Mysore J. Agric. Sci., 53 (4) : 22-27 (2019)

Standardization of Direct Shoot Regeneration and Glyphosate Sensitivity of Finger Millet (*Eleusine coracana* (L.) Gaertn.)

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Abstract

A simple and efficient way of plant regeneration has been established using in vitro- derived, actively growing shoot apical meristems (SAMs) in finger millet (GPU 28). Shoot apical meristems from three - day old seedlings were evaluated for the efficiency of direct shoot induction on varied concentrations and combinations, of 6-benzy laminopurine (BAP) and 2, 4 - dichlorophenoxyacetic acid (2, 4D). The highest shoot induction was observed in medium supplemented with 3 mg/L BAP (13.05). An increase in the number of shoots per explants was observed when SAMs were sub - cultured in a fresh MS medium once in every 2 weeks. The regenerated shoots rooted easily in half strength MS medium containing 2.8 μM indole-3-acetic acid and successfully acclimated in the field. To study glyphosate sensitivity SAMs were cultured on MS medium supplied with different concentration (1, 2, 3, 4 and 5 mg/L) of glyphosate and 3 ppm/L BAP. At 5 ppm of glyphosate, SAMs did not show any response for shoot initiation and all the SAM explants were turned brown due to cell death. Based on this investigation 5 ppm of glyphosate was chosen as lethal dose. This study was used to select the - transformed explants and to allow the selective growth of transformed tissues on the MS selection medium.

Keywords: SAMs, Regenerations and Calus

Finger millet is the staple diet food that supply a major portion of calories and protein to large segment of populations in tropical dry land regions. Finger millet has superior nutritional value compared to rice and wheat. Finger millet is grown worldwide more than 4 million hectares with an annual production of at least 4.5 million tons of grain. Other than 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).

Plant tissue culture technology is an important biotechnological tool used for efficient transfer of genetic traits to plants regeneration under various biotic and abiotic stresses (Kumar *et al.*, 2015). In cereal crops several regeneration and genetic transformation protocols are available and finger millet has got little attention compared to rice, maize, wheat and oats (Ceasor and Ignacimlthu, 2009). The current finger millet regeneration system depends on somatic embryogenesis, predominantly involving a callus phase and is limited by low regeneration frequencies and long regeneration period (Dey *et al.,* 2012).

Direct organogenesis is an effective approach to minimize somaclonal variation omitting the callus induction and subculturing cycles (Arockiasamy and Ignacimuthu, 2007). Direct plant regeneration by culturing shoot apical meristems (SAMs) is a simple and promising tool for plant regeneration because of rapid and effective regeneration capacity. The present research revealed a rapid and efficient direct plant regeneration system for the Indian finger millet genotype 'GPU28' using invitro-derived shoot apical meristem as explants, without an intermediate callus phase. For multiple shoot induction and regeneration, the concentration and combination of plant growth regulators (PGR) were optimized and MS media concentration for rooting, was also standardized. This procedure is rapid, reliable, reproducible and can immensely be used for genetic transformation of finger millet in the future.

Material and Methods

Plant Materials and Explants Preparation

The mature seeds of finger millet were pretreated with sterile water for 30 minutes in order to remove the husk. These were surface sterilized with 0.1 per cent (w/v) mercuric chloride ($HgCl_2$) for 5 minutes and further rinsed three times, in each 1 minutes, with sterile distilled water. Surface-sterilized seeds (GPU28) were inoculated on Murashige and Skoog (MS; Murashige and Skoog, 1962) basal medium containing 3 per cent (w/v) sucrose, 0.8 per cent (w/v) agar-agar type 1. The pH of the media was adjusted to 5.8 before auto claving (121 °C and 15 lbs per sq. inch). For shoot meristem initiations seeds were incubated in dark at 26 ± 2 °C for 3 days.

Shoot Induction and Elongation

Aseptically grown 3-days-old SAMs (Shoot apical meristem) were excised and utilized as explants. The SAMs (4-6 mm) were cultured on shoot induction medium (SIM) comprising of MS basal medium supplemented with 30 per cent sucrose and various concentrations and combinations of cytokinins and auxin as shown in Table 1. The medium pH was adjusted to 5.8 before adding 0.8 per cent (w/v) agar-agar type I. The culture bottles were incubated in the growth room in 16/8 hours light/dark at 26±2 °C for 14 days. The shoots formed after 14 days were transferred to fresh SIM (Shoot induction medium) and incubated in growth chamber with 16/8 hours light/dark at 26±2 °C for a further 14 days to elongate the induced shoot. The percentages of the number of shoots that formed in each shoot clump and the mean number of shoots induced in each explants were calculated following 28 days of culture. After 28 days elongated shoots were transferred to half-strength MS medium with 1.5 per cent (w/v) sucrose and indole-3-acetic acid (IAA; 1.4 µM). Cleri Gel 3 per cent (w/v) was used for solidifying rooting media. The cultures were incubated for 10 days at 28 °C in 16/8 h light/dark and 75/50 per cent relative humidity (RH). Rooted shoots were hardened in pots containing sterile soilrite and peat (1:1 w/w), and established in the field up to flowering and seed setting.

