

An Efficient Plant Regeneration System for *Sorghum bicolor* — a Valuable Major Cereal Crop

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Abstract

An efficient, rapid and large-scale *in vitro* clonal propagation of agronomically important Indian cereal crop genotypes (NSH27 & K5) of *Sorghum bicolor* (L.) Moench. by enhanced shoot proliferation in shoot tip segments was designed. MS medium fortified with plant growth regulators and coconut water markedly influenced *in vitro* propagation of *Sorghum bicolor*. *In vitro* plantlet production system has been investigated on Murashige and Skoog (MS) medium with the synergistic combination of 6-benzyladenine (22.2 μM), kinetin (4.6 μM), adenine sulphate (2.8 μM), 5% coconut water and 3% sucrose which promoted the maximum number of shoots as well as beneficial shoot length. Subculturing of shoot tip segments on a similar medium enabled continuous production of more than 100 healthy shoots with similar frequency. When the healthy shoot clumps were cultured on MS medium fortified with 6-benzyladenine (22.2 μM), kinetin (4.6 μM), adenine sulphate (2.8 μM), α -naphthaleneacetic acid (2.7 μM), ascorbic acid (30.0 μM) and 5% coconut water, a rapid production of axillary and adventitious buds was developed after 8 wk culture. More than 300 shoots were produced 10 wk after culture. Rooting was highest (100%) on half strength MS medium containing 22.8 mM IAA. Micropropagated plants established in garden soil, farmyard soil and sand (2:1:1) were uniform and identical to the donor plant with respect to growth characteristics. These plants grew normally without showing any traits.

Key words: *ex vitro*, hardening, *In vitro*, plant growth regulators, shoot regeneration

Introduction

Sorghum is the most important cereal crop for a long time and occupies a major place in food grain production and as forage for domestic animals. The crop is well adapted to tropical and subtropical areas throughout the world with vast areas under its cultivation. It is an important food crop in Africa, Central America, and southern Asia, and is the fifth major cereal crop grown in the world (470,000 km² harvested in 1996). African slaves introduced sorghum into the U.S. in the early 17th century, where most of the world's sorghum is now produced. *Sorghum* species are sources of fiber, fuel and secondary products and also used in alcohol industry (sweet Sorghum) as it contains high amount of starch. In addition to its principle used as flour, preparation of porridge and unleavened bread.

Tissue culture, an important area of biotechnology can be used to improve the productivity of planting material through enhanced availability of identified planting stock with desired traits. The idea of cell and tissue culture were put forth by a German Scientist Haberlandt in 1902. The greatest advances in increasing regeneration frequency had come from the realization that cereal tissue cultures (Ogura and Shimada, 1978; Shimada and Yamada, 1979; Inoue and Maeda, 1980; Nabors et al., 1982).

Micropropagation is the true to type propagation of selected genotype using *in vitro* culture technique. This technique provides a rapid reliable system for a production of large number of genetically uniform disease free plantlets. Micropropagation procedure for the multiplication of desired or selected genotypes of *Panicum virgatum* (Alexandrova et al., 1996). Shoot tip culture was important system for micropropagation. An efficient clonal propagation procedure for six rice varieties cultivated in Argentina was developed

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by using shoot tip cultures, and the genetic stability of the micropropagated plants was verified by isozyme analysis (Medina et al., 2004). Micropropagation was successfully utilized by Meristematic shoot segments excised from mature embryo-derived seedlings of barely (Sharma et al., 2004). A simple, genotype-independent and efficient method for plant regeneration using shoot tip explants of pearl millet was established (Mythili et al., 2001).

In sorghum, plant regeneration (via callus) has been described using various explants (Thomas et al., 1977; Gamborg et al., 1977; Ma et al., 1987). In sorghum, immature inflorescence was used as potential explants for regeneration (Elkonin et al., 1996; Raghavendra Rao et al., 2000). Somatic embryogenesis was achieved from shoot tip explants of sorghum (Seetharama et al., 2000)

Therefore, *S. bicolor* has been few reports to date on micropropagation from shoot tips (Zhong et al., 1998). The purpose of this study was to develop an *in vitro* propagation method from shoot tip of *S. bicolor*, an economically important Indian cereal crop (NSH27 & K5) for crop improvement. In the present work we have, for the first time, established for a rapid and reproducible method for high-frequency axillary and adventitious shoot proliferation from shoot tip segments, followed by successive establishment of regenerated plants in soil. We have also examined the morphological and growth characteristics of *S. bicolor*. The present communication is aimed at standardizing protocols for high frequency regeneration, which is useful for chitinase genetic engineering experiments.

Materials and Methods

Plant material and disinfections

Seeds of *Sorghum bicolor* (L.) Moench genotype NSH27 and K5 were obtained from Tamil Nadu seed germination testing laboratory, Tiruchirappalli, Tamil Nadu, India. The seeds were kept in running tap water for one hour and then washed with an aqueous solution of 2% (v/v) Teepol (Reckitt Benckiser, India) for 3 min followed by rinsing with distilled water and 70% (v/v) ethanol for 1 min and washing with autoclaved sterile distilled water three to five times. The seeds were then surface-disinfected with 0.2% (w/v) aqueous mercuric chloride solution for 10 min and finally rinsed with autoclaved distilled water (five to seven changes). The seeds were grown in MS basal medium as well as in moistened cotton. The shoot tip segments derived from 5 d-old aseptic seedlings were used as explants. The shoot tip segments (0.5-1.0 cm) were isolated from proximal end of shoot to meristematic region in an aseptic condition. The shoot tip

segments were then further trimmed at proximal ends prior to inoculation on culture media with respective PGRs.

