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Enhanced plant regeneration involving somatic embryogenesis from shoot tip explants of *Sorghum bicolor* (L. Moench)

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ABSTRACT

A highly efficient protocol was developed for plant regeneration through somatic embryogenesis from shoot tip explants of Sorghum bicolor by combining the plant growth regulators,- 2,4-dichlorophenoxy acetic acid and kinetin along with casein hydrolysate and L-proline in Murashige and Skoog (MS) medium. The frequency of embryogenic callus formation was found to be highest with 99%, when shoot tip explants were cultured on Murashige and Skoog (MS) medium supplemented with 2.5 mg/L 2,4-dichlorophenoxy acetic acid, 0.25 mg/L kinetin and 500 mg/L of casein hydrolysate. The highest mean number of 33.3 somatic embryos was obtained, when the embryogenic callus was subcultured on Murashige and Skoog medium supplemented with 2.5 mg/L 2,4-dichlorophenoxy acetic acid, 0.25 mg/L kinetin, and 500 mg/L of casein hydrolysate and 500 mg/L of L-proline. The somatic embryos were transferred to regeneration medium supplemented with different plant growth regulators and a highest regeneration of 21.4 plantlets per embryogenic callus was achieved in Murashige and Skoog medium supplemented with 4 mg/L benzyl aminopurine. The regenerated shoots were transferred onto different rooting media containing the auxins-indole-3- acetic acid, indole-3-butyric acid and 1-napthalene acetic acid, respectively each supplemented at the concentrations of 0.5, 1.0 and 2.0 mg/L in half-strength Murashige and Skoog medium with 0.8 gm/L activated charcoal. The highest number of roots and root length of 12.4 and 5.7 cm respectively, were obtained in half-strength Murashige and Skoog medium supplemented with 1.0 mg/L indole-3-butyric acid and 0.8 gm/L activated charcoal. The in vitro grown plantlets transferred to green house were morphologically similar to in vivo plants with survival rates up to 70%.

Keywords: *Sorghum bicolor*; shoot tip explants; somatic embryogenesis; plant regeneration.

INTRODUCTION

Sorghum bicolor is an important agronomical cereal crop, belonging to the Family Poaceae, providing food security to around 400 million people in developing countries of Asia, Africa and Latin America. It is the fifth most important cereal crop after wheat, rice, maize and barley in terms of production [1]. World annual sorghum production is over 60 million tones of which Africa produces about 20 million tones. This makes sorghum quantitatively the second most important cereal grain in Africa after maize [2]. In India, sorghum is the fourth important cereal crop after wheat, rice and maize and is the third important cereal for consumption after rice and wheat [3]. Sorghum, being the best-suited cereal for semi-arid regions, is also used as fodder and in the production of alcoholic beverages. Hence, sorghum has gained substantial popularity among the farmers due to its greater adaptability in terms of tolerance to drought, salts, heavy metals [4] and various forms of utilization like grain, green fodder, stover, silage and hay to suit the diverse needs of farming systems [5]. In recent years, sorghum has gained the attention of researchers due to its small genome size and its potential as a biofuel crop has secured a promising place for this crop in global agricultural improvement plans [6]. The sorghum genome (~730 Mb) has recently been

sequenced, providing a greater understanding of genomics-assisted breeding in this crop and thereby making it an attractive model for functional genomics [7].

The improvement of sorghum for agronomic and quality traits, such as pest and disease resistance and grain protein quality, has been carried out by traditional plant breeding methods and improved culture management practices [8]. The application of biotechnological tools in sorghum has been hampered by its extreme recalcitrance to produce transgenic plants *in vitro*. Recalcitrance in sorghum tissue culture is due to the release of phenolics, lack of regeneration in long term *in vitro* cultures, and a high degree of genotype dependence [9]. Hence, sorghum has been categorized as one of the more difficult plant species to manipulate for tissue culture and transformation [10]. However, tissue culture and plant regeneration in sorghum have been considerably successful with certain explants [11-13].

In sorghum, *in vitro* response of mature seeds is poor and therefore, the choice of a suitable explant becomes a prerequisite for tissue culture-based transformation processes. Several reports are available pertaining to high plant regeneration from mature and immature embryos, immature inflorescences, anthers and leaf segments [14]. Callus induction and regeneration from shoot apices of sorghum have been reported by Zhong *et al.* [15], Harshavardhan *et al.* [16] and Syamala and Prathibha [17]. Other reports revealed somatic embryogenesis from shoot tip explants [18], leaf segments [19] and immature inflorescence explants of sorghum [20]. Formation of somatic embryos has become an important aspect of regeneration, particularly for transgenesis in most of the important cereal crops, which in turn emphasizes the establishment of efficient embryogenic cultures [21]. Any further improvements in somatic embryogenesis- based regeneration protocol will be helpful for the efficient transgenic plant production in sorghum. In this context, the current study reports the development of a rapid and reproducible protocol for efficient somatic embryogenesis and plantlet regeneration from shoot tip explants of sorghum with a view to increase the efficiency of genetic transformation in future.