Table 1

Effect of plant growth regulators on direct plant regeneration

Treatments	Concentration and composition of ingredients
T1	MS basal salts, 3mg/L BAP, 500 mg/L casein enzymichydrolysate,750 mg/L proline, 30 g/L of sucrose, 8 g/L (*w/v*) agar-agar type-1 and pH 5.8
T2	MS basal salts, 3 mg/L BAP, 1 mg/L 2,4 D, 500 mg/L casein enzymichydrolysate, 750 mg/L proline, 30 g/L of sucrose, 8 g/ L (*w/v*) agar-agar type-1 and pH 5.8
T3	MS basal salts, 2 mg/L BAP, 1 mg/L 2,4 D, 500 mg/L casein enzymichydrolysate, 750 mg/L proline, 30 g/L of sucrose, 8 g/L (*w/v*) agar-agar type-1 and pH 5.8
T4	MS basal salts, 2 mg/L BAP, 0.19mg/L 2,4 D, 500 mg/L casein enzymichydrolysate, 750 mg/L proline, 30 g/L of sucrose, 8 g/L (*w/v*) agar-agar type-1 and pH 5.8
T5	MS basal salts, 4 mg/L BAP, 1 mg/L 2,4 D, 500 mg/L casein enzymichydrolysate, 750 mg/L proline, 30 g/L of sucrose, 8 g/ L (*w/v*) agar-agar type-1 and pH 5.8

Glyphosate Sensitivity Assay

To determine the concentration of glyphosate for the effective selection of transformed plants, 3 days old seedling- derived SAMs were cultured on MS medium (pH 5.8) supplied with different concentrations (1, 2, 3, 4 and 5 mg/L) of glyphosate and plant growth regulators (PGRs) (Table 2). The cultures were maintained in a growth chamber with a 16/8 h light/dark cycle using 50 µmol photons m^{-2} s^{-1} of cool white fluorescent illumination and 75/50 per cent RH at 28°C for 12 days. SAM explants cultured on MS medium devoid of glyphosate were kept as control. The percentage of explant survival was calculated after 12 days of incubation in the light.

TABLE 2

Standardization of glyphosate concentration for screening transgenics

Treatments	Concentration and composition of ingredients
T1	MS basal salts, 3 mg/L BAP, 500 mg/L casein enzymichydrolysate,750 mg/L proline, 30 g/L of sucrose, 8 g/L (*w/v*) agar-agar type-1 and pH 5.8
T2	MS basal salts, 3 mg/L BAP, 1 mg/L glyphosate, 500 mg/L casein enzymichydrolysate, 750 mg/L proline, 30 g/L of sucrose, 8 g/L (*w/v*) agar-agar type-1 and pH 5.8
T3	MS basal salts, 3 mg/L BAP, 2 mg/L glyphosate, 500 mg/L casein enzymichydrolysate, 750 mg/L proline, 30 g/L of sucrose, 8 g/L (*w/v*) agar-agar type-1 and pH 5.8
T4	MS basal salts, 3 mg/L BAP, 3 mg/L glyphosate, 500 mg/L casein enzymichydrolysate, 750 mg/L proline, 30 g/L of sucrose, 8 g/L (*w/v*) agar-agar type-1 and pH 5.8
T5	MS basal salts, 3 mg/L BAP, 4 mg/L glyphosate, 500 mg/L casein enzymichydrolysate, 750 mg/L proline, 30 g/L of sucrose, 8 g/L (*w/v*) agar-agar type-1 and pH 5.8
T6	MS basal salts, 3 mg/L BAP, 5 mg/L glyphosate, 500 mg/L casein enzymichydrolysate, 750 mg/L proline, 30 g/L of sucrose, 8 g/L (*w/v*) agar-agar type-1 and pH 5.8

RESULTS AND DISCUSSION

Effects of Induction of Shoot Apical Meristems (SAMs)

Surface - sterilized mature seeds of finger millet (GPU 28) were inoculated on plant growth regulator free MS media, for shoot meristem initiation in dark for 3 days. When 3 days old meristemic shoot tips, consisting of apex and part of mesocotyl were excised and cultured on MS basal medium containing various concentration of BAP and 2, 4 D (Table 3) to determine the effect of growth regulator for direct organogenesis.