Culture conditions

Single shoot tip segments were cultured on Murashige and Skoog (1962) basal medium (MS) supplemented with 3% (w/v) sucrose (Himedia, India) for culture initiation and served as explant sources for subsequent experiments. The pH of the medium (supplemented with respective growth regulators) was adjusted to 5.8 with 1 N NaOH or 1 N HCl before gelling with 0.8% (w/v) agar (Himedia, Mumbai, India). In all the experiments, the chemicals used were of analytical grade (Himedia, Kelco, Merkard and Sigma). The medium was dispensed into culture vessels (Borosil, Mumbai, India) and autoclaved at 105 kPa and 121°C for 15 min. The disinfected explants were implanted vertically on the culture medium (test tubes (150×25 mm) containing 15 ml medium) and plugged tightly with non-absorbent cotton. All the cultures were incubated at 25±2°C under 16 h photoperiod of 45 – 50 $\mu\text{mol m}^{-2} \text{s}^{-1}$ irradiance provided by cool white fluorescent tubes (Philips, India) and with 55 – 60% relative humidity (RH). All subsequent subcultures were done at 3-wk intervals.

Effect of basal media

MS (Murashige and Skoog, 1962) basal medium (hormone free) was evaluated for their effects on *in vitro* growth and development of *Sorghum bicolor*. The basal medium was consisting of 3% (w/v) sucrose and gelling with 0.8% (w/v) agar.

Effect of cytokinins

Shoot tip segments were cultured on MS medium containing 3% (w/v) sucrose and with 0.8% (w/v) agar and supplemented with different concentrations and combinations of plant growth regulators, including 2.2-26.6 μM BAP; 2.3-23.2 μM Kin; 22.2 μM BAP + 2.3-13.9 μM Kin; 22.2 μM BAP + 1.4 μM Ads + 4.6-9.2 μM Kin and 22.2 μM BAP + 2.8 μM Ads + 4.6-9.2 μM Kin.

Effect of cytokinins and coconut water

Shoot tip segments were cultured on MS medium containing 3% (w/v) sucrose with 0.8% (w/v) agar and supplemented with 5% (w/v) coconut water and different concentrations and combinations of plant growth regulators, including 8.8-26.6 μM BAP; 6.9-23.2 μM Kin; 22.2 μM BAP + 4.6-9.2 μM Kin; 22.2 μM BAP + 1.4 μM Ads + 4.6-9.2 μM Kin and 22.2 μM BAP + 2.8 μM Ads + 4.6-9.2 μM Kin.

Effect of BAP and auxins

Shoot tip segments were cultured on MS medium containing 3% (w/v) sucrose with 0.8% (w/v) agar and supplemented with different combinations and concentrations of plant growth regulators, including 4.4-26.6 μM BAP + 2.7 μM NAA; 4.4-26.6 μM BAP + 2.5 μM IBA; 4.4-26.6 μM BAP + 2.9 μM IAA.

Rooting medium

Elongated shoots were excised from each culture passage and transferred to half-strength (1/2 MS) MS medium containing 3% (w/v) sucrose and 0.8% (w/v) agar. The medium was further supplemented with 2.8-28.5 μM indoleacetic acid (IAA), 2.5-24.6 μM indolebutyric acid (IBA) or 2.7-26.9 μM naphthaleneacetic acid (NAA) individually.

Acclimatization and transfer of plantlets to soil

Plantlets with well-developed roots were removed from the culture medium and after washing the roots gently under running tap water, plantlets were transferred to plastic pots (10 cm diameter) containing autoclaved garden soil, farmyard soil and sand (2:1:1). Each were irrigated with 1/8 MS basal salt solution devoid of sucrose and inositol every 4 d for 2-wk. The potted plantlets were covered with porous polyethylene sheets for maintaining high humidity and were maintained inside the culture room conditions. The relative humidity was reduced gradually, and after 30 d the plantlets were transplanted to botanical evaluation garden and kept under shade in a net house for further growth and development. The morphological and growth characteristics were examined.

Statistical analysis

Experiments were set up in a Randomized Block Design (RBD) and each experiment usually had 10 replication and was repeated at least three times. Ten to fifteen explants were used per treatment in each replication. Observations were recorded on the frequency (number of cultures responding for axillary and adventitious shoot proliferation) and the number of shoots per explant, shoot length, roots per shoot and root length respectively. The treatment means were compared using Duncan's Multiple Range Test (DMRT) at a 5% probability level according to Gomez and Gomez (1976).

