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Original Article

HIGH FREQUENCY CALLUS INDUCTION AND PLANT REGENERATION FROM SHOOT TIP EXPLANTS OF SORGHUM BICOLOR L. MOENCH

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ABSTRACT

Objective: *Sorghum bicolor* is a relatively recalcitrant crop that is less amenable to *in vitro* manipulation by tissue culture and transformation techniques. In this study, a robust protocol was developed for attaining high frequency callus induction and plant regeneration from the shoot tip explants of *Sorghum bicolor.*

Methods: The shoot tips were cultured in the presence of 2,4-dichlorophenoxyacetic acid (2,4-D) alone, and in combinations with benzylaminopurine, kinetin and 1-napthaleneacetic acid at varying concentrations in Murashige Skoog (MS) media to determine the optimal media for callus induction. Further, thidiazuron (TDZ) in the concentration range of 0.1-2.0 mg/L was supplemented in MS media to study its effect on plant regeneration.

Results: The highest frequency of embryogenic callus (74.8%) was obtained on MS medium supplemented with 2.0 mg/L of 2,4-D and 0.5 mg/L of kinetin. The embryogenic calli on transfer to regeneration medium supplemented with 1.0 mg/L of thidiazuron (TDZ) in MS media showed the highest regeneration of shoots with 12.2 (mean) shoots per embryogenic callus. The histochemical analysis of callus showed the formation of shoot apex from the meristematic centres of calli. The regenerated shoots were rooted on half-strength MS medium containing 1.0 mg/L of Indole-3-butyric acid (IBA). The well rooted plantlets developed after 2 weeks were subjected to hardening and acclimatization with a survival frequency of 68%. The *in vitro* regenerated plants were observed to be phenotypically similar to *in vivo* plants.

Conclusion: The development of rapid and reproducible *in vitro* regeneration protocols would enable the isolation and characterization of secondary metabolites from sorghum under controlled environment that could be evaluated for their pharmacological efficacy.

Keywords: Embryogenic callus, Shoot tips, Regeneration, 2,4-D, Kinetin, TDZ.

INTRODUCTION

Sorghum is an agronomic annual crop belonging to the family Poaceae, and most closely related to the *Saccharum* among the cultivated grasses. Sorghum is a stable subsistence dry-land crop, used as human food with the dried stalks used for fodder, building materials and fuel in Asia and Africa, while sorghum grain is used as animal feed in USA, China and South America [1]. Worldwide, it is the fifth major cereal crop and in India, sorghum ranks third both in area and in production after rice and wheat. Sorghum cultivation in India covers an area of 7.92 million hectares with a total production of 7.92 million tones with an average productivity of 1020 kg/hectare [2]. The global changes in weather, temperature and water availability has subsequently increased the focus towards the cultivation of dryland crops such as sorghum [3, 4].

The success of the transformation techniques in sorghum depends on the availability of optimal tissue culture protocols. However, sorghum is reported to be one of the most difficult plant species to manipulate through tissue culture and transformation [5]. Earlier reports on callus induction and plant regeneration from immature inflorescence [6, 7], shoot segments [8], shoot tips [9, 10] and from mature embryos of sorghum [11] have attained only limited success in plant regeneration in sorghum and practical difficulties still exist in establishing and propagating regenerable cultures for longer periods [12].

The physiological status of the explant material, medium composition, culture conditions and the genotype of the donor plants are the major factors that determine the morphogenic potential of the plant cells during *in vitro* culture [13, 14, 15]. Explants derived from mature seeds are considered an excellent source material for biotechnological applications due to easy storage and accessibility to large amounts of uniform quality explant material [16, 17]. Shoot tip explants are readily available and contain actively dividing meristematic cells that could easily sustain *in vitro* adaptability with increased potential to establish morphogenic cultures.

Plant regeneration in sorghum is generally achieved by the induction of embryogenic calli on a 2,4-dichlorophenoxyacetic acid (2,4-D) containing medium followed by plant regeneration on a medium without growth regulator or supplemented with cytokinin and/or auxin. Further, the hormonal milieu of callus induction medium affects regeneration of plants in plant regeneration medium [7]. In our study, 2,4-D at different concentrations were studied for callus induction and its optimal concentration was combined with various cytokinins and 1-napthaleneacetic acid (NAA) at varying concentrations to identify the synergistic combination that triggered the maximum callusing ability of shoot tip explants in the basal nutrient medium. In addition, the effect of the addition of thidiazuron (TDZ) at different concentrations, as the sole growth regulator for shoot regeneration was also examined.

