In vitro plant regeneration and mass propagation system for *Sorghum bicolor* –a valuable major cereal crop

P. Baskaran[1*] and N. Jayabalan [1]

[1]Plant Tissue Culture & Molecular Biology Division, Department of Plant Science, School of Life Sciences, Bharathidasan University, Tiruchirappalli-620 024, Tamil Nadu, India.

Baskaran, P. and Jayabalan, N. (2005). *In vitro* plant regeneration and mass propagation system for *Sorghum bicolor*-a valuable major cereal crop. Journal of Agricultural Technology 1(2): 345-363.

A rapid, improved and large-scale *in vitro* clonal propagation of important Indian cereal crop genotypes (K8 and 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-Naphthalene acetic acid (2.7 µM), Ascorbic acid (30 µM) and 5% coconut water, a rapid production of axillary and adventitious buds was developed after eight weeks culture. More than 275 shoots were produced ten weeks after culture. Rooting was highest (100%) on half strength MS medium containing 22.8 µM 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 morphological traits.

Key words: *ex vitro*, hardening, *in vitro*, plant growth regulators, shoot regeneration, *Sorghum bicolor*

Abbreviations: Ads-Adenine sulphate; BAP-6-Benzyladenine; IAA-Indoleacetic acid; IBA-Indolebutyric acid; Kin-Kinetin; MS-Murashige and Skoog; NAA-α-Naphthalene acetic acid.

Introduction

Sorghum is one of the most important cereal crops 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

*Corresponding author: P. Baskaran; e-mail: Baskaran_research@yahoo.co.in

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 United States in the early 17th century, where most of the world's sorghum is now produced. *Sorghum* species are sources of fibre, fuel and secondary products and is 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 use 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 could be produced (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 genotypes using an *in vitro* culture technique. This technique provides a rapid reliable system for a production of large number of genetically uniform disease free plantlets. Micropropagation procedures for multiplication of desired or selected genotypes of *Panicum virgatum* are available (Alexandrova *et al*., 1996). Shoot tip culture is an important system for micropropagation. An efficient clonal propagation procedure for six rice varieties cultivated in Argentina was developed 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 inflorescences were 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)

There have been few reports on micropropagation of *S. bicolor* 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 (K8 and 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 K8 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. Five days old shoot tip segments were selected from the *in vitro* raised plants for culture initiation.

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 121ºC 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±2ºC under 16 hour photoperiod of 45-50 µmol m^{-2} 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 week 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 diam.) 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 days for 2 week. 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 day 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 replications and was repeated at least three times. Ten to 15 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* (K8 and K5) were excised from *in vitro* five day old culture raised seedlings (Fig. 1A). These explants were cultured on MS basal medium (without growth regulators) for shoot regeneration. The shoot tip explants did not promoted shoot differentiation. In the MS basal medium, the explants appeared healthy and grew normally at five weeks. In the present study, the explants served as five week cultures, but produced dark brown pigments in the medium. The exudates on the medium completely inhibited explant growth as well as shoot tip explants which declined rapidly.

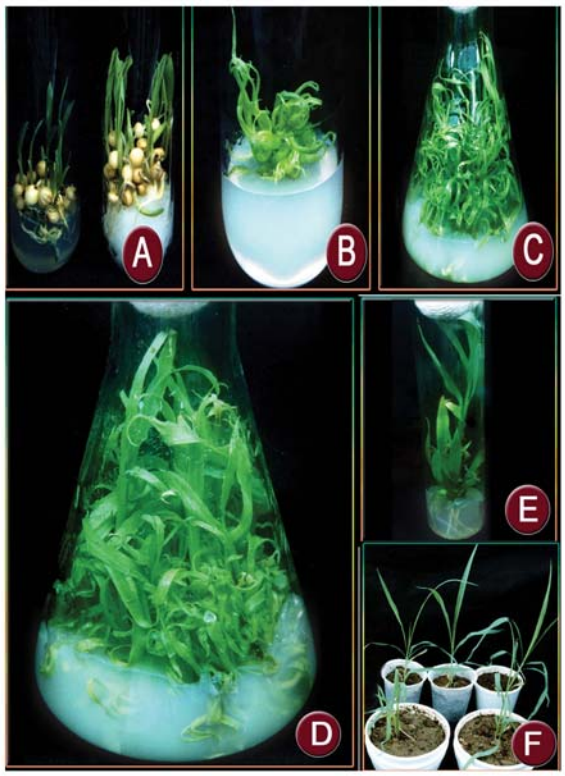

Figure 1. *In vitro* plantlet formation of *Sorghum bicolor* (L.) Moench: (A) Five day old seedlings on MS basal medium. (B) Induction of multiple shoots from shoot tip segment on MS + 22.2 μM BAP + 2.8 μM Ads + 4.6 μM Kin + 5% coconut water (after 4 week). (C) Induction of multiple shoots from shoot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