In our study, we have used two different auxins namely 2,4-dichlorophenoxy acetic acid (2,4-D) and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) for Embryogenic calli (EC) induction and somatic embryogenesis, with varied concentrations to identify the auxin and its optimal concentration that could effectively maximize callus induction and the formation of somatic embryos from shoot tip explants. Further, the combined effect of auxins along with kinetin, casein hydrolysate and subsequently amino acids (L-arginine, L-glutamine, L-proline) at varying concentrations were also analysed to substantiate that there is always interaction among the plant growth regulators (PGRs) and growth stimulators to increase the efficiency of *in vitro* response.

MATERIALS AND METHODS

Plant material and explant preparation: The seeds of *Sorghum bicolor* cultivar M35-1 were procured from International Crops Research Institute for the Semi-arid Tropics (ICRISAT), Andhra Pradesh, India. The seeds were washed in 0.02% Tween-20 for 5 min followed by rinsing in sterile distilled water. They were then surface sterilized using 70% ethanol for 1 min followed by immersing in 0.1% mercuric chloride for 5 min. The seeds were then washed effectively with sterile distilled water for 5 times to remove the surface sterilants, after which they were inoculated in 90 mm petriplates (30 seeds per plate) containing Murashige and Skoog (MS) [22] basal medium supplemented with 3% sucrose and 0.8% agar. The pH of the pre-agar MS media was adjusted to 5.8 using 0.1 M NaOH before autoclaving. The petriplates were incubated at 25±2°C in the dark for germination. Shoot tips of 4-6 mm in size, from 5- day-old seedlings were excised aseptically using a sterile scalpel blade, to be used as the explants in this study.

Embryogenic callus (EC) induction: The shoot tip explants were inoculated in the callus induction medium containing MS basal salts supplemented with 3% sucrose, 0.8% agar and plant growth regulators – 2,4-D or 2,4,5-T at 2.0, 2.25, 2.5 or 3.0 mg/L. Further, the combined effect of auxin and cytokinin on callus induction was studied by supplementing kinetin at 0.2, 0.25 or 0.5 mg/L along with the above auxins at a concentration that gave the higher embryogenic callus formation in MS medium. These cultures were incubated at 25±2°C in the dark for about 4 weeks with routine inspection every day. The frequency of EC formation in each concentration and combination of PGRs was determined and recorded after 4 weeks of incubation in dark. Similarly, the effect of addition of casein hydrolysate on EC formation was examined in a separate experiment by supplementing MS medium containing 2.5 mg/L 2,4-D and 0.25 mg/L kinetin with 300, 500 or 1000 mg/L of casein hydrolysate respectively and the results were recorded after 4 weeks of incubation in dark.

Somatic embryogenesis: The EC obtained in callus induction medium were further subcultured routinely after 2 weeks of culture onto fresh medium of the same composition, until any change in the morphology of the callus was observed. The mean frequency of EC that formed somatic embryos was counted after 7 weeks of incubation in the

dark at 25±2°C. The effect of addition of casein hydrolysate on somatic embryo formation was examined in a separate experiment by supplementing MS medium containing 2.5 mg/L 2,4-D and 0.25 mg/L kinetin with 300, 500 or 1000 mg/L of casein hydrolysate respectively. Further, the effect of addition of amino acids on somatic embryo formation was also studied in another experiment by including L-arginine, L-glutamine and L-proline separately at a concentration range of 300-600 mg/L, in MS media containing 2.5 mg/L of 2,4-D, 0.25 mg/L of kinetin and 500 mg/L of casein hydrolysate. The cultures were incubated at 25±2°C in the dark and the mean number of somatic embryos formed was calculated after 7 weeks of incubation.

Microscopic analysis: The EC tissue was fixed in a fixative solution of formalin, acetic acid and ethanol (FAA) (1:1:8 v/v) for 48 hours. The samples were dehydrated in xylene and ethanol series and then infiltrated and embedded in paraffin wax. Thin sections (4-6 µm thick) were cut using a rotary microtome. The sections were heat fixed to 3-aminopropyltriethoxysilane (APES)-coated glass slides, dewaxed and stained with Haematoxylin and Eosin and observed under an electron microscope to study the somatic embryo development.

