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Rapid and efficient plant regeneration from shoot apical meristems of finger millet [*Eleusine coracana* (L.) Gaertn.] via direct organogenesis

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A simple and efficient plant regeneration system via direct organogenesis was established in finger millet using *in vitro* derived shoot apical meristems. Six varieties; GBK-043128, GBK-043094, GBK-043050, GBK-043137, GBK-043122 and GBK-043124 were evaluated. MS medium was used for cotyledonary germination. Maximum number of shoots (84.33%) was observed in variety GBK-043128 while GBK-043094 had the least germination efficiency (62. 67%). Shoot apical meristems from three-day old seedlings were evaluated for their potency of shoot induction on varied 6-benzylaminopurine (BAP) concentrations. Highest shoot induction was observed in medium supplemented with 1.75 mg/l BAP in GBK-043050 (3.00) whereas GBK-043094 (1.28) had the least response in medium supplemented with 1.0 mg/l BAP. To induce rooting, *in vitro* regenerated plants cultured on MS medium was supplemented with different concentrations of indole-3- acetic acid. The highest response in root induction, with a larger number of roots (10.28), was observed in MS medium supplemented with 4.0 µM IAA. Statistical analysis indicated that plant regeneration response varied greatly among the varieties. *In vitro* germinated plants were successfully transferred to the greenhouse after hardening, with 300 shoots developing into fertile plants, which were indistinguishable with wild type plants. This plant regeneration system has potential for production of transgenic finger millet crops.

Key words: Direct organogenesis, finger millet, root induction, shoot apical meristems.

INTRODUCTION

Finger millet [*Eleusinecoracana* (L.) Gaertn.] is an important cereal crop worldwide which is cultivated on more than 4 million hectares with an annual production of at least 4.5 million tons of grain. Due to its ease of

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cultivation, low fertilizer requirements and high adaptability, finger millet is considered a food security and major staple food crop for millions of subsistence and rural communities in semi-arid regions of Asia and Africa (Chivenge et al., 2015). Besides its direct use as table and feed stock, finger millet is also a candidate for the production of renewable plant products such as ethanol (Tekaligne et al., 2015). Compared to other cereals, finger millet has outstanding nutritional qualities as the grain is rich in calcium, phosphorus, iron, cysteine, tyrosine, tryptophan and methionine (Latha et al., 2005). Finger millet is primarily consumed as porridge in Africa but in South Asia as bread, soup, roti (flat bread) and to make beer. Other new food merchandise made of finger millet that have become common among younger generation include vermicelli, pasta, noodles, sweet products, snacks and different bakery products.

Despite its major importance, finger millet has not received significant attention from plant biotechnologists. The plant's growth and productivity is greatly constrained by agronomic practices, pests and diseases, low soil fertility, labour intensity, high weed infestation, low yielding varieties, lodging, poor attitude to the crop, salinity and drought emanating from climate change (Mgonja et al., 2007). In light of the utilization of finger millet-based items, interest for the crop is expanding over the world (Pathi et al., 2013) and enhancing and developing finger millet varieties with desirable qualities and tolerance to various environmental pressures through genetic engineering is therefore a critical need. However, for genetic engineering to occur, a reliable and effective plant regeneration system have to be initially established. Plant tissue culture technology is thus a significant biotechnological instrument for the transfer of genetic traits to overcome crop yield losses due to various biotic and abiotic stresses (Kumar et al., 2015b). Finger millet has long been considered recalcitrant for plant tissue culture (Gupta et al., 2017). To the best of the researchers' knowledge, the current finger millet regeneration systems available were based on somatic embryogenesis and are limited by low regeneration frequencies and long regeneration periods (Dey et al., 2012).

Direct and indirect shoot organogenesis from different explants are reportable as effective explants for several plant species. Direct organogenesis regeneration from explants, omitting the callus induction segment, is admirable notably in modern plant tissue culture technology wherever reducing costs of regeneration systems, minimising somaclonal variation and increasing rapidity are fundamental components of consideration (Burner and Grisham, 1995). The success of plant regeneration especially through direct regeneration is dependent on choice of explant and maturation and conversion of the explant into plants. Direct regeneration therefore, represents a promising tool for plant regeneration because is a rapid and an effective approach. The present work reports a rapid and efficient direct plant regeneration system for African finger millet varieties using *in vitro*-derived shoot apical meristems as explants, without an intermediate callus phase. For multiple shoot induction and regeneration, the concentration of cytokinin was optimized. This procedure is rapid, reliable and reproducible and can immensely be used for genetic transformation of finger millet in the future.