Influence of genotype on shoot regeneration

Multiple shoot induction was tested using shoot explants of *S. bicolor* (K8 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 K8, multiple shoot initiation was observed within 15 days of culture per shoot tip explant. In this culture system, K5 genotypes initiated multiple shoots after 25 days of culture with lower shoots (data not shown). A maximum number of shoots was observed in K8

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 ± SE)	Shoot length (cm) (mean ± SE)
BAP			
2.2	65.7^{g}	6.5±0.5^{h}	2.0±0.05^{ef}
4.4	77.6^{f}	10.3±0.65^{g}	2.4±0.2^{e}
6.6	80^{e}	15.0±0.52^{f}	2.6±0.27^{de}
8.8	88.6^{d}	20.0±0.61^{e}	3.0±0.32^{d}
13.3	90^{c}	27.0±0.69^{d}	3.7±0.38^{c}
17.8	92.5^{b}	34.2±0.7^{c}	4.2±0.35^{b}
22.2	100^{a}	42.5±0.8^{a}	5.2±0.3^{a}
26.6	79.1^{ef}	36.3±0.64^{b}	4.0±0.2^{bc}
Kin			
2.3	50^{g}	4.0±0.42^{g}	1.3±0.14^{ef}
4.6	54.4^{f}	6.8±0.5^{f}	1.5±0.2^{e}
6.9	60.5^{e}	11.0±0.56^{e}	2.0±0.22^{de}
9.2	63.2^{d}	14.4±0.62^{d}	2.3±0.3^{c}
13.9	68^{bc}	19.0±0.74^{c}	2.9±0.38^{a}
18.5	76^{a}	24.2±0.58^{a}	2.5±0.25^{b}
23.2	70.8^{b}	21.6±0.42^{b}	2.1±0.21^{d}
BAP (22.2) + Kin			
2.3	68^{e}	46.2±0.62^{d}	5.8±0.67^{a}
4.6	78.6^{bc}	58.4±0.7^{b}	5.2±0.7^{b}
6.9	94.^{a}	68.2±1.02^{a}	4.6±0.68^{c}
9.2	78.^{b}	50.6±0.94^{c}	4.2±0.52^{cd}
13.9	72.4^{d}	40.0±0.8^{e}	3.0±0.38^{e}
BAP (22.2) + Ads (1.4) + Kin			
4.6	94.^{b}	69.8±1.02^{b}	5.0±0.64^{a}
6.9	100^{a}	74.4±1.2^{a}	4.2±0.57^{b}
9.2	90.4^{c}	58.0±1.08^{c}	3.2±0.52^{c}
BAP (22.2) + Ads (2.8) + Kin			
4.6	100^{a}	78.6±1.38^{a}	4.0±0.42^{a}
6.9	94^{b}	69.0±1.2^{b}	3.8±0.34^{ab}
9.2	82.4^{c}	56.0±1.4^{c}	2.8±0.26^{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 week of culture.

after 8 weeks of culture (Table 1). Genotype K8 had high shoot frequency and faster shoots regeneration potential. For further studies, we have selected the genotype of K8 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 week of culture. Callus could remove from the shoot clumps at 3 week culture and then cultured on same media. The shoots were grown normally at 8 week culture. The dark brown pigments on the medium completely inhibited multiple shoot growth as well as shoots were declined vigorously after 10 week 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 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 concentrations grew slower. A 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 weeks 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). 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 Adenine Sulphate (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 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 day of culture. In each combination, the cultures, which were maintained for a long time (after 8 weeks), resulted in gradual browning, defoliation and decaying of leaves of the shoot. The successive subculture was carried out at 3 - week intervals, and plants exhibited normal appearance. The observations indicate that these media are at concentrations favourable 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) + 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.