Plant regeneration: The somatic embryos obtained after 7 weeks of culture were transferred to culture flasks containing MS basal medium supplemented with different concentrations and combinations of benzyl aminopurine (BAP), kinetin (KN), thidiazuron (TDZ) and naphthalene acetic acid (NAA) to determine the optimal concentration and combination of PGRs for efficient regeneration. The cultures were subsequently incubated at 25±2°C under an alternating photoperiod of 16 h light/8 h dark at 40-50µmol $m^{-2}s^{-1}$. The regenerated shoots were further subcultured after a week onto the same medium containing 0.8 g/L activated charcoal to control the growth retardation of plantlets caused by phenolic exudation. The percentage of EC responded and the mean number of plantlets per EC was calculated after 2 weeks of incubation in light.

Rooting and Hardening: The regenerated shoots were transferred to root induction medium containing half-strength MS medium supplemented with 0.8 g/L activated charcoal and different concentrations of the three auxins viz. indole-3-acetic acid (IAA), indole-3-butyric acid (IBA) and NAA at 0.5, 1.0, 2.0 mg/L to determine the type and concentration of auxin suitable for efficient rooting. The mean number of roots and root length (cm) were measured after 2 weeks of incubation in light. The rooted plantlets were then transferred to polycups containing sterilized vermiculite after 2 weeks and maintained *in vitro* until they were grown to a length of approximately 8-10 cm. The healthy plantlets were finally transferred to pots to be maintained in green house.

Data analysis and interpretation**:** The experimental design included three replicates for each experiment and each experiment was repeated three times. For callus induction, 20 shoot tip explants were used per treatment and the mean percentage of EC frequency (number of explants forming EC / total number of explants x 100) was calculated after 4 weeks of incubation in dark. In somatic embryogenesis, only the EC was considered and the percentage of somatic embryogenesis along with the mean number of somatic embryos formed from each EC was calculated after 7 weeks of incubation in the dark. For plant regeneration, the number of plantlets produced by each EC was calculated after a further 2 weeks of incubation in light. In root induction, the percentage of plantlets responded and the mean number of roots and their length (cm) were calculated after 2 weeks of incubation. For statistical analysis, one-way analysis of variance (ANOVA) using SPSS software (Version 12.0) was employed to assess the significant differences in the mean values of different treatments. Comparisons between the mean values were made using Fisher's least significant difference (LSD) [23, 24] at 5% level using the statistical package for social science.

RESULTS

Embryogenic callus induction: The mature seeds of sorghum cultivar M35-1 produced initial shoots after 5 days of culture in MS basal medium. Shoot tip explants cultured on callus induction medium produced callus at cut ends after one week of incubation (Figure 1A). In the callus induction medium containing 2.5 mg/L 2,4-D, the shoot tip explants produced white to creamy, translucent calli after 2 weeks of incubation in the dark at 25±2°C . In 2,4,5-T containing MS medium for callus induction, rigid irregular white calli were produced and with increasing concentrations of 2,4,5-T, rhizogenic calli were predominant along with non-embryogenic calli. The EC induction frequency was observed to be higher in 2,4-D supplemented MS medium when compared to 2,4,5-T supplemented MS medium. Further, addition of KN to 2.5 mg/L 2,4-D containing MS medium enhanced the EC frequency with nodular structure initiation in callus. In addition, the inclusion of casein hydrolysate at 500 mg/L in the medium along with the above PGRs showed a significant increase in EC induction frequency (Table 1) (Figure 1B). Hence, MS medium supplemented with 2.5 mg/L 2,4-D, 0.25 mg/L KN and 500 mg/L casein hydrolysate proved to be the optimal medium for EC induction with 99% frequency of EC formation.

Table 1 : Effect of auxin, cytokinin and casein hydrolysate on embryogenic callus induction and somatic embryogenesis in *Sorghum bicolor* cultivar M35-1