TABLE 3

Shoot induction of finger millet seeds on various concentrations of cytokinins and auxin after 14 days of incubation

Treatments (ppm/L)	Survival (%)	Multiple shoot induction (%)	Multiple shoot number
T1	100	100	1.35
T2	100	20	0.20
T3	20	5	0.05
T4	100	100	1.20
T5	50	100	0.10
Mean	74	65	0.58
Min	20	5	0.05
Max	100	100	1.35
C.D	13.60	10.39	0.12
SE (m)	4.47	3.40	0.04
SE (d)	6.32	4.83	0.05
C.V	12.09	14.53	14.08

The finger millet (GPU 28) exhibited remarkably different regeneration responses depending on the growth regulator type and concentration in the medium. A combination of BAP and 2, 4 D was tried. The best shoot induction and survival percentage was observed in medium supplemented with 3 mg / L BAP (Table 3) after 14 days of incubation. The addition of lower concentration of 2, 4 D along with BAP increased shoot induction, while higher concentration 2, 4 D was used along with BAP decreased the efficiency of shoot induction.

The successful use of SAM explants in this study implies that they are a better choice for plant regeneration in cereals compared to other explants. Direct shoot regeneration without callus phase is the most preferred pathway for plant regeneration in finger millet. In our study, BAP (3 ppm) was found to be more effective in direct shoot induction from SAM explants of finger millet. Pande *et al*. (2015) reported 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 µM 2, 4-D in combination with 750 mg/L proline, 500 mg/l casein enzymatic hydrolysate and 2 mg/l glycine.

Effect of Plant Growth Regulators on Multiple Shoot Induction

Marked differences in multiple shoot induction rates were observed among the different concentrations and combinations of BAP and 2, 4 D. However, shoot survival response and number of shoots per explants varied based on the concentration and combination of BAP and 2, 4 D. After 28 days of incubation, more number of multiple shoots were observed in medium containing 3mg/L BAP (Table 4). Statistical analysis of variance indicated that significant difference was observed among the different concentration of BAP and 2, 4 D in plant regeneration response. Induction medium supplemented with 3mg/L BAP exhibited significantly better response. When MS + 4mg/L BAP + 1mg/L 2,4 D and MS + 2mg/L BAP + 1mg/L 2, 4 D was used in media the response was 0 per cent respectively on 28th day in plant regeneration response. When MS media was supplemented with 2mg/L BAP and 0.19 mg/L 2, 4 D the response was 75 per cent on 28th day in plant regeneration response. It was observed that by increasing the concentration of 2, 4 D direct organogenesis duration was increased. The regenerated shoots rooted easily and showed nodule like structure. The roots were produced on the medium containing IAA. The plantlets were transferred to the soil (Plate 1).

TABLE 4

Shoot induction of finger millet seeds on various concentrations of cytokinins and auxin after 28 days of incubation

Treatments (ppm/L)	Survival (%)	Multiple shoot number
T1	100	13.05
T2	15	0.20
T3	0	0.00
T4	75	1.35
T5	0	0.00
Mean	38	2.92
Min	0	0.00
Max	15	13.50
C.D	9.619	0.096
SE(m)	3.162	0.032
SE(d)	4.472	0.045
C.V	16.644	2.16

