6 ^b	11.5 ± 0.5 ^c	9.7 ± 0.6 ^c	5.6 ± 0.8 ^{bc}	4.9 ± 1.1 ^b
2.5	700	0.5	2.0	150	8.6 ± 0.6 ^c	10.7 ± 0.3 ^d	16.7 ± 0.4 ^d	12.8 ± 0.7 ^d	6.5 ± 0.5 ^{cd}	5.2 ± 0.4 ^b
5	700	0.5	2.0	150	9.7 ± 0.3 ^c	12.4 ± 0.4 ^e	18.9 ± 0.3 ^e	15.9 ± 0.9 ^e	7.2 ± 0.4 ^d	6.6 ± 0.2 ^c
7	700	0.5	2.0	150	5.9 ± 0.6 ^{ab}	8.7 ± 0.6 ^c	9.4 ± 0.7 ^b	7.9 ± 1.0 ^b	5.2 ± 0.7 ^{ab}	3.7 ± 1.0 ^{ab}
10	700	0.5	2.0	150	4.8 ± 0.5 ^a	5.9 ± 0.7 ^a	7.7 ± 0.6 ^a	6.1 ± 0.2 ^a	4.4 ± 0.4 ^a	3.1 ± 0.7 ^a

For each activated charcoal concentration, 10 days old shoots of the six foxtail millet genotypes were tested in MS(I) medium containing 8.9 μM BAP, 2.7 μM NAA, 700 mg L⁻¹ pro, 0.5 mg L⁻¹ cys, 2 mg L⁻¹ gly and 150 mg L⁻¹ arg. The Percentage of multiple shoot induction, elongation and mean length of shoots were calculated after 12 days of incubation in dark. The data correspond to the average of three replicates. Values represent the mean ± SE (analyzed by SPSS version 17.0)

Means followed by the same letter in rows are not significantly different at $p \leq 0.05$ based on Duncan's multiple range test

regenerated plants (Fig. S1). No variation was seen between in vitro grown and the plantlets derived from the seeds.

Discussion

In this current study, LBs have been used as a preliminary explant for successful direct multiple shoot induction and also improve plant regeneration ability of six important Indian foxtail millet genotypes. The procedure reported here has best regeneration capacity over previously published reports of foxtail millet via organogenesis. Monocot plants consist of a competence of dedifferentiated cells in the basal meristem that creates leaf base segments a prime organization in cereals (Wernicke and Brettell 1982). LBs are a suitable system for the biotechnological studies of in vitro and in vivo developmental responses and the cell interactions. In oats, LBs have been used to achieve

transgenic plants with higher regeneration potential (Matsuda et al. 1998).

LBs are the most important prerequisites for in vitro direct shoot formation. Multiple shoot clumps regeneration from shoot tips was first reported in maize (Zhong et al. 1992). Regeneration of shoot meristematic cultures from seed derived mature embryo was successfully reported in pearl millet, wheat and finger millet (Devi et al. 2000; Yu et al. 2012; Satish et al. 2016b). Shoot apex explants has also been efficiently used as an initial explant source to retrieve stably transformed plants in sorghum (Rao et al. 1995), oats (Choi et al. 2000), barley (Manoharan and Dahleen 2002), wheat (Moghaieb et al. 2010), maize (Huang and Wei 2004), rice (Arockiasamy and Ignacimuthu 2007; Dey et al. 2012) and finger millet (Ceasar and Ignacimuthu 2011). Somatic embryos derived from mature seeds are used as a primary source of explants in foxtail millet for callus induction (Reddy and Vaidyanath 1990; Rao et al. 1988; Satish et al. 2016a) and showed the strong influence of genotype (Satish et al. 2016a, b). However,

Fig. 3 Effect of IAA on root induction from regenerated shoots of foxtail millet on half strength MS medium after 10 days of incubation in light

this is the first report on the direct plant regeneration system developed from in vitro LBs of six foxtail millet

genotypes, thus useful for shoot induction and plant regeneration studies.

Fig. 4 Schematic representation of the complete protocol for the regeneration of foxtail millet plants

The most important advantage in the direct regeneration of plants is avoidance of callus interference phase, which requires several subcultures. In order to induce direct shoots in foxtail millet, four different cytokinins namely TDZ, Kin, BAP and zeatin at different concentration were tested individually. Previously, in many plant species, appropriate types and concentration of cytokinins have been found to promote cell expansion and cell division in tissue culture (Ružić and Vujović 2008). In our study, compared to other cytokinins, BAP supplemented medium responded well for shoot initiation with the maximum of survival rate. These results were consistent with previous direct regeneration study in finger millet by our group (Satish et al. 2015). The shoot induction in rice and millets is generally achieved by TDZ or BAP (Ceasar and Ignacimuthu 2008; Satish et al. 2015; Dey et al. 2015). The lower concentration of TDZ showed a very less number of shoot induction than BAP, whereas higher concentration of TDZ is toxic and cause damage to the shoot induction (Hutchinson et al. 2004). In our previous report, BAP only responded well for foxtail millet regeneration (Satish et al. 2016a). The combined use of PGRs in MS medium stimulated the shoot induction frequency and also produced the maximum shoots efficiently than the individual PGRs. The synergistic effect of BAP in combination with auxins has