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 ± SE)	Shoot length (cm) (mean ± SE)
BAP			
8.8	92.6^{c}	22.0±0.42^{e}	3.5±0.32^{d}
13.3	94^{b}	30.6±0.53^{d}	4.3±0.38^{c}
17.8	100^{a}	42.4±0.6^{b}	4.8±0.42^{b}
22.2	100^{a}	51.0±0.67^{a}	6.0±0.48^{a}
26.6	82.1^{d}	38.2±0.46^{c}	4.6±0.38^{bc}
Kin			
6.9	62.5^{e}	13.0±0.4^{e}	2.0±0.24^{cd}
9.2	69.4^{d}	16.2±0.56^{d}	2.2±0.3^{c}
13.9	73^{bc}	22.0±0.62^{bc}	3.6±0.46^{a}
18.5	82.2^{a}	32.0±0.46^{a}	2.8±0.28^{b}
23.2	74^{b}	24.6±0.4^{b}	2.0±0.26^{cd}
BAP (22.2) + Kin			
4.6	80^{c}	64.0±0.5^{b}	4.8±0.6^{ab}
6.9	97.2^{a}	78.2±0.72^{a}	5.1±0.72^{a}
9.2	90.5^{b}	60.6±0.64^{c}	4.3±0.54^{c}
BAP (22.2) + Ads (1.4) + Kin			
4.6	94^{b}	82.2±1.02^{b}	5.0±0.9^{a}
6.9	100^{a}	92.6±1.14^{a}	4.3±0.82^{b}
9.2	90.2^{c}	70.0±1.1^{c}	3.4±0.58^{c}
BAP (22.2) + Ads (2.8) + Kin			
4.6	100^{a}	110.0±1.56^{a}	4.8±0.3^{a}
6.9	97.6^{b}	101.2±1.42^{b}	3.6±0.28^{b}
9.2	90.2^{b}	88.4±1.34^{c}	2.8±0.14^{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 week of culture.

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 four week culture (Fig. 1B). 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 week culture. In each auxin, we observed that IBA and IAA produced green compact callus at proximal cut ends slowly even at 30 days of culture. However, NAA developed green compact callus within 20 days of culture. These calli were promoted shoot regeneration after 4 week culture. In this culture, NAA and IBA promoted root induction slowly even in a 50 day culture. However, IAA promoted strengthens as well as lengthy 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 four week, 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 basal callus was not formed at proximal end of the explant. Shoot clumps had high shoot differentiation within 4 weeks of culture. The subculture was maintained at 5 week intervals. The exudation (brown pigment) was arrested by ascorbic acid subculturing was necessary at 5 week intervals. The culture was maintained at 8 weeks without subculture and the exudation inhibited the shoot growth and shoots declined. In this medium, more than 250 shoots were formed at axillary and adventitious shoots from multiple shoot clumps after 8 weeks of culture (Fig. 1C). More than 275 shoots were observed after 10 weeks of culture (Fig. 1D).

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 ± SE)	Shoot length (cm) (mean ± SE)
NAA (2.7) + BAP			
4.4	70.2^{g}	11±0.12^{g}	2.8±0.16^{e}
6.6	74.4^{f}	18.4±0.24^{f}	3.1±0.28^{de}
8.8	76.3^{e}	24.8±0.3^{e}	3.4±0.3^{d}
13.3	80^{d}	30±0.38^{d}	4.1±0.32^{c}
17.8	92^{b}	42.6±0.4^{b}	4.5±0.36^{b}
22.2	95.6^{a}	60.4±0.52^{a}	5.2±0.43^{a}
26.6	82^{c}	38±0.36^{c}	4.2±0.37^{bc}
IBA (2.5) + BAP			
4.4	72^{g}	8±0.14^{g}	1.8±0.14^{g}
6.6	74.5^{f}	12.5±0.23^{f}	2.3±0.21^{f}
8.8	82.6^{d}	21±0.36^{de}	2.9±0.28^{e}
13.3	86^{c}	24.8±0.42^{c}	3.6±0.32^{cd}
17.8	90^{b}	26.6±0.46^{b}	4.8±0.38^{a}
22.2	93^{a}	34.2±0.68^{a}	4.5±0.36^{ab}
26.6	80.6^{de}	21±0.5^{d}	3.8±0.28^{c}
IAA (2.9) + BAP			
4.4	65.2^{g}	9.8±0.24^{f}	1.9±0.1^{e}
6.6	69.4^{f}	13.4±0.27^{e}	2.4±0.28^{de}
8.8	73^{e}	22.2±0.46^{d}	2.6±0.34^{d}
13.3	77.6^{d}	28±0.48^{c}	3.8±0.38^{bc}
17.8	81.2^{c}	32.2±0.54^{bc}	4.8±0.42^{a}
22.2	93^{a}	41±0.52^{a}	4±0.36^{b}
26.6	85^{b}	34.8±0.38^{b}	3.4±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 week of culture.