Concentration of PGRs (mg/L)			Casein hydrolysate (mg/L)	* % of EC frequency from Shoot tip (Mean± SD)	% of somatic embryogenesis from EC (Mean± SD)	Number of Somatic embryos per EC (Mean± SD)
2,4-D	2,4,5-T	KN				
2.0	0.0	0.0	0.0	45.4 ±0.5h	20.1 ± 0.3j	5.5 ± 0.5f
2.25	0.0	0.0	0.0	60.1 ± 0.3g	25.1 ± 0.3i	6.2 ± 0.4f
2.5	0.0	0.0	0.0	75.1 ± 0.3e	45.1 ± 0.3g	8.2 ± 0.4e
3.0	0.0	0.0	0.0	65.3 ± 0.4f	10.2 ± 0.4l	4.5 ± 0.5g
0.0	2.0	0.0	0.0	40.2 ± 0.4i	15.2 ± 0.4k	2.3 ± 0.4ij
0.0	2.25	0.0	0.0	25.3 ± 0.4k	10.2 ± 0.4l	1.5 ± 0.5j
0.0	2.5	0.0	0.0	20.5 ±0.7l	5.4 ± 0.6m	3.4 ± 0.8h
0.0	3.0	0.0	0.0	10.2 ± 0.4m	0.6 ± 0.5n	0.5 ± 0.5k
2.5	0.0	0.2	0.0	85.2 ± 0.4d	60.8 ± 0.4e	11.6 ± 0.5c
2.5	0.0	0.25	0.0	95.5 ± 0.5b	90.2 ± 0.6b	19.8 ± 0.4b
2.5	0.0	0.5	0.0	75.2 ± 0.4e	50.3 ± 0.4f	8.6 ± 0.5e
0.0	2.0	0.2	0.0	60.6 ± 0.8g	35.2 ± 0.4h	2.9 ± 0.3hi
0.0	2.0	0.25	0.0	35.3 ±0.6j	20.6 ± 0.8j	3.2 ± 0.4h
0.0	2.0	0.5	0.0	25.3 ± 0.6k	10.1 ± 0.3l	2.1 ± 0.3ij
2.5	0.0	0.25	300	90.3 ± 0.9c	80.5 ±0.9c	12.3 ± 0.4c
2.5	0.0	0.25	500	*99.9 ±0.3a*	*95.5 ±0.9a*	*28.2 ±0.4a*
2.5	0.0	0.25	1000	95.3 ± 0.6b	70.4 ± 0.8d	10.6 ± 0.5d

**For each treatment, 20 shoot tip explants were used.*

Means followed by the same letter are not significantly different at 0.05% levels based on Fisher's LSD test. The optimum response is shown in italics.

Somatic embryogenesis: The EC subcultured onto the same medium routinely after every 2 weeks of culture, were found to be healthy presenting a creamy to light yellow, translucent mass with slight globular appearance (Figure 1C). By the end of 5th week somatic embryos were initiated from the ECs under dark culture conditions. Maintaining the EC in 2,4-D by regular transfer to fresh nutrient media regulated the gradual development of somatic embryos from a perpetually meristematic (embryogenic) state (Figure 1D). The percentage of somatic embryogenesis was found to be 95% in MS medium supplemented with 2.5 mg/L 2,4-D, 0.25 mg/L KN and 500 mg/L casein hydrolysate, with a mean number of 28.2 somatic embryos per EC. The absence of casein hydrolysate in the above medium resulted in a decrease in the somatic embryos to a mean number of 19.8, thereby indicating its significant role in somatic embryo formation (Table 1). Further, the addition of amino acids (L-arginine, L-glutamine or L-proline) to the above medium promoted the formation of somatic embryos, wherein 500 mg/L of L-proline was more stimulatory followed by L-glutamine and L-arginine, each at 400 mg/L concentration. Thus, the highest number of 33.3 somatic embryos was obtained on MS medium supplemented with 2.5 mg/L 2,4-D, 0.25 mg/L kinetin, 500 mg/L casein hydrolysate and 500 mg/L L-proline (Table 2).

Table 2: Effect of amino acids on somatic embryo formation in MS medium containing 2.5 mg/L 2,4-D, 0.25 mg/L KN and 500 mg/L casein hydrolysate

Concentration of aminoacids (mg/L)			Number of Somatic embryos per EC (Mean± SD)
L-Arginine	L-Glutamine	L-Proline	
0	0	0	28.2 ± 0.4fg
300			28.5 ± 0.5ef
400			30.1 ± 0.3c
500			27.3 ± 0.5h
600			14.4 ± 0.1k
	300		29.1 ± 0.8de
	400		31.7 ± 0.3b
	500		20.2 ± 0.4i
	600		15.7 ± 0.2j
		300	28.6 ± 0.2ef
		400	29.8 ± 0.5cd
		500	*33.3 ±0.6a*
		600	27.6 ± 0.3gh

Means followed by the same letter are not significantly different at 0.05% levels based on Fisher's LSD test. The optimum response is shown in italics.