MATERIALS AND METHODS

Plant material and explants preparation

Six Kenyan preferred finger millet varieties were used: GBK-043128, GBK-043094, GBK-043050, GBK-043137, GBK-043124 and GBK-043122. The seeds of these varieties were procured from Kenya Agricultural Research and Livestock Organization, Gene Bank, Nairobi, Kenya. The seeds were surface sterilized by washing them with sterile distilled water followed by incubating them with 70 % (v/v) ethanol for two min then transferred to 20% sodium hypochlorite containing a drop of Polysorbate 20 for 23 min. Surface sterilized seeds were rinsed thrice with double distilled water and germinated aseptically on Murashige and Skoog (MS) basal medium (Murashige and Skoog, 1962) supplemented with 30% sucrose. The medium pH was adjusted to 5.8 before adding 3 g/l gelrite, followed by autoclaving at 121°C for 15 min under 15 kPa. The cultures were incubated at 25±2°C in the dark for germination for three days. The germination percentage of the six varieties was calculated after 3 days of culture.

Shoot induction and elongation.

Aseptically grown 3-day-old shoot tips, comprising of the apex and a part of mesocotyl, were excised and utilized as explants. The shoot tips (4-6mm) were cultured on shoot induction medium (SIM) comprising of MS basal medium supplemented with 30% sucrose and different concentrations of 6-benzylaminopurine (BAP) (1, 1.5, 1.75 and 2 mg/l). The culture bottles were incubated in the growth room with 16/8-hours light/dark at 26±2°C for 12 days. Twelve days after culture, the formed shoots were transferred to fresh SIM and incubated in the growth room with 16/8-hours light/dark at 26±2°C for a further 12 days to elongate the induced shoots. The percentages of the number of shoots that formed in each shoot clump and the mean number of shoots induced in each explant were calculated following 24 days of culture.

Root induction

The elongated shoots (5-6 cm long) were transferred to rooting medium comprising of MS basal medium supplemented with 30% sucrose and various concentrations of indole-3- acetic acid (IAA;1, 2, 3, 4 and 5 µM). The plantlets were then incubated in the growthroom under light16/8-hours light/dark at 26±2°C to induce rooting. The total number of roots initiated per shoot was calculated after 28 days of culture.

Hardening and acclimatization

Rooted plants were washed with double distilled water to remove medium on the plantlets. The plants were thereafter transferred into peat moss in plastic cups (11×15 cm) for hardening for 5 days

Table 1. Germination efficiencies of six finger millet varieties.

Variety	Number of seeds	Germination efficiency
GBK-043137	100	84.33± 0.048[a]
GBK-043128	100	82.33±0.034[a]
GBK-043124	100	72.70±0.10[ab]
GBK-043122	100	62.67±0.013[b]
GBK-043094	100	62.67±0.013[b]
GBK-043050	100	80.00± 0.012[a]

Means (± SE) followed by different alphabets in each column are significantly different (P ≤0.05) using Fishers LSD.

following which the plants were then transferred to soil in pots and incubated in greenhouse for acclimatization. The plants were watered regularly and data on survival rate of plants recorded after 4 weeks of culture. Plant survival rate was calculated as:

$$Survival\ rate = \frac{surviving\ plants}{total\ plantlets}\ x\ 100$$

Experimental design and data analysis

A completely randomized block design with three replications of 15 explants per replication was employed. MS basal medium excluding plant growth regulators was used as a negative control. Observations for any morphological changes formed on the cultures were made periodically and recorded at regular intervals. Data on the number of rooted shoots were chronicled after 28 days of culture on rooting medium. After 30 days of transfer of the plantlets to plastic cups, the survival percentage was recorded. The variability in data was expressed as mean ± standard error (SE). The data collected were analysed using one-way analysis of variance (ANOVA) with Minitab statistical computer softwarev.17. Means were separated using the Fisher's protected LSD test at a confidence level of 95% ($p \leq 0.05$).