MATERIALS AND METHODS

Plant material

The seeds of *Sorghum bicolor* CO-S-28 procured from Tamilnadu Agricultural University, Coimbatore, India, were used to initiate the study. Mature viable seeds were collected and soaked for one hour prior to inoculation. The seeds were then washed with a drop of diluted tween-20 detergent solution for 10 minutes and subsequently rinsed in sterile distilled water for 3 times. The surface sterilization was done by immersing the seeds in 70% ethanol for 30 seconds, followed by a sterile water wash and subsequent immersing in 0.1% mercuric chloride for 5 minutes followed by vigorous rinsing in sterile distilled water for 3-5 times. The seeds were then dried using sterile Whatman #1 filter papers; inoculated in (90 X 60) mm petridishes containing Murashige Skoog (MS) basal

medium [18] and incubated in dark at 28±2ºC. The shoot tips excised from the 5-days-old seedlings were used to initiate the callus cultures.

Callus induction medium

For callus induction from shoot tip explants, the MS medium was supplemented with 2,4-D in varying concentrations ranging from 1.0-4.0 mg/L to identify the optimal concentration that would result in higher embryogenic callus (EC) induction frequency. Further, to the optimal concentration of 2,4-D, varying concentrations of benzylaminopurine (BAP), kinetin (0.2-0.5 mg/L) and NAA (0.1-1.0 mg/L) were supplemented individually to study the combinatorial effect of 2,4-D with cytokinins and auxin on EC induction frequency. The excised shoot tip explants were inoculated in the above concentrations and combinations of phytohormones with 25 explants per treatment, for attaining maximum EC induction frequency and the cultures were maintained in dark incubation at 28±2ºC for a period of 3 weeks. The percentage of frequency of EC induction was calculated after 3 weeks.

Shoot induction from Embryogenic callus

The embryogenic calli formed were then transferred to regeneration medium that contained TDZ at a concentration range of 0.1-2.0 mg/L supplemented respectively in MS medium. The cultures were incubated at 28±2ºC in light with an intensity of 50 $\mu m^{-2}S^{-1}$ photosynthetic photon flux density for about 4 weeks to stimulate shoot development. A regular subculture on to the same regeneration medium including 0.8 gm/L activated charcoal was performed after 2 weeks to minimize the growth retardation by phenolic exudation. The mean percentage of responsive embryogenic calli and the mean number of individual shoots formed per embryogenic callus were calculated.

Histological study

For histological 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 then dehydrated in xylene and ethanol series, infiltrated and embedded in paraffin wax. Thin sections of about 4-6 µm thickness 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.

Rooting and Hardening

The individual shoots formed after 4 weeks were transferred to rooting medium that contained half-strength MS medium supplemented with 1.0 mg/L of Indole-3-butyric acid (IBA) and 0.8 gm/L activated charcoal. The rooted plantlets were transferred to polycups containing sterile sand and vermiculite (1:1) and routinely nurtured with liquid MS medium. The plantlets were then transferred to potted soil in green house.

RESULTS AND DISCUSSION

The shoot tip explants derived from 5 days-old *in vitro* seedlings, cultured in 2,4-D containing MS medium for callus induction were responsive in regard to the efficient initiation of callus. Both embryogenic and non-embryogenic calli were formed in all the tested concentrations of 2,4-D. However, the higher number of embryogenic callus was observed with 2.0 mg/L of 2,4-D after 3 weeks of culture. When 1.0 mg/L 2,4-D produced non-friable calli that could not proliferate further and turned into rhizogenic calli forming white roots (Figure 1C), the embryogenic calli formed in 2.0 mg/L of 2,4-D were compact and slimy. The non-embryogenic calli were predominating at higher concentrations of 2,4-D and further, it was observed that dried necrotic browning callus was formed at 4.0 mg/L of 2,4-D as a result of phenolic exudation. In cereals, the efficiency of 2,4-D at low levels in callus induction has been reported and its higher concentrations were found to be less effective in the formation for embryogenic callus [19, 20]. Our results also indicated a similar trend, with 2.0 mg/L of 2,4-D as the optimal concentration for embryogenic callus formation and to this, varying concentrations of BAP, kinetin and NAA were added to study the combinatorial effect. Among these combinations, 2.0 mg/L of 2,4-D and 0.5 mg/L of kinetin was found to be the most efficient combination for induction of embryogenic calli from shoot tip explants, yielding a frequency of about 74.8% (Table 1; Figure 1A&B). Previous reports have also shown that a combination of 2,4-D with cytokinins like kinetin will improve the embryogenic callus induction [7, 10].

Table 1: Effect of auxins and cytokinins on embryogenic callus formation frequency in sorghum

Concentration of PGRs (mg/L)				% of EC frequency from Shoot tip
2,4-D	BAP	KIN	NAA	(Mean± SD)
1.0				27.6±0.4 h
2.0				46.4±0.9 f
3.0				32.5±0.1gh
4.0				26.8±0.6 hi
2.0	0.2			52.3±0.6 d
2.0	0.25			57.7±0.8 c
2.0	0.5			62.2±0.5 b
2.0		0.2		60.1±0.2 bc
2.0		0.25		71.5±0.3 ab
2.0		0.5		*74.8±0.7 a*
2.0			0.1	49.0±0.6 e
2.0			0.5	52.7±0.1d
2.0			1.0	33.5±1.1g

For each treatment, 25 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.