Microscopic analysis: Microscopic analysis of EC revealed the formation of somatic embryos and their subsequent globular development, followed by the shoot apex formation (Figure 2). Further in the microtome sections of EC, the cells in the growing region of calli were smaller in size and densely clustered with embryogenic meristematic centres in an organized nodule, containing prominent nuclei. The non-embryogenic cells were found to be loosely arranged, elongated or bulbous cells lacking nuclei.

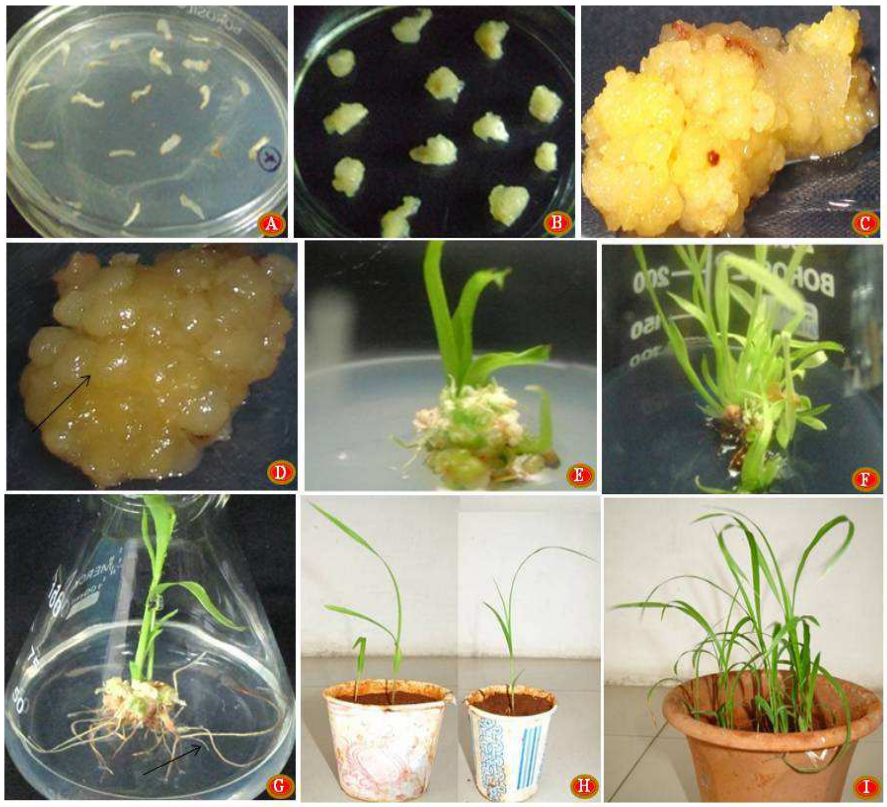
Plant regeneration: The regeneration potential of somatic embryos studied in three different PGRs revealed that BAP at a concentration of 4 mg/L was highly effective in contributing to maximum average number of 21.4 plantlets (Table 3). The initiation of green shoot buds was prominently observed in 4 mg/L BAP after one week of incubation in light (Figure 1E). However with the increase in the number and length of plantlets, the medium presented a more brownish appearance due to phenolic exudation. But this effect was significantly controlled by the addition of 0.8 g/L activated charcoal in the subculture medium and this in turn increased the number of plantlet formation (Figure 1F). The regeneration response in TDZ (1 mg/L) was also promising initially, but got drastically reduced due to phenolic exudation. But, the regeneration efficiency of somatic embryos tested in other PGRs and their combination was insignificant and specifically in BAP, KN and NAA combination (Table 3), it was also observed that formation of thick rigid roots suppressed the shoot formation.

Table 3: Effect of auxin and cytokinin on plant regeneration from somatic embryos in *Sorghum bicolor* cultivar M35-1

Concentration of PGRs (mg/L)				% of embryogenic callus responded (Mean ±SD)	Number of plantlets per embryogenic callus (Mean ±SD)
BAP	KN	NAA	TDZ		
0.0	0.0	0.0	0.0	21.3 ± 0.9f	1.8 ± 0.4f
4.0	0.0	0.0	0.0	*97.8 ±0.4a*	*21.4 ±0.5a*
0.0	2.0	0.0	0.0	46.3 ± 0.3d	12.8 ± 0.2c
0.0	0.0	0.0	1.0	67.9 ± 0.7b	16.2 ± 0.4b
1.0	0.0	0.0	1.0	60.7 ± 0.2c	11.6 ± 0.7d
2.0	1.0	0.2	0.0	30.4 ± 0.6e	8.5 ± 0.5e

Different levels indicate significantly different values at a probability level of P=0.05 based on Fisher's LSD test. The optimum response is shown in italics.