RESULTS

Effects of MS medium on germination

The seeds of the six selected finger millet varieties were germinated on MS basal medium containing with 30% sucrose and different germination efficiencies recorded after three days of incubation in dark (Figure 1A). GBK-043137 had the highest germination efficiency of 84.33% followed by GBK-043128, GBK-043050 and GBK-043124 with seed germination efficiencies of 82.33, 80 and 72.70% respectively. Varieties GBK-043122 and GBK-043094 had the least germination efficiencies of 62.67% (Table 1). Significantly higher difference in germination was observed for varieties GBK-043137, GBK-043128, GBK-043050, GBK-043124, GBK-043122 and GBK-043094 (Table 1). However, for varieties GBK-043137, GBK-043128, GBK-043050 there was significantly lower difference in germination rate (Table 1).

Effect of induction of shoot apical meristems

When 3-day-old meristemic shoot tips, consisting of the apex and part of mesocotyl were cultured on MS basal medium containing various concentrations of BAP, shoot induction was observed within 1 day of incubation. After one day of incubation, the explants induced shoot which were white in color (Figure 1B) and formed a single leaf-like structure which thereafter became green (Figure 1C) and formed multiple shoots after 24 days (Figure 1D). The six finger millet varieties tested exhibited remarkably different regeneration responses depending on the concentration of BAP. The best shoot induction was observed in medium containing 1.75 mg/l BAP (Table 2). Shoot apical meristems explants of all finger millet varieties tested responded well to different concentrations of BAP tested in the shoot induction medium (Figure 1B). However, shoot induction response and number of shoots per explant also varied based on the variety and BAP concentration in the shoot induction medium. Statistical analysis of variance indicated that significant differences were observed among the varieties in plant regeneration response. Induction medium supplemented with 1.75 mg/l BAP exhibited significantly better response of shoot induction than the other BAP concentration tested, ranging from 3.00 to 1.28 shoots per explant. The variety GBK-043050, showed significantly superior response of shoot induction; GBK-043128, GBK-043124, GBK-043137 and GBK-043122 produced a moderate response; GBK-043094 produced a significantly lowest response of 1.62 shoots per explant in shoot induction medium supplemented with 1.75 mg/l BAP (Table 2). The lowest performing media was 1.0 mg/l on GBK-043094 with an average number of shoots of 1.28±0.13 (Table 2). The height of the plant varied between 5 and 6 cm.

Similarly, shoot clumps developed on all varieties when sub-cultured in shoot induction medium containing BAP. The shoot clumps produced were significantly more shoots and the shoots were also longer. The best response was observed in MS basal medium containing 1.75 mg/l BAP with 12 total shoots (Table 3). However, shoot clumps sub-cultured to the same induction medium containing 1.0 mg/l BAP and no response of shoot