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 ± SE)	Root length (cm) (mean ± SE)
Half strength MS+IAA			
2.8	78^{e}	25±0.25^{d}	2.7±0.24^{c}
5.7	82.5^{d}	27±0.32^{c}	3.2±0.21^{bc}
11.4	89^{c}	31±0.52^{bc}	3.4±0.3^{b}
17.1	93^{b}	33±0.4^{b}	3.8±0.24^{ab}
22.8	100^{a}	35±0.52^{a}	4.2±0.2^{a}
28.5	100^{a}	26.3±0.48^{cd}	2.4±0.16^{d}
Half strength MS + IBA			
2.5	62^{f}	11±0.22^{d}	1.4±0.15^{de}
4.9	69.4^{de}	13±0.26^{cd}	1.6±0.12^{cd}
9.8	70.7^{d}	15.5±0.34^{bc}	1.5±0.08^{d}
14.8	75^{c}	16±0.36^{b}	2.1±0.13^{ab}
19.7	82^{a}	18±0.32^{a}	2.4±0.28^{a}
24.6	76^{b}	13.8±0.46^{c}	1.7±0.14^{c}
Half strength MS + NAA			
2.7	68^{f}	15±0.4^{e}	2.2±0.25^{cd}
5.4	74^{e}	17±0.38^{d}	2.5±0.2^{c}
10.7	80^{d}	21±0.48^{c}	2.8±0.31^{bc}
16.1	85.2^{bc}	22.9±0.32^{bc}	3±0.24^{ab}
21.5	92.6^{a}	27±0.46^{a}	3.2±0.12^{a}
26.9	86.8^{b}	23±0.4^{b}	2.9±0.16^{b}

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 days of culture.

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 callus formation. Half-strength MS medium supplemented with different concentrations of IAA or IBA or NAA induced roots from shoots within 20 days 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. 1E). Mild callus was produced in 2.8-11.4µµM 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 hardening on garden soil, farmyard soil and sand (2:1:1) for 3 weeks (Fig. 1F). However, the survival decreased to 98 and 72.4% respectively, after 4-10 week of acclimatization (Fig. 2). 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.

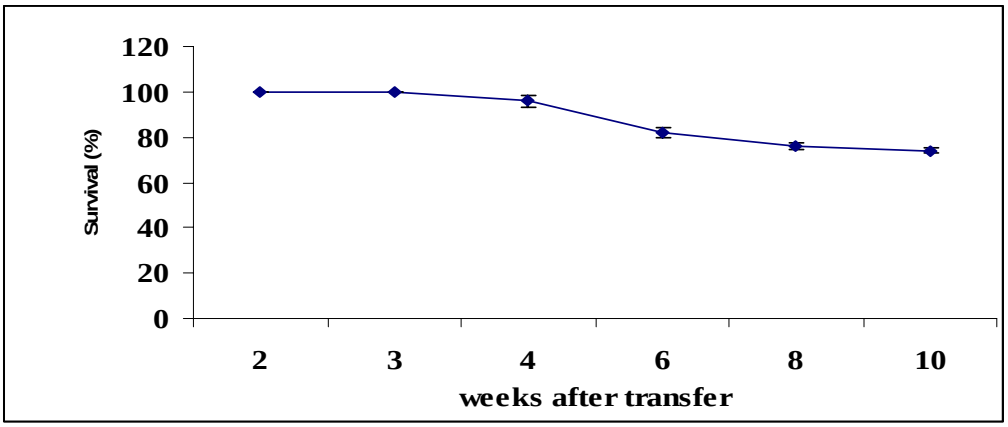

Fig. 2. Frequency of *ex vitro* survival of acclimatized microplants of *Sorghum bicolor*. Values shown were proportions of three replicates of 25 microplants. Error bars represent the standard error.

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 the 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 was 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 *et al*., 1998). Genotype-independent for plant regeneration used 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 a dark brown pigment. Similar observations have 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, 2005). Shoot initiation was observed when shoot tip explants were cultured medium containing plant growth regulators. Therefore, a higher concentration of cytokinin (BAP or Kin) gave an effective response. Higher concentration of BAP were effective for shoot multiplication in *S. bicolor*. Similar observations 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) was promoted shoot multiplication in rice (Medina *et al*., 2004).

In this context, an interesting feature was that 8 week exposure to BAP, Ads and Kin combination was sufficient to achieve optimal shoot multiplication on *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 enriches 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, pers. comm.).