When the cultures were transferred from callusing media to different plant regeneration media containing TDZ at concentrations from 0.1 - 2.0 mg/L and kept in light, greening of compact embryogenic calli was observed within 6–7 days of transfer, in 1.0 mg/L of TDZ (Figure 1D). This green compact callus slowly started regenerating shoots and multiple shoots were also frequently observed (Table 2; Figure 1E&F). TDZ can significantly improve morphogenic response from callus derived from a wide range of explants concerning frequency of shoot formation, number of shoots per explant and the time needed for shoot induction compared to other cytokinins [21]. It was also found that addition of TDZ to other cytokinins had a synergistic effect and produced more shoots in sorghum [22]. In this study, addition of TDZ at 1.0 mg/L was observed to be significantly efficient in producing more shoots from each embryogenic callus. However, with the gradual increase in the length and number of shoots, browning of medium due to phenolic exudation was commonly observed and was partly overcome by the inclusion of 0.8 gm/L of activated charcoal in regeneration medium during subculture. It was also found that the morphogenic response of calli was lost at higher concentrations of TDZ (2.0 mg/L) leading to the formation of necrotic calli.

Table 2: Effect of TDZ on shoot induction from embryogenic callus of sorghum

*Concentration of TDZ (mg/L)	% of embryogenic callus responded (Mean ±SD)	Number of plantlets per embryogenic callus (Mean ±SD)
0.1	45.4±1.2 d	3.4±0.3 e
0.5	57.5±0.4 c	7.9±0.2 c
1.0	*86.2±0.7 a*	*12.2±0.7a*
1.5	65.5±0.7 b	10.4±0.5 b
2.0	62.1±1.5 bc	4.8±0.1 d

*In each treatment, 20 embryogenic calli 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.

The histological examination revealed the embryogenic cells to be small with clustered meristematic cells and highly cytoplasmic. The formation of multiple shoot apices was observed in regenerating calli after 2 weeks in light (Figure 1J&K). The additive effect of IBA on *in vitro* rooting of sorghum has been frequently reported [12, 23, 24]. When IBA at 1.0 mg/L in half-strength MS medium was used for root induction, nearly 82% shoots generated roots within 2 weeks. The rooted plantlets after hardening in polycups were transferred to green house with 68% survival rate (Figure 1G-I).

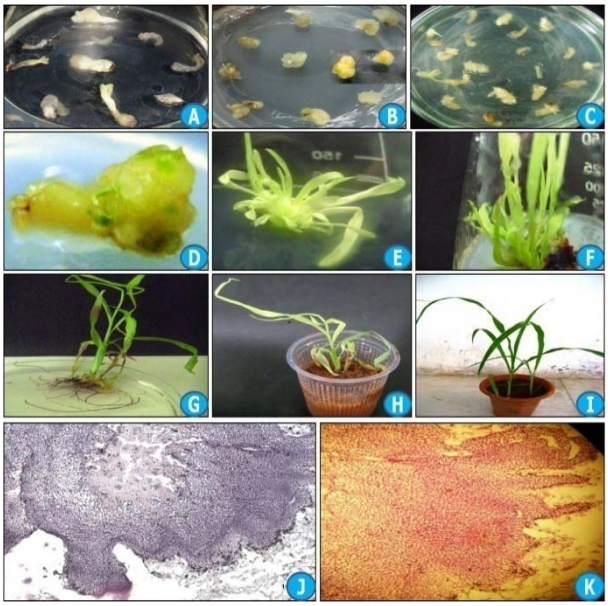

Fig. 1: Callus induction and plant regeneration in *Sorghum bicolor* CO-S-28.

A) Callus initiation in 2,4-D (2.0 mg/L) and Kinetin (0.5 mg/L) after one week.

B) Embryogenic calli formed in (2.0 mg/L) and Kinetin (0.5 mg/L) after 3 weeks.

C) Non-embryogenic rhizogenic calli formed in 2,4-D (1.0 mg/L)

D) Initiation of shoot buds in regeneration medium containing TDZ (1.0 mg/L)

E & F) Multiple shoot formation from embryogenic callus in regeneration medium containing TDZ (1.0 mg/L).

G) Rooting of regenerated shoots in IBA (1.0 mg/L) H & I) Hardening and acclimatization of the plantlets in poly cup and potted soil respectively.

J) Microtome section of callus showing densely clustered meristematic tissues with scutellar development.

K) Multiple shoot apices viewed under light microscope in regenerating 5 weeks-old calli.

CONCLUSION

Highly robust protocols for efficient regeneration in sorghum would enable to overcome the recalcitrance and genotype-specific response of sorghum in tissue culture and this in turn would promote its agronomical quality and productivity by simplifying the application of transformation techniques for genetic improvement. Further, such rapid and reproducible regeneration protocols would enable the isolation and characterization of active metabolites in sorghum under controlled environment that could be evaluated for their pharmacological potential.

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