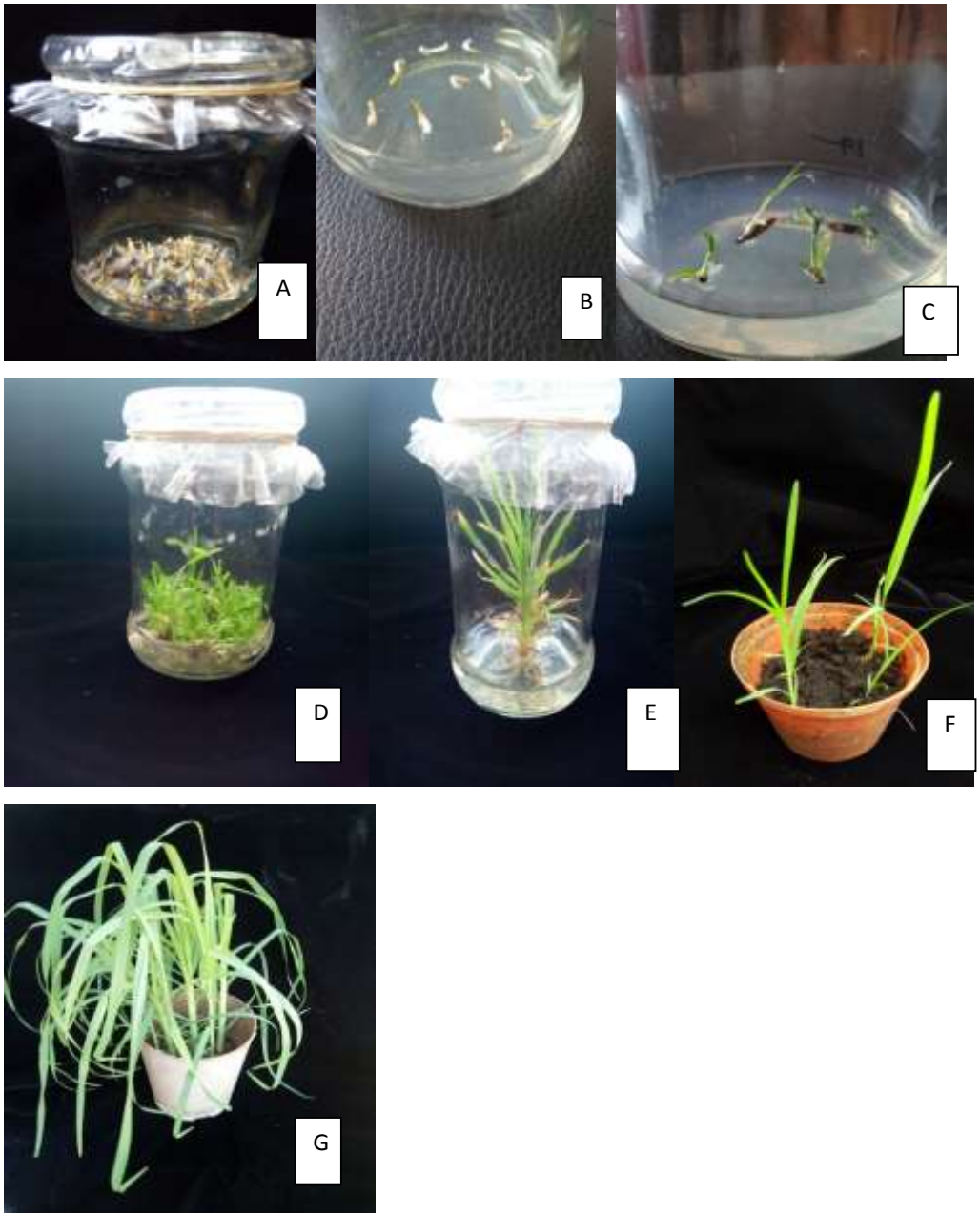

Figure 1. (A) Three day-old finger millet seedlings germinated on plant growth-regulators free MS medium; (B) Initiation of shoots from shoot apical meristems(4-6 cm) inoculated on MS medium containing BAP; (C) Shoots formed in 12 days; (D) Multiple shoots formed in 24 days; (E) Root development in MS medium containing IAA; (F) Acclimated plantlets in plastic cups containing sterile peat moss; (G) Two weeks after hardening off on peat moss

multiplication was observed on MS basal medium lacking plant growth regulators. The shoot multiplication response also varied among the 6 varieties evaluated (Table 3).

Root induction

Rooting occurred after two weeks when the elongated shoots (5-6 cm in height) were cultured on MS basal varied responses of rooting based on the variety and concentration of IAA. Majority of the varieties produced optimal root induction response on MS supplemented with 3 µM IAA. The variety GBK 043124 showed significantly higher response of root induction (8.89 roots); GBK043137, GBK043128, GBK043122 and GBK043094 produced moderate response; GBK 043050

Table 2. Shoot induction on BAP after 12 days of six finger millet varieties.

Variety	BAP (mg/l)			
	1.0	1.5	1.75	2.0
GBK-043137	1.70±0.23^{b}	1.98±0.11^{ab}	2.02±0.11^{b}	1.88±0.28^{b}
GBK-043128	1.55±0.08^{b}	2.22±0.11^{ab}	2.40±0.31^{ab}	1.60±0.21^{b}
GBK-043124	1.67±0.32^{b}	1.96±0.15^{ab}	2.01±0.15^{b}	2.02±0.16^{ab}
GBK-043122	1.84±0.29^{ab}	1.75±0.17^{b}	2.07±0.41^{ab}	1.72±0.07^{b}
GBK-043094	1.28±0.13^{b}	1.89±0.29^{ab}	1.62±0.29^{b}	1.74±0.13^{b}
GBK-043050	2.38±0.26^{a}	2.71±0.65^{a}	3.00±0.39^{a}	2.61±0.39^{a}
Control	0.00±0.00^{c}	0.00±0.00^{c}	0.00±0.00^{c}	0.00±0.00^{c}

Means (± SE) followed by different alphabets in each column are significantly different (P ≤0.05) using Fishers LSD

Table 3. Shoot multiplication on BAP after 24 days of six finger millet varieties.

Variety	BAP (mg/l)			
	1	1.5	1.75	2.0
GBK-043137	3.60±0.15^{a}	4.13±0.19^{b}	4.43±1.44^{cd}	3.77±0.23^{c}
GBK-043128	3.30±0.32^{a}	3.63±0.09^{bc}	10.33±0.88^{ab}	6.00±0.58^{bc}
GBK-043124	2.00±0.00^{b}	3.00±0.00^{c}	8.00±1.53^{bc}	7.33±0.33^{ab}
GBK-043122	3.83±0.32^{a}	3.43±0.55^{bc}	4.13±0.63^{cd}	4.00±0.57^{bc}
GBK-043094	3.00±0.58^{a}	3.47±0.29^{bc}	4.00±1.08^{d}	3.70±0.59^{c}
GBK-043050	3.67±0.29^{a}	5.00±0.00^{a}	12.00±2.31^{a}	10.00±2.89^{a}
Control	0.00±0.00^{c}	0.00±0.00^{d}	0.00±0.00^{e}	0.00±0.00^{d}

Means (± SE) followed by different alphabets in each column are significantly different (P ≤0.05) using Fishers LSD.

produced the significantly low response of 4.25 roots. However, root induction in varieties GBK-043124 and GBK-043050 was highest achieved in 4 and 2 µM with 10.28 and 5.70 roots, respectively. Root induction was least achieved in MS medium supplemented with 1 and 5 µM across the six varieties (Table 4).

Hardening and acclimatization

The rooted plants were transferred to peat moss and maintained in growthroom for 5 days. Following the 5 days culture in growthroom, the plants were transferred to soil in plastic pots with 100% survival rate and incubated in the greenhouse where the plants were watered regularly. All plant regenerated via direct organogenesis grew well and exhibited phenotypic homogeneity and same growth characteristics when compared to field-grown finger millet plants derived from seeds (Figure 1G).

DISCUSSION

Genetic engineering techniques are increasingly becoming important to achieve rapid improvements in finger millet cultivars. To successfully achieve this objective and efficiently produce bioengineered crops, improvements to the existing laborious and time-consuming protocols for *in vitro* regeneration need to be established. In this study, procedures for rapid and effective shoot-regeneration of six Kenyan farmer preferred finger millet varieties that have potential for application in genetic engineering experiments was optimized. In order to establish rapid and efficient plant regeneration procedures that could be used in genetic engineering experiments, hormone regimes which have previously been reported in successful finger millet transformation were tested. The choice of shoot organogenesis over somatic embryogenesis is because it is fast and also circumvents prolonged callus stages, therefore minimising chances of somaclonal variation (Karp, 1991). More so, shoot apical meristems are also easily handled compared to other explants and can be induced to produce multiple shoots (Arockiasamy and Ignacimuthu, 2007).

The shoot apical meristems derived from mature seeds was used as an initial explant for efficient and reproducible direct regeneration protocol for finger millet. The successful use of shoot apical meristems explants in this study implies that they are a better choice for plant regeneration in cereals when compared to other explants.

Table 4. Root induction on IAA after 28 days of six finger millet varieties.

Variety	IAA (µM)				
	1	2	3	4	5
GBK-043137	4.33±0.17^{ab}	7.13±0.35^{a}	8.03±0.42^{ab}	4.50±0.25^{b}	3.39±0.59^{ab}
GBK-043128	3.70±0.29^{bc}	6.50±0.12^{ab}	7.86±0.29^{ab}	4.65±0.80^{b}	2.63±0.18^{bcd}
GBK-043124	4.54±0.27^{a}	6.74±0.54^{ab}	8.89±0.11^{a}	10.28±0.20^{a}	4.00±00^{a}
GBK-043122	3.88±0.07^{abc}	4.41±0.51^{c}	7.60±0.48^{b}	4.81±0.51^{b}	3.23±0.44^{abc}
GBK-043094	2.51±0.39^{d}	4.44±0.50^{c}	6.25±0.25^{c}	5.25±0.30^{b}	2.47±0.15^{cd}
GBK-043050	3.48±0.42^{c}	5.70±0.49^{b}	4.25±0.53^{d}	3.15±0.52^{c}	2.01±0.13^{d}
Control	0.00±0.00^{e}	0.00±0.00^{e}	0.00±0.00^{d}	0.00±0.00^{d}	0.00±0.00^{e}

Means (±SE) followed by different alphabets in each column are significantly different (P ≤0.05) using Fishers LSD.

This study therefore confirms previous reports by Ramakrishnan et al. (2014) and Ceasar and Ignacimuthu (2010). Mature seeds derived explants are better source material for tissue culture research than others because of the availability of seeds, ease of storage of seeds, and homogeneity quality of the explants (Yang et al., 2013). To induce shoots, different concentrations (1.0, 1.5, 1.75 and 2.0 mg/l) of BAP were tested separately. Cytokinins play important role in shoot growth. BAP is commonly used for in vitro regeneration of cereals and other monocot plants (Ramakrishnan et al., 2013). BAP concentration of 1.75 mg/l was found to be more effective in direct shoot induction of six finger millet varieties. The genotype dependent variations in shoot induction among finger millet varieties observed in this study were also noted in previous studies for monocot cereal plants (Pazuki and Sohani, 2013).

Shoot clumps obtained from the shoot induction medium were subcultured to shoot elongation medium with various concentrations of BAP. The medium supplemented with 1.75 mg/l BAP produced more number of shoots of 10.33 shoots per explant. To the best of the researchers' knowledge, this is the highest number ever reported in finger millet. Report by Pande et al. (2015) stated that optimal multiple shoot induction response was recorded on MS basal media supplemented with 3.0 mg/l of BAP, while Satish et al. (2015) reported 8.3 shoots per explants of finger millet variety 'CO(Ra)-14' in MS basal medium containing 17.6 µM 6 BAP, 0.9 µM2,4-dichlorophenoxyacetic acid (2,4-D) in combination with 750 mg/l proline, 500 mg/l casein enzymatic hydrolysate and 2 mg/l glycine. When compared to other monocots, Pilahome et al. (2014) reported 30.33 shoots per explant of Sakon Nakhon in MS medium containing 50 µM 6-BAP in rice while Muoma et al. (2008) reported 5.7 shoots from inbred lines of Kenyan maize KAT and TLO8 from shoot apices on MS basal medium containing 26.64 µM BAP, 296 µM adenine and 9 µM 2,4-D. Earlier research work on maize genotypes from stated 4.3 shoots (CM300) and 1 to 3 shoots (LM5) in MS medium supplemented with 4.4 µM BA and 2.8 µM IAA from 14-day-old immature embryos (Rakshit et al., 2010; Manivannan et al., 2010). Similarly, research work by Pathi et al. (2013) described 9 shoots from mature embryo in maize genotype HQPM-1 on MS basal medium containing 8.8 µM BA, 4.6 µM Kinetin and 2.6 µM 1-naphthaleneaceticacid (NAA).

The direct regeneration system reported in here is rapid, effective and proficient and offers mass multiplication of finger millet within 7 weeks. Results from various work on other plants including *Zea mays* (Ramakrishnan et al., 2014), *Curcuma attenuata* (Kou et al., 2013), *Hippophae rhamnoides* (Sriskandarajah and Lundquist 2009), *Metabriggsia ovalifolia* (Ma et al., 2010), *Primulina tabacum* (Yang et al., 2012), and *Pulsatilla koreana* (Lin et al., 2011) using the two-stage tissue culture system has also proven it to be efficient in other plants.

In order to achieve the *in vitro* rooting of regenerated shoots, indole-3- acetic acid was used at different concentrations (1, 2, 3, 4 and 5 µM). This study found 3 µM to be the best concentration for four finger millet varieties; GBK-043137, GBK-043128, GBK-043122, GBK-043094. Varieties GBK-043124 and GBK-043050 however, showed best root induction at 4 and 2 µM, respectively. Interestingly poor rooting was observed in 1 and 5 µM across all the varieties. These results indicate that *in vitro* rooting in finger millet can be induced with IAA at concentrations between 2 and 4 µM. Peat moss was used for acclimatization and hardening of the rooted plants because of its high water retention capacity recorded in previous study (Ngetich et al., 2018). Consequently, all the plants were successfully acclimatized and hardened with 100% survival rate. This high survival rates could also be attributed to the well-developed root system and greenhouse conditions. Plants were regenerated within 52 days.

Conclusion

This study outlines a rapid efficient, simple and

reproducible protocol for shoot regeneration of several finger millet varieties using shoot apical meristems as explants. The system developed in vitro plant regeneration protocol is potentially useful for plant genetic transformation and gene function studies of finger millet.

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CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ABBREVIATIONS

BAP, 6-Benzylaminopurine; **IAA**, Indole-3-acetic acid; **MS**, Murashige and Skoog basal medium; **SAM**, shoot apical meristems.

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