Figure 1. Somatic embryogenesis and plant regeneration in *Sorghum bicolor* cultivar M35-1



A. *Initiation of callus from excised shoot tip explants after one week incubation in the dark on MS medium supplemented with 2.5 mg/L 2,4-D and 0.25 mg/L KN,* ***B.*** *Embryogenic callus formation after 4 weeks of incubation in the dark on MS medium supplemented with 2.5 mg/L 2,4-D, 0.25 mg/L KN and 500 mg/L casein hydrolysate,* ***C.*** *Enlargement of embryogenic callus formed after 4 weeks of incubation in the dark on MS medium supplemented with 2.5 mg/L 2,4-D, 0.25 mg/L KN and 500 mg/L casein hydrolysate,* ***D.*** *Maturation of somatic embryos formed after 7 weeks of incubation in the dark on MS medium supplemented with 2.5 mg/L 2,4-D, 0.25 mg/L KN and 500 mg/L casein hydrolysate,* ***E.*** *Initiation of shoots from somatic embryos after one week of culture in the light on MS medium supplemented with 4.0 mg/L BAP,* ***F.*** *Regeneration of plantlets after two weeks of culture in the light on MS medium supplemented with 4.0 mg/L BAP,* ***G.*** *Root induction after two weeks of incubation in the light on half-strength MS medium supplemented with 1.0 mg/L IBA,* ***H.*** *Hardening of the plantlets in poly cups containing sterile vermiculite and* ***I.*** *Acclimatization of the regenerated in vitro plants in potted soil.*

Rooting and Hardening: The excised shoots were tested for their rooting efficiency in half strength MS media supplemented with 0.5, 1.0 and 2.0 mg/L IAA, IBA and NAA individually, of which 1.0 mg/L IBA was found to be optimal PGR producing a maximum mean number of 12.4 roots and a mean root length of 5.7 cm. Further, it was observed that in all the three auxins used, 1.0 mg/L was found to give the maximum response with respect to the other tested concentrations (Table 4).

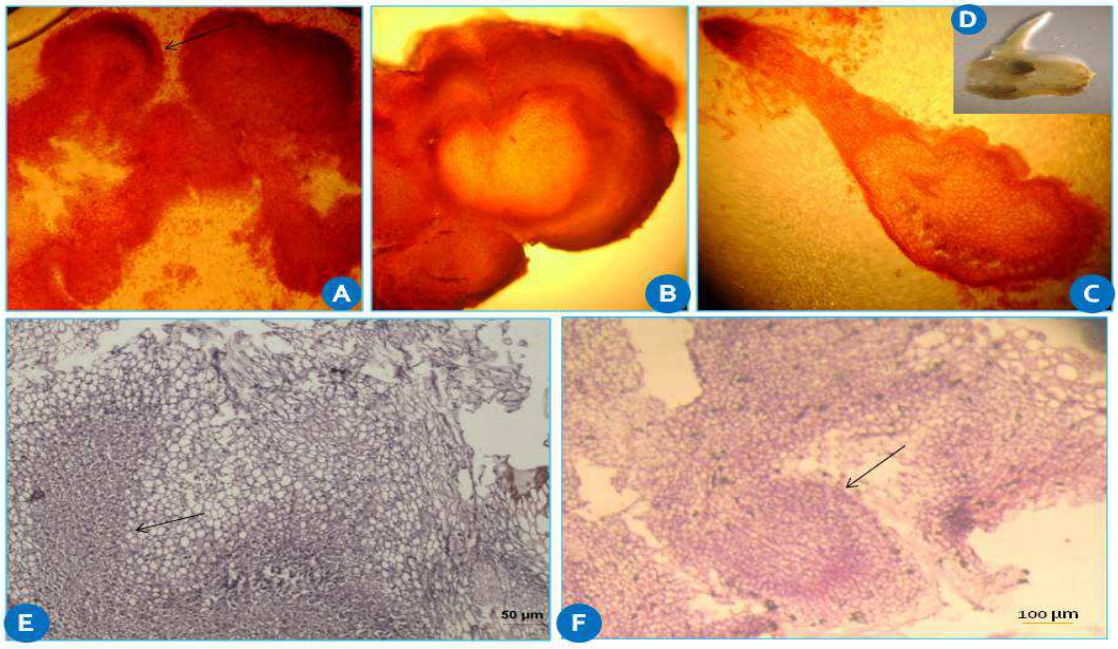
The rooting was initiated after a week; after 2 weeks the rooted plantlets transferred to polycups containing sterilized vermiculite, were observed to be grown to a length of approximately 10 cm under in *vitro* conditions. About 10 ml of sterile, concentrated liquid MS medium was added at regular intervals of 3 days to nourish the plantlets during the course. The healthy plantlets were transferred to pots and maintained in green house (Figure 1I). The acclimatized *in vitro* plantlets were morphologically similar to *in vivo* grown plants and the matured plants showed similar growth characteristics.