Results

Effect of basal media on shoot regeneration

Shoot tip explants of *S. bicolor* (NSH27 & K5) were excised

from 5 d-old *in vitro* raised seedlings (Fig. 2A). These explants were cultured on MS basal medium (without growth regulators) for shoot regeneration. The shoot tip explants were not promoted shoot differentiation. In the MS basal medium, the explants appeared healthy and grew normally at 5-wk culture. In the present study, the explants served as 5-wk but it was produced dark brown pigment in the medium 5-wk after culture. The exudates on the medium completely inhibited explant growth as well as shoot tip explants were declined vigorously.

Influence of genotype on shoot regeneration

Multiple shoot induction was tried using shoot explants of *S. bicolor* (NSH27 and K5) on MS medium containing various concentrations of cytokinin (BAP and Kin). Multiple shoot response was found to be dependent on the genotype of the *S. bicolor*. In genotype NSH27, multiple shoot initiation was observed within 15 d of culture per shoot tip explant. In this culture system, K5 genotypes initiated multiple shoots after 25 d of culture with lower shoots (data not shown). A maximum number of shoots was observed in NSH27 after 8-wk of culture (Table 1). Genotype NSH27 had high shoot frequency and faster shoots regeneration potential. For further studies, we have selected the genotype of NSH27 only.

Effect of cytokinins on shoot regeneration

Various concentrations of BAP (2.2-26.6 μM), Kin (2.3-23.2 μM) and Kin (2.3-13.9 μM) with BAP (22.2 μM each) were tried for shoot induction and to determine the multiplication potential of shoot tip segments. In each concentration of BAP or Kin or combination with different concentrations of Kin and 22.2 μM BAP induced the multiple shoots. Higher concentration of BAP (22.2 μM) or Kin (18.5 μM) in the medium, revealed an increase in the number of axillary and adventitious shoots as well as shoot length (Table 1). The shoots were small when the concentrations of BAP or Kin were raised. Initially, white-greenish compact callus was formed directly at cut ends of shoot tip segments containing MS medium fortified with BA (4.4-13.3 μM) or Kin (4.6-13.9 μM), but this callus later turned dark brown and the shoots declined after 4-wk of culture. Callus could remove from the shoot clumps at 3-wk culture and then cultured on same media. The shoots were grown normally at 8-wk culture. The dark brown pigments on the medium completely inhibited multiple shoot growth as well as shoots were declined vigorously after 10-wk culture. More number of morphologically distinct multiple shoots were developed from the shoot tip in MS medium containing BAP combined with Kin. Multiple shoots developed with a combination of BAP (22.2 μM) and

Table 1. Influence of cytokinins on shoot regeneration from shoot tip explants of *S. bicolor*.

Plant growth regulators (μM)	Shoots sprouting frequency (%)	Number of shoots/ explant (mean \pm SE)	Shoot length (cm) (mean \pm SE)
BAP			
2.2	66.7 ^g	6.7 \pm 0.52 ⁿ	2.3 \pm 0.09 ^{ef}
4.4	76.6 ^f	11.3 \pm 0.61 ^g	2.6 \pm 0.24 ^e
6.6	80.0 ^e	17.4 \pm 0.56 ^f	2.7 \pm 0.27 ^{de}
8.8	86.6 ^d	23.0 \pm 0.64 ^e	3.1 \pm 0.34 ^d
13.3	90.0 ^c	30.0 \pm 0.69 ^d	4.0 \pm 0.36 ^c
17.8	93.5 ^b	41.6 \pm 0.72 ^c	4.5 \pm 0.35 ^b
22.2	100 ^a	52.5 \pm 0.78 ^a	5.7 \pm 0.32 ^a
26.6	78.1 ^{ef}	44.5 \pm 0.60 ^b	4.2 \pm 0.20 ^{bc}
Kin			
2.3	55.0 ^g	4.5 \pm 0.48 ^g	1.5 \pm 0.19 ^{ef}
4.6	58.0 ^f	7.0 \pm 0.52 ^f	1.8 \pm 0.23 ^e
6.9	61.5 ^e	12.0 \pm 0.60 ^e	2.1 \pm 0.28 ^{de}
9.2	65.7 ^d	16.3 \pm 0.64 ^d	2.5 \pm 0.31 ^c
13.9	70.2 ^{bc}	20.0 \pm 0.70 ^c	3.0 \pm 0.36 ^a
18.5	79.0 ^a	27.0 \pm 0.56 ^a	2.8 \pm 0.29 ^b
23.2	72.0 ^b	24.2 \pm 0.45 ^b	2.2 \pm 0.27 ^d
BAP (22.2) + Kin			
2.3	70.0 ^e	58.0 \pm 0.68 ^c	6.8 \pm 0.87 ^a
4.6	78.0 ^{bc}	64.6 \pm 0.74 ^b	5.6 \pm 0.72 ^b
6.9	98.4 ^a	72.0 \pm 1.08 ^a	5.4 \pm 0.63 ^{bc}
9.2	80.5 ^b	56.8 \pm 1.01 ^{cd}	4.9 \pm 0.40 ^c
13.9	74.2 ^d	42.4 \pm 0.94 ^e	3.4 \pm 0.18 ^d
BAP (22.2) + Ads (1.4) + Kin			
4.6	96.0 ^d	68.0 \pm 1.06 ^d	5.2 \pm 0.74 ^a
6.9	100 ^a	78.6 \pm 1.24 ^a	4.6 \pm 0.67 ^b
9.2	92.4 ^c	62.0 \pm 1.02 ^c	3.6 \pm 0.56 ^c
BAP (22.2) + Ads (2.8) + Kin			
4.6	100 ^a	81.6 \pm 1.54 ^a	4.2 \pm 0.47 ^a
6.9	95.0 ^b	70.0 \pm 1.28 ^{bc}	3.8 \pm 0.36 ^{ab}
9.2	84.4 ^c	72.0 \pm 1.48 ^b	3.0 \pm 0.28 ^c

Treatment means followed by different letters within PGR and response variable combinations are significantly different from each other ($p < 0.05$); comparison by DMRT.