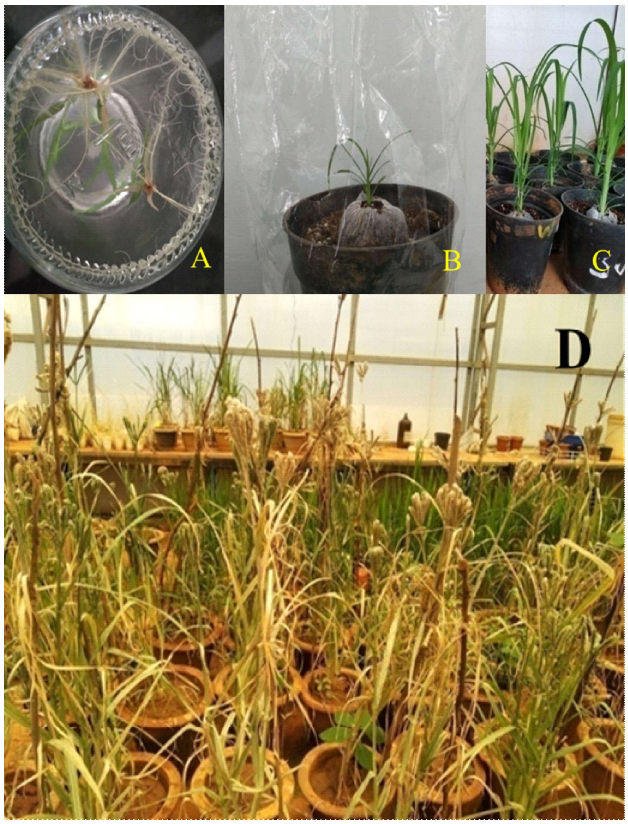

Plate 1 : Different stages of finger millet growth and hardening

A) Rooting of transgenic plant (B) and (C) Growth of healthy plantlets well rooted putative transformant of finger millet transfer to green house; (D) Fertile transgenic finger millet plant.

The induction of shoots in cereals and millets is commonly achieved by BAP or thidiazuron (TDZ) (Bayer *et al.,* 2014; Dosad and Chawla, 2016). Ramakrishna *et al.* (2013) reported that BAP is one of the active plant growth regulator frequently used for tissue culture of monocotyledon plants. In our study SAMs from 3 days old seedlings were found to be optimal resulting in an excellent regeneration response in 3 BAP supplemented with 3 mg/L BAP. In this study comparison of several concentration and combination of the plant growth regulators has shown that the efficiency for multiple shoot formation is regulated by

balance between particular types of cytokinins and auxins. Previously a large number of studies focused on embryogenic calli mediated plant regeneration in cereals inclusive of finger millet (Sharma *et al.,* 2011) barley (Xue *et al.,* 2010) and rice (Ramesh *et al.,* 2009) has been published. However, the results of present study suspect that this more complex regeneration procedure may not be required for finger millet (GPU 28).

Glyphosate Sensitivity Assay

The SAMs inoculated on MS medium containing 3 mg/L BAP and devoid of glyphosate were viable and initiated multiple shoots after 12 days of incubation (Fig 1). The frequency of shoot initiation and multiple shoot induction decreased drastically with the increasd concentration of glyphosate in MS medium (Table 5). There was a significant difference among the frequency of regeneration for glyphosate concentration between 0 ppm to 5 ppm. At above 3 ppm glyphosate, there was a complete decrease in chlorophyll pigmentation coupled with arrest in shoot regeneration and growth. At 5 ppm concentration of glyphosate in MS media SAMs did not show any response for shoot initiation and all the SAM explants were turned brown due to cell death (Plate 2). Based on this investigation 5 ppm of glyphosate was chosen as lethal dose. This study was used to select the non - transformed explants and to allow the selective growth of transformed SAMs on MS medium.

TABLE 5

Details of sensitivity test for shoot apical meristems of finger millet (GPU 28) to glyphosate on shoot regeneration medium

Treatments (ppm/L)	Survival (%)
T1	8.00
T2	8.00
T3	7.33
T4	1.33
T5	0.67
T6	0.00
Mean	5.07
Min	0.00
Max	8.00
C.D	1.038
SE(m)	0.333
SE(d)	0.471
CV	13.674

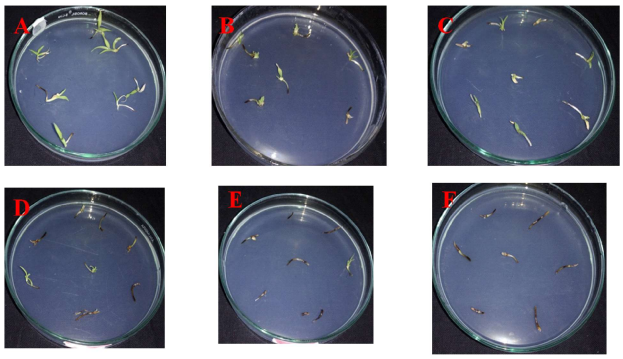

Plate 2: Glyphosate sensitivity of regeneration of shoot apical meristems derived from 5 days old seedlings of finger millet (GPU 28).

A- Multiple shoot induction from shoot apical meristems of GPU 28 on MS medium containing 3ppm BAP, 500 mg l^{-1} casein enzymichydrolysate and 30 g l^{-1} of sucrose. B-F Re-generation of 5 days old shoot apical meristems from GPU 28 in MS medium containing 3ppm BAP, 500 mg l^{-1} casein enzymichydrolysate, 30 g l^{-1} of sucrose along with 1, 2, 3, 4, 5 ppm glyphosate for 12 days.

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(*Received :* August, 2019 *Accepted :* September, 2019)