Table 4: Effect of auxins on root induction in *Sorghum bicolor* cultivar M35-1

Concentration of PGRs (mg/L)			% of shoots responded (Mean ± SD)	Number of roots (Mean ± SD)	Root length (cm) (Mean ± SD)
IAA	IBA	NAA			
0.0	0.0	0.0	0.0 ± 0.0j	0.0 ± 0.0f	0.0 ±0.0f
0.5			65.0 ± 0.2e	3.6± 0.3e	2.3 ± 0.8de
1.0			85.0 ± 0.4b	9.2± 0.8b	4.9± 0.2a
2.0			50.0 ± 0.2g	3.3 ± 0.6e	1.6± 0.1e
	0.5		60.0 ± 0.6f	6.2 ± 0.7c	3.9 ±0.3b
	1.0		*90.0 ±0.8a*	*12.4 ±0.5a*	*5.7 ±0.4a*
	2.0		40.0 ± 0.8h	3.0 ± 0.4e	3.4 ±0.5bc
		0.5	70.0±0.5d	5.1±0.1d	2.9±0.7cd
		1.0	80.0±0.2c	8.5±0.8b	3.9±0.3b
		2.0	20.0±0.6i	3.1±0.2e	1.9±0.1e

Means followed by the same letter are not significantly different at 0.05% levels based on Fisher's LSD test. The optimum response is shown in italics.

Figure 2. Microscopic examination of section of an Embryogenic callus



A. *Formation of somatic embryo from embryogenic callus,* ***B.*** *Globular stage of somatic embryo,* ***C.*** *Formation of shoot apex from somatic embryo,* ***D.*** *Shoot initiation from somatic embryo,* ***E.*** *Microtome sections of embryogenic callus showing embryogenic cells that contain organized nodule of meristematic cells with prominent nuclei (arrow) and* ***F.*** *the development of globular embryo (arrow).*

DISCUSSION

Developing *in vitro* culture methods for efficient regeneration of sorghum has been a challenging task since sorghum is categorized to be an extremely recalcitrant cereal crop; the rate of response is also genotype-dependant

[9]. However, several studies have reported regeneration protocols from various explant sources of sorghum including mature and immature embryos [25], immature inflorescences [20], leaf segments [19] and shoot apex [15]. In particular, shoot apical meristem has been used effectively to develop regeneration systems and to recover stably transformed maize, wheat, rice, barley, sorghum and millet [26]. The current study used shoot tip explants to develop a rapid, reproducible and efficient protocol for deriving high plant regeneration through somatic embryogenesis, which in turn may improve transformation capabilities in future. Shoot tips excised from 5-day old *in vitro* grown seedlings proved to be better explants as the callus initiation was observed after a week in the presence of specific PGRs and the EC induction frequency was uniformly rapid in the M35-1 cultivar, when compared to mature embryo explants.

In vitro response is greatly influenced by the content and balance of PGRs. Preliminary attempts for initiating callus from seed and shoot tip explants with low concentrations (< 2.0 mg/L) of 2,4-D and 2,4,5-T elicited only non-embryogenic calli, presenting a hard and snowy white non-proliferative appearance. Hence, for the EC induction, two auxins, 2,4-D and 2,4,5-T in the concentration ranges of 2.0-3.0 mg/L were tested separately and in combination with KN (0.2-0.5 mg/L). The EC induction frequency was found to be 75% with 2.5 mg/L of 2,4-D alone; however, the inclusion of 0.25 mg/L KN in the above medium increased the EC formation to 95%. Previous reports by Maheswari *et al*. [27] also showed that the inclusion of a low concentration of cytokinin in the callus induction medium containing 2,4-D promoted the induction of EC in sorghum genotypes. Further, the genotypic limitations of plant regeneration in sorghum can be overcome by supplementing a strong cytokinin like KN with 2,4-D in the callus induction medium [28]. Casein hydrolysate can be used as a relatively cheap source of a mixture of amino acids [29]. These amino acids serve as a source of reduced nitrogen required for plant metabolism. The present study revealed that the addition of casein hydrolysate to the above PGRs improved the efficiency of EC formation up to 99%. When EC was further subcultured on the same medium, it resulted in a much significant response with respect to nodular formation and subsequent development of globular structures of somatic embryos. These observations are consistent with the previous reports of cell differentiation into the embryogenic mode starting very early in shoot tip culture and continuing as long as they are cultured on the same medium [18]. In contrast, EC without subculture showed browning of callus due to phenolic exudation.