Data recorded after 8-wk of culture.

Kin (6.9 μM) grew faster, while those initiated in BAP (2.2-26.6 μM) or Kin (2.3-23.2 μM) and BAP (22.2 μM) plus other Kin concentration grew slower. White-greenish compact callus developed directly from the cut ends of multiple shoot clumps containing MS medium fortified with BAP (6.6-13.3 μM) and Kin in the range 4.6-23.2 μM within 4-wk of culture. A significant shoot sprouting frequency and number of shoots was obtained in MS medium supplemented with BA (22.2 μM) + Kin (6.9 μM) but the shoot lengths differed (Table 1). The MS medium containing BAP (22.2 μM) and a low concentration of Kin (2.3 μM) increased the shoot length but caused a low frequency of axillary shoot formation. With an increase in concentration of Kin (6.9 μM) in the medium,

the number of axillary and adventitious shoots also increased significantly (Table 1). But the higher the concentration of BAP (26.6 μM) or Kin (23.2 μM) or BAP (22.2 μM) + Kin (9.2-13.9 μM) in the medium, revealed a decrease in the number of shoots as well as shoot length. However, the shoot length was best with BA (22.2 μM) + Kin (2.3 μM) (Table 1).

Effect of cytokinins and adenine sulphate on shoot regeneration

Shoot tip explants of *S. bicolor* were cultured on MS medium supplemented with BAP (22.2 μM), Kin (4.6-9.2 μM)

and addition of Ads (1.4-2.8 μM) for shoot regeneration. Among the growth regulators, MS medium fortified with BAP (22.2 μM) + Kin (4.6 μM) + Ads (2.8 μM) was found to be the best response for shoot regeneration (Table 1). In the present study, higher concentrations of cytokinin reduced the shoot number as well as shoot length. The higher concentration of BAP (22.2 μM) + Kin (9.2 μM) + Ads (1.4 or 2.8 μM) in the medium, revealed a decrease in the number of shoots as well as shoot length. Excised explants cultured on MS medium formed white compact callus at the proximal ends of the shoot tip explant after 30 d of culture. In each combination, the cultures, which were maintained for a long time (after 8 wk), resulted in gradual browning, defoliation and decaying of leaves of the shoot. The successive subculture was carried out at 3-wk intervals, and plants exhibited normal appearance. The observations indicate that these media are at concentrations favorable for promoting shoot proliferation in *Sorghum bicolor*.

Effect of cytokinins and coconut water on shoot regeneration

Synergistic effects of cytokinin and coconut water on shoot regeneration were assayed. MS medium was supplemented with different combinations and concentrations of shoot multiplication medium (8.8-26.6 μM BAP + 5% coconut water; 6.9-23.2 μM Kin + 5% coconut water; 22.2 μM BAP + Kin (4.6-9.2 μM) + 5% coconut water; 22.2 μM BAP+ Kin (4.6-9.2 μM) + Ads (1.4 or 2.8 μM) + 5% coconut water). *In vitro* growth and development of *Sorghum bicolor* was highly influenced by concentrations of growth regulators and coconut water added to the culture medium. Addition of coconut water (5% (w/v)) increased the shoot number as well as shoot length (Table 2). The most significant shoot sprouting frequency and number of shoots was obtained in MS medium supplemented with BAP (22.2 μM) + 5% coconut water; Kin (18.5 μM) + 5% coconut water; BAP (22.2 μM) + Kin (6.9 μM) + 5% coconut water and BAP (22.2 μM) + Kin (6.9 μM)

Table 2. Influence of cytokinins and 5% coconut water on shoot regeneration from shoot tip explants of *S. bicolor*.

Plant growth regulators (μM)	Shoots sprouting frequency (%)	Number of shoots/ explant (mean \pm SE)	Shoot length (cm) (mean \pm SE)
BAP			
8.8	90.6 ^c	26.0 \pm 0.46 ^e	3.5 \pm 0.30 ^d
13.3	94.0 ^b	32.0 \pm 0.59 ^d	4.2 \pm 0.36 ^c
17.8	100 ^a	45.6 \pm 0.62 ^b	4.8 \pm 0.35 ^b
22.2	100 ^a	57.5 \pm 0.68 ^a	6.0 \pm 0.40 ^a
26.6	86.1 ^d	42.5 \pm 0.54 ^c	4.6 \pm 0.28 ^{bc}
Kin			
6.9	64.5 ^e	14.0 \pm 0.40 ^e	2.2 \pm 0.26 ^{cd}
9.2	70.4 ^d	18.3 \pm 0.54 ^d	2.4 \pm 0.34 ^c
13.9	74.3 ^{bc}	22.0 \pm 0.60 ^{bc}	3.4 \pm 0.42 ^a
18.5	84.0 ^a	30.0 \pm 0.54 ^a	2.8 \pm 0.27 ^b
23.2	76.0 ^b	22.2 \pm 0.42 ^b	2.1 \pm 0.22 ^d
BAP (22.2) + Kin			
4.6	86.0 ^c	69.0 \pm 0.52 ^d	5.0 \pm 0.67 ^{ad}
6.9	99.2 ^a	80.0 \pm 0.74 ^a	5.2 \pm 0.73 ^a
9.2	92.5 ^b	65.4 \pm 1.06 ^c	4.2 \pm 0.50 ^c
BAP (22.2) + Ads (1.4) + Kin			
4.6	96.0 ^d	87.2 \pm 1.06 ^d	5.0 \pm 0.92 ^a
6.9	100 ^a	94.5 \pm 1.24 ^a	4.0 \pm 0.78 ^b
9.2	94.2 ^c	73.0 \pm 1.18 ^c	3.2 \pm 0.42 ^c
BAP (22.2) + Ads (2.8) + Kin			
4.6	100 ^a	118.0 \pm 1.56 ^a	4.6 \pm 0.34 ^a
6.9	100 ^a	108.0 \pm 1.42 ^b	3.4 \pm 0.30 ^b
9.2	93.6 ^b	87.6 \pm 1.34 ^c	2.5 \pm 0.16 ^c

Treatment means followed by different letters within PGR and response variable combinations are significantly different from each other ($p < 0.05$); comparison by DMRT.

Data recorded after 8-wk of culture.

+ Ads (1.4 μM) + 5% coconut water (Table 2). The MS medium containing BAP (22.2 μM), Kin (13.9 μM), BAP (22.2 μM) + Kin (6.9 μM) and BAP (22.2 μM) + Kin (4.6 μM) + Ads (1.4 or 2.8 μM) and inclusion of coconut water (5% (w/v)) resulted in higher shoot length. However, an increase in the concentration of BAP or Kin in the medium resulted in a decrease in shoot number as well as shoot length. Green compact basal callus was formed from the cut ends and later turned deep black. The exudates (brown pigments) on the medium completely inhibited multiple shoot growth as well as shoots were declined vigorously. The maximum number of shoot induction was obtained in MS medium supplemented with BAP (22.2 μM) + Kin (4.6 μM) + Ads (2.8 μM) + 5% coconut water increased the shoot number and moderately increased shoot length after 4-wk culture (Fig. 2B). The shoot length varied significantly in all combinations and concentrations of shoot multiplication medium (Table 2). Addition of coconut water (10-15%) significantly affected the axillary shoot bud formation, except for shoot length (data not shown).

Effect of BAP, auxins and coconut water on shoot regeneration

The responses of *in vitro* cultures to different concentrations of BAP with NAA (2.7 μM) or IBA (2.5 μM) or IAA (2.9 μM) and 5% coconut water added to the medium were frequently tested. Among the three auxins, NAA proved to be better for shoot regeneration than IBA and IAA (Table 3). Satisfactory shoot proliferation from shoot tip explants was obtained on MS + BAP (22.2 μM) + NAA (2.7 μM) + 5% coconut water. However, IBA (2.5 μM) and IAA (2.9 μM) resulted in lower numbers of shoots; shoot lengths also varied. The exudates (brown pigments) on the medium completely inhibited multiple shoot growth as well as shoots were declined vigorously after 8-wk culture. In each auxin, we observed that IBA and IAA produced green compact callus at proximal cut ends slowly even at 30 d of culture. However, NAA developed green compact callus within 20 d of culture. These calli were promoted shoot regeneration after 4-wk culture. In this culture, NAA and IBA promoted root induction slowly even at 50 d of culture. However, IAA promoted the elongation of

Table 3. Influence of BAP, auxins and coconut water on shoot regeneration from shoot tip explants of *S. bicolor*.

Plant growth regulators (μM)	Shoots sprouting frequency (%)	Number of shoots/ explant (mean \pm SE)	Shoot length (cm) (mean \pm SE)
NAA (2.7) + BAP			
4.4	72.2 ^g	12.6 \pm 0.18 ^g	2.0 \pm 0.14 ^e
6.6	75.2 ^f	19.4 \pm 0.26 ^f	2.8 \pm 0.26 ^{de}
8.8	78.3 ^e	26.0 \pm 0.34 ^e	3.2 \pm 0.28 ^d
13.3	81.0 ^d	33.0 \pm 0.39 ^d	4.0 \pm 0.32 ^c
17.8	94.0 ^b	44.6 \pm 0.42 ^b	4.6 \pm 0.35 ^b
22.2	96.5 ^a	62.2 \pm 0.54 ^a	5.4 \pm 0.38 ^a
26.6	84.2 ^c	36.4 \pm 0.34 ^c	4.0 \pm 0.24 ^{bc}
IBA (2.5) + BAP			
4.4	74.0 ^g	9.0 \pm 0.16 ^g	1.5 \pm 0.10 ^g
6.6	75.4 ^f	13.0 \pm 0.25 ^f	2.1 \pm 0.18 ^f
8.8	83.5 ^d	20.0 \pm 0.38 ^e	2.8 \pm 0.26 ^e
13.3	85.0 ^c	25.4 \pm 0.40 ^c	3.4 \pm 0.30 ^{cd}
17.8	90.0 ^b	28.8 \pm 0.48 ^b	4.6 \pm 0.36 ^a
22.2	94.0 ^a	36.2 \pm 0.64 ^a	4.2 \pm 0.34 ^{ab}
26.6	82.5 ^{de}	24.4 \pm 0.54 ^{cd}	3.6 \pm 0.28 ^c
IAA (2.9) + BAP			
4.4	68.3 ^g	10.4 \pm 0.20 ^f	1.8 \pm 0.18 ^e
6.6	70.5 ^f	15.0 \pm 0.27 ^e	2.2 \pm 0.24 ^{de}
8.8	73.0 ^{de}	24.6 \pm 0.40 ^d	2.6 \pm 0.26 ^d
13.3	75.6 ^d	29.0 \pm 0.46 ^c	3.6 \pm 0.34 ^{bc}
17.8	80.7 ^c	31.6 \pm 0.52 ^{bc}	4.8 \pm 0.38 ^a
22.2	92.0 ^a	42.0 \pm 0.56 ^a	4.0 \pm 0.28 ^b
26.6	85.0 ^b	32.2 \pm 0.48 ^b	3.2 \pm 0.22 ^c

Treatment means followed by different letters within PGR and response variable combinations are significantly different from each other ($p < 0.05$); comparison by DMRT.

Data recorded after 8-wk of culture.

roots. It was difficult to isolate shoots with roots from each auxin containing shoot multiplication medium (MS + 4.4-13.3 μM BAP). This auxin concentration appeared to damage the roots, which were difficult to harden.

Effect of ascorbic acid on rapid shoot production

After 4-wk, shoot clumps (22.2 μM BAP + 2.8 μM Ads + 4.6 μM Kin and 5% coconut water) were cultured on MS basal medium fortified with BAP (22.2 μM) + Kin (4.6 μM) + Ads (2.8 μM) + NAA (2.7 μM) + Ascorbic acid (30.0 μM) + 5% coconut water for rapid shoot regeneration. In the present study, the callus was not formed at proximal end of explant. Shoot differentiation was promoted within 4-wk culture of inoculation. The subculture was maintained at 5-wk intervals. The exudation (brown pigment) was arrested by ascorbic acid but the subculture was necessary at 5-wk intervals. The culture was maintained at 8-wk without subculture and the exudation inhibited the shoot growth as well as shoots declined. In this medium, more than 250 shoots were formed at axillary and adventitious shoots from multiple shoot clumps after 8-wk culture (Fig. 2C). More than 300

shoots were observed after 10-wk culture (Fig. 2D).

Effect of auxins on rooting of shoots

Excised shoots were rooted on half-strength MS medium with different types of auxins. We have found that in *Sorghum bicolor* that reducing MS salt strength to one-half normally enhanced rooting frequency but also reduced callusing. Half-strength MS medium supplemented with different concentrations of IAA or IBA or NAA induced roots from shoots within 20 d of culture. Among the three auxins tested the number of roots and root lengths varied (Table 4). Half-strength MS medium fortified with IAA (22.8 μM) was found to be more effective for root induction than IBA and NAA (Table 4; Fig. 2E). Mild callus was produced in 2.8-11.4 μM IAA, 2.5-14.8 μM IBA, 2.7-10.7 μM NAA.

Hardening of regenerated plants and examination of morphological characters

Plantlets were successfully acclimatized without growth chamber facility. 100% of plantlet survival was seen after

Table 4. Influence of different auxins and half strength MS medium on rooting of *in vitro* – formed shoots of *Sorghum bicolor*.

Plant growth regulators (μM)	Percentage shoots showing root regeneration	Number of roots/shoot (mean \pm SE)	Root length (cm) (mean \pm SE)
Half strength MS +			
IAA			
2.8	80.0 ^e	26.0 \pm 0.25 ^d	2.8 \pm 0.25 ^c
5.7	84.5 ^d	28.0 \pm 0.31 ^c	3.1 \pm 0.20 ^{bc}
11.4	90.0 ^c	30.0 \pm 0.54 ^{bc}	3.2 \pm 0.31 ^b
17.1	95.0 ^b	32.0 \pm 0.42 ^b	3.4 \pm 0.22 ^{ab}
22.8	100 ^a	34.0 \pm 0.51 ^a	3.6 \pm 0.18 ^a
28.5	100 ^a	27.3 \pm 0.48 ^{cd}	2.1 \pm 0.15 ^d
Half strength MS +			
IBA			
2.5	65.0 ^f	12.0 \pm 0.21 ^d	1.2 \pm 0.15 ^{de}
4.9	68.4 ^{de}	13.0 \pm 0.28 ^{cd}	1.4 \pm 0.12 ^d
9.8	69.7 ^d	14.5 \pm 0.31 ^{bc}	1.5 \pm 0.08 ^{od}
14.8	75.0 ^c	16.0 \pm 0.37 ^b	2.0 \pm 0.13 ^{ab}
19.7	80.0 ^a	18.0 \pm 0.30 ^a	2.1 \pm 0.28 ^a
24.6	77.0 ^b	13.3 \pm 0.41 ^c	1.6 \pm 0.14 ^c
Half strength MS +			
NAA			
2.7	70.0 ^f	16.0 \pm 0.42 ^e	2.4 \pm 0.25 ^{cd}
5.4	75.0 ^e	18.0 \pm 0.38 ^d	2.6 \pm 0.20 ^c
10.7	80.0 ^d	20.0 \pm 0.46 ^c	2.9 \pm 0.31 ^b
16.1	84.3 ^{bc}	21.9 \pm 0.31 ^{bc}	3.0 \pm 0.24 ^{ab}
21.5	90.1 ^a	26.0 \pm 0.43 ^a	3.2 \pm 0.12 ^a
26.9	85.9 ^b	22.0 \pm 0.41 ^b	3.0 \pm 0.16 ^{ab}