In this study, BAP and auxins also 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). The exudation was inhibited regeneration frequency, growth and development of *S. bicolor*. High levels of phenolic oxidation appear to be widespread across many monocot species (Smith, 2000). To overcome exudation, ascorbic acid was investigated. Incorporation of ascorbic acid (30 µM) to the medium prevented exudation, enhanced production of healthy and higher frequency of shoot regeneration was achieved in *S. bicolor*. Addition of antioxidant to the medium was effective in preventing the exudates and resulted healthy shoot regeneration in other species (Komalavalli and Rao, 2000; Farooq *et al*., 2002).

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, 2005). Half strength MS salt with IAA (22.8 µM) showed highest root formation. Similar response was observed in *S.bicolor* (Saradamani *et al*., 2003; Baskaran and Jayabalan, 2005). 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, 2005).

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 is great potential for improvement of this crop by biotechnological approaches such as using chitinase genetic transformation.

Acknowledgements

The first author is thankful to B. Muthukumar and J. Britto for their critical appraisal of the paper and to Tamil Nadu seed germination testing laboratory for providing the seed. The author wishes to thank P. Velayutham and G.G. Gideon for helpful discussion and critical reading of the manuscript and Joseph for the valuable help in photograph of this manuscript.

References

Ahmad, A., Zhong, H., Wang, W. and Sticklen, M.B. (2002). Shoot apical meristem: *in vitro* regeneration and morphogenesis in wheat (*Triticum aestivum* L.). In Vitro Cell Developmental Biology-Plant 38: 163-167.

Alexandrova, K.S., Denchev, P.D. and Conger, B.V. (1996). Micropropagation of switchgrass by node culture. Crop Science 36: 1709-1711.

Archana, M. and Paramjit, K. (2003). Genotype dependent somatic embryogenesis and regeneration from leaf base cultures of *Sorghum bicolor*. Journal of Plant Biochemistry and Biotechology 12: 53-56.

Bajaj, Y.P.S., Reghunath, B.R., and Gopalakrishnan, P.K. (1993). *In vitro* culture studies and clonal propagation. In: *Biotechnology in Agriculture and Forestry* (*Medicinal and Aromatic Plants) Elettaria cardamomum Maton (cardamom)*: *Aromatic compounds* (ed. Y.P.S. Bajaj) Springer-Verlag, Berlin: 132-147.

Baskaran, P. and Jayabalan, N. (2005). A simple approach to improve plant regeneration from callus culture of *Sorghum bicolor* for crop improvement. Journal of Agricultural Biotechnology 1: 179-192.

Brain, K.W. and Richard, A.C. (1993). Clonal propagation of pink ginger *in vitro*. Horticultural Science 28: 1203.

Choi, Y.E., Yang, D.C., Yoon, E.S. and Choi, K.T. (1998). Plant regeneration via adventitious bud formation from cotyledon explants of *Panax ginseng* C.A. Meyer. Plant and Cell Reproduction 17: 731-736.

Constantine, D. (1978). *Round Table Conference*. Gembloux, Belgium. 134p.

Deshpande, S.R., Josekutty, P.C. and Prathapasenan, G. (1998). Plant regeneration from axillary buds of a mature tree of *Ficus religiosa*. Plant and Cell Reproduction 17: 571-573.

Dunstan, D.I., Short, K.C. and Thomas, E. (1978). The anatomy of secondary morphogenesis in cultured scutellum tissues of *Sorghum bicolor*. Protoplasma 97: 251-260.

Elkonin, L.A., Lopushanskaya, R.F. and Pakhomova, N.V. (1996). Embryogenic callus of Sorghum (*Sorghum bicolor* (L.) Moench) by amino acids. Maydica 40: 153-157.

Farooq, S.A., Farooq, T.T. and Rao, T.V. (2002). Micropropagation of *Annona squamosa* L. using nodal explants. Pakistan Journal of Biological Science 5: 43-46.

Gamborg, O.L., Shyluk, J.P., Brar, D.S. and Constable, F. (1977). Morphogenesis and plant regeneration from callus of immature embryos of sorghum. Plant Science Letters 10: 67-74.

Gomez, K.A. and Gomez, A.A. (1976). *Statistical Procedures for Agricultural Research with Emphasis on Rice*. International Rice Research Institute. Los Baños, Philippines.

Haberlandt, G. (1902). Culturversuche mit isolierten Pfanzenzellen. Sitzungsberichte der