been studied in several plant species (Rency et al. 2017). In this study, the addition of IAA, IBA or NAA separately to the medium containing BAP (8.9 μM) showed increased frequency of shoot induction and the most efficient response was achieved at BAP (8.9 μM) and NAA (2.7 μM). Our results were reliable with Park and Sivanesan (2015) in anjuga on MS medium fortified with (8.9 μM) BAP and (2.7 μM) NAA. The mean percentage of primary shoot induction after 10 days of incubation in light was superior on MS medium containing 8.9 μM BAP with 2.7 μM NAA of six foxtail millet genotypes. In earlier studies, a BAP and NAA together has been shown to lead to efficient shoot induction and plant regeneration in foxtail millet (Wang et al. 2011), kodo millet (Kaur and Kothari 2004) and rice (Karthikeyan et al. 2009). In this study, BAP alone produced less shoot initiation, but could not produce the rapid multiple shoot regeneration where the optimal ratio of the BAP (8.9 μM) in combination of NAA (2.7 μM) gave higher frequency of shoot propagation. Similarly, Karakas et al. (2016) reported that the highest number of shoots was obtained from nodal explants of Goat's rue with a 4.4 μM BA and 1.3 μM NAA combination.

In this study, addition of amino acids and AC in MS(I) medium improved multiple shoot induction and

plant regeneration in all foxtail millet genotypes tested. Previously, Pro, Gly and Arg have been efficaciously used for high-frequency somatic embryogenesis and regeneration of sorghum (Rao et al. 1995), rice (Ozawa and Komamine 1989) and foxtail millet genotypes (Satish et al. 2016a). The supplementation of additives in the media has been found to result in significant improvement in adventitious shoot bud induction, proliferation and maturation of shoots. The addition of cytokines, carbohydrates and amino acids in the media enhances the maturation and regeneration of embryogenic callus in foxtail millet genotypes (Satish et al. 2016a). Amino acids served as an organic nitrogen source in in vitro cultures have enhanced the multiple shoot proliferation and regeneration in many plant species (Asad et al. 2009). The addition of exogenous amino acids have induced the different morphogenetic responses (Vasanth et al. 2006). Asad et al. 2009 reported that PGRs with various amino acid significantly promoted the multiple shoot proliferation. Ramakrishnan et al. (2014) have used Gly to stimulate the proliferation of multiple shoots in maize similarly, Cys and Pro promoted the high frequency plant regeneration in rice (Grewal et al. 2006; Sivakumar et al. 2010). AC increases the efficiency of root growth and improves the morphogenic response of in vitro cultured plants (Dharishini et al. 2015). Chutipaijit and Sutjaritvorakul (2018) reported that lower concentrations of AC diminishes the oxidation of phenolic compounds in tissue culture medium and augments the regeneration of rice plants. Moreover, few reports of AC revealed its primitive effect on culture establishment and the regeneration potential (Burner and Grisham 1995; Kunitake et al. 1995). However, none of these authors used AC for precocious plant development. Though, the present study deciphers, AC augmented regeneration efficiency through inducing multiple shoot induction in foxtail millet genotypes. This study confesses that the frequency of multiple shoot induction, proliferation and elongation increased rapidly in all six Indian foxtail millets studied when concentration of amino acids and AC was increased steadily in the culture medium as compared to the media without amino acid supplements.

In previous reports, the in vitro morphogenesis has been influenced by different genotypes in millets and cereals (Ceasar and Ignacimuthu 2008; Satish et al. 2016a). Here, six different Indian genotypes of foxtail millet were studied for their ability to undergo in vitro development and successive plant regeneration. All the foxtail genotypes studied were efficient in shoot induction, multiple shoot proliferation and plant regeneration on various culture conditions tested. However, the frequency of in vitro regeneration response was found to be genotype independent and in this study 'CO5' genotype persisted to be the most responsive in all the experiments.

In the present study, two different types of molecular marker techniques RAPD and ISSR have been used to analyse the somaclonal variation among field grown and in vitro derived six Indian foxtail millet genotypes. Previously, two or more molecular marker techniques have employed to assess the genetic fidelity and diversity in various crops (Gupta et al. 2010; Rajendran et al. 2016; Satish et al. 2016c). However, using DNA molecular marker techniques is ideal to ensure that the in vitro regenerated plants are true to the parent type (Martins et al. 2004). RAPD markers are genetically dominant and arbitrary and are used to identify inter- and intra-specific variation in many plant species. They are often based on non-coding regions of DNA (Agarwal et al. 2015; Satish et al. 2016a). The total number of amplified bands for each primer varied from 5 to 15 in 'CO5', 4 to 10 in 'CO7', 4 to 8 in 'K-221-1', respectively. The banding patterns of the RAPD and ISSR primers are shown in supplementary figure 1. The resulted banding profile of RAPD and ISSR marker analysis revealed that the plants derived from seeds and randomly selected in vitro regenerants were similar. This clearly indicated the genetic fidelity of in vitro regenerated plants.

Conclusion

We report a highly efficient direct multiple shoot bud induction and plant regeneration system from in vitro derived LBs for six genotypes of foxtail millet. This study is novel for foxtail millet due to true-to-type proliferation within a short span and the relatively high regeneration frequency. Besides this, the study may put forward some good fortune in the molecular analysis or genetic improvement of foxtail millet.

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Authors' contributions PR and MR conceived and designed research. PR carried out the experiments. SP performed the molecular marker analysis. ASR and RR analyzed data. PR prepared the

manuscript, LS and MR proofread the manuscript. All authors read and approved the manuscript for publication.

Compliance with ethical standards

Conflict of interest The authors declare that there is no conflict of interest.

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