Treatment means followed by different letters within medium treatment and response variable combinations are significantly different from each other ($p < 0.05$); comparison by DMRT. Data recorded after 30 d of culture.

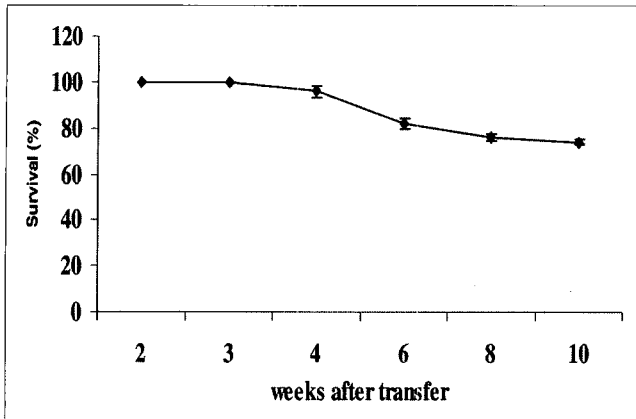


Figure 1. The frequency of *ex vitro* survival of acclimatized microplants of *Sorghum bicolor*. Values shown were proportions of three replicates of 25 microplants. Error bars represent the standard error.

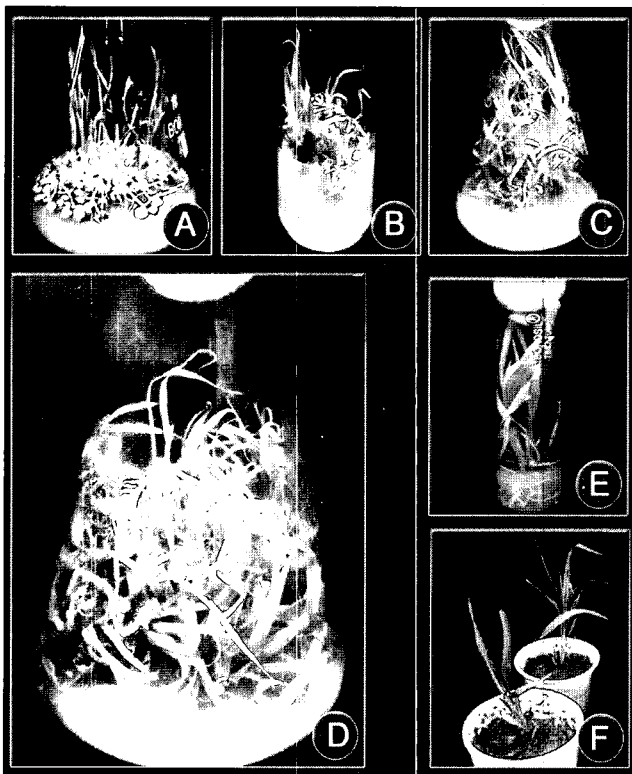


Figure 2. *In vitro* plantlet formation of *Sorghum bicolor*: (A) Five old seedlings on MS basal medium. (B) Induction of multiple shoots from shoot tip segment on MS + 22.2 μ M BAP + 2.8 μ M Ads + 4.6 μ M Kin + 5% coconut water (after 4 wk). (C) Induction of multiple shoots from shoot tip segment on MS medium + BAP (22.2 μ M) + Kin (4.6 μ M) + Ads (2.8 μ M) + NAA (2.7 μ M) + Ascorbic acid (30.0 μ M) + 5% coconut water (after 8 wk). (D) Proliferation of axillary and adventitious shoots from subcultured multiple shoot clump after 10 wk. (E) Regeneration of roots from shoots of *S. bicolor* cultured on half-strength MS medium containing 22.8 μ M IAA. (F) *In vitro* regenerated plantlets of *S. bicolor* transferred to plastic cup containing garden soil, farmyard soil and sand (2:1:1).

hardening on garden soil, farmyard soil and sand (2:1:1) for 3 wk (Fig. 2F). However, the survival decreased to 98.0 and 72.4% respectively, after 4-10 wk of acclimatization (Fig. 1). There was no detectable variation among the acclimatized plants with respect to morphological and growth characteristics. All the micropropagated plants were free from external defects.

Discussion

Influence of genotype on shoot regeneration

In this study, we utilized an efficient and reproducible method to obtain a high frequency of well-developed *S. bicolor* regenerants from shoot tip culture. Our study has described the efficiency of multiple shoot regeneration depending on the genotype of *S. bicolor* from shoot tip. Patil and Kuruvinashetti (1998) also reported that influence of genotypes with regard to the frequency of regeneration of plantlets in rabi sorghum cultivars. A strong genotype dependent response was observed in *S. bicolor* and the genotype 296 B was found to be most responsive as compared to the other genotypes (Archana and paramjit, 2003). *In vitro* regeneration dependent on four different wheat genotypes (Ahmad et al., 2002). However, all genotypes showed a similar differentiation and regeneration pattern at a high frequency (Hagio, 1994; Heng, 1998). Genotype-independent for plant regeneration using shoot tip explants of pearl millet (Mythili et al., 2001).

Influence of growth regulators on shoot regeneration

In this study, shoot multiplication was inhibited by the production of dark brown pigment. Similar observation has also been described by others (Gamborg et al., 1977; Dunstan et al., 1978; Wernicke and Brettell, 1980; Vasil and Vasil, 1981; Oldach et al., 2001; Baskaran and Jayabalan, 2005a). Shoot initiation was observed when shoot tip explants were cultured to plant growth regulators containing medium. Therefore, higher concentration of cytokinin (BAP or Kin) responded effectively. Higher concentration of BAP was effective for shoot multiplication in *S. bicolor*. Similar observation was reported in *S. bicolor*, *P. virgatum* and *Oryza sativa* (Heng et al., 1998; Alexandrova et al., 1996; Medina et al., 2004). BAP activity has been shown to be superior to common adenine-based cytokinins (Patil and Kuruvinashetti, 1998; Sarma and Rogers, 1998; Syamala and Devi, 2003). Since then, BAP has been successfully used in many tissue culture protocols of other cereals (Vasil and Vasil, 1981;

Oldach et al., 2001). In organogenesis, the shoot meristems were directly cultured on a high-cytokinin medium comprising BAP, resulted six to eight shoots per meristem in *Zea mays* (Sairam et al., 2003). MS medium supplemented with BAP and Kin combination was effective for shoot multiplication of *S. bicolor*. WP medium supplemented with BAP and Kin gave the greatest response to initiation and multiplication (Latha et al., 1998). Cytokinin combination (BAP and Kin) promoted shoot multiplication in rice (Medina et al., 2004).

In this context, an interesting feature was that 8-wk exposure to BAP, Ads and Kin combination was sufficient to achieve optimal shoot multiplication of *S. bicolor*. Similarly, addition of adenine sulphate to the medium was found beneficial for maximum shoot bud induction in other plant species (Deshpande et al., 1998; Vila et al., 2003). In the present study, addition of 5% coconut water to cytokinin enriched medium enhanced maximum number of shoots. Coconut water has been shown to stimulate shoot proliferation in many species of plants (Kumar, 1999). Several reports confirmed the beneficial effect of coconut water for micropropagation in other plant species (Reghunath and Bajaj, 1992; Bajaj et al., 1993; Brain and Richard, 1993; Sajina et al., 1997; Wondyifraw and Surawit, 2004; Baskaran and Jayabalan, 2005b).

In this study presented, BAP and auxins induced shoot regeneration. Synergistic effect of shoot regeneration was found to be more in BAP with NAA. Similar response was also observed in other plant species (Madhumita et al., 2002). However, BAP and 2, 4-D combination was highly effective for adventitious buds differentiation (Heng et al., 1998). BAP combined with IBA promoted best shoot regeneration (Choi et al., 1998).

Influence of auxins on root formation

In our study, reducing MS salt and IAA induced high frequency of root formation. Similarly, the promotory effect of reducing the salt concentration of MS on *in vitro* rooting of shoots has been described in several reports (Constantine, 1978; Skirvin et al., 1980; Baskaran and Jayabalan, 2005a). Half strength MS salt with IAA (22.8 μ M) showed highest root formation. Similar response was observed in *S. bicolor* (Saradamani et al., 2003; Baskaran and Jayabalan, 2005a). However, IBA or NAA was used for root induction (Heng et al., 1998; Sarma and Rogers, 1998; Syamala and Devi, 2003). In the field trial, regenerated plants did not show any variation. Similar response was also observed in *S. bicolor* (Seetharama et al., 2000; Baskaran and Jayabalan, 2005a).

In conclusion, a reproducible, quick and large-scale micropropagation protocol was established from shoot tip of *S. bicolor*. Axillary and adventitious shoot proliferation from

shoot tip was dependent on the interaction between plant growth regulators concentrations in the medium. A rapid multiplication rate could be obtained by a cytokinins ratio associated with auxin, adenine sulphate, ascorbic acid and coconut water.

In this study, by using genotype and subculture, a simple and highly effective method for the successful shoot formation was obtained. The results would suggest that shoot tip have possible effects of enhancing shoot initiation and plant regeneration followed by successive establishment of regenerated plants in soil with no observable morphological and growth characters variations. This protocol has great potential for improvement of this crop by biotechnological approaches such as using chitinase genetic transformation.

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