In somatic embryogenesis, the effective role of 2,4-D in combination with KN and casein hydrolysate was established with a yield of 28.2 somatic embryos per EC. This is in agreement with the earlier findings where the combined use of auxins and cytokinins induced somatic embryogenesis in callus cultures of cereals [30, 31]. Similar effects of 2,4-D (2.0 mg/L) and KN (0.5 mg/L) on somatic embryogenesis was also reported in the same cultivar using immature inflorescence explants [20]. Occasionally, certain explants bearing somatic embryos were spotted with brown pigments but its impact on the somatic embryo development was insignificant; yet, stating that the regeneration capability may be significantly influenced by such brown phenolic exudates.

Amino acids increase the levels of reduced nitrogen that can stimulate somatic embryo development. The requirement for reduced nitrogen in embryo induction is that very young embryos lack nitrate reductase which reduces nitrate to nitrite [32]. In our study, the addition of L-proline drastically improved the somatic embryo formation in MS medium containing 2.5 mg/L 2,4-D, 0.25 mg/L KN and 500 mg/L casein hydrolysate. Since sorghum is a high nitrogen feeding crop, it was expected that addition of L-glutamine and L- arginine to the culture medium would have a dominating role in somatic embryogenesis when compared to L-proline. But however, the results proved that L-proline was more stimulatory compared to the other two amino acids, L-glutamine and L-arginine. The stimulatory effect of L-proline on somatic embryo maturation and plantlet formation has been studied in kodomillet [33], maize [34] and sorghum [35]. Further, L-proline has been shown to function in osmoregulation, morphogenesis and to regulate salt tolerance in sorghum [36].

The regeneration potential was also influenced by the presence of growth regulators in the medium. It was observed that the presence of 2,4-D in the medium suppressed the shoot initiation, thereby maintaining the somatic embryoids even after transfer to light. However, with the subsequent removal of 2,4-D in the regeneration medium, the green shoot bud initiation was rapid in the presence of BAP and other tested PGRs in light. Besides, the number of plantlets was higher in 4 mg/L BAP thereby showing it to be the congenial supplement in MS medium for regeneration followed by TDZ (1.0 mg/L). The diminishing effect on shoot proliferation caused by phenolic exudation was considerably reduced by the addition of 0.8 g/L activated charcoal. Also, regular subculture of explants helped to eliminate the necrosis induced by leached phenolics [37]. The promotary effect of activated charcoal on morphogenesis may be mainly due to its irreversible adsorption of inhibitory compounds in the culture medium and substantially decreasing the toxic metabolites, phenolic exudation and brown exudate accumulation [38].

Several reports are available confirming the effective role of auxins in root formation. Further, reducing the salt concentration in MS medium particularly macronutrients including potassium during *in vitro* rhizogenesis, utilizes and prevents the accumulation of salt complex by guard cells in the stomata. Therefore, the plantlets on transfer to the soil show high water contents in leaves due to minimum water loss through stomata [39]. NAA and IBA are known to induce rhizogenesis in many cereals such as rice, maize, and triticale [40, 41]. Earlier reports have shown that cultured immature inflorescence explants of sorghum cultivars produced roots on MS medium containing 1.0 mg/L IAA [42] and the rooting of shoots derived from long-term maintained EC was obtained in half-strength MS medium containing 1.0 mg/L NAA [43]. It has been reported that NAA significantly promoted root proliferation, whereas IAA and IBA notably stimulated root elongation in sorghum [44]. In our study, the roots were induced from *in vitro* shoots within 15 days of culture in half-strength MS medium supplemented with IAA, IBA or NAA. Among the three auxins tested for rooting, the percentage of root formation, number of roots and root length varied with varying concentrations (0.5-1.0 mg/L) and was observed to be higher in 1.0 mg/L IBA. The roots in IBA were observed to be distinct as slender whitish structures with visible root hairs. The plantlets on transfer to green house showed promising growth with 80% survival rate. The plants were grown to maturity and no variations were observed among the acclimatized plants with respect to morphological and growth characteristics.

This study describes an effective and rapid protocol using shoot tip explants of *Sorghum bicolor* as the source for high plant regeneration via somatic embryogenesis. This protocol may provide an easy access to genetic transformation studies in future with a scope to increase the potential of the plant in agronomic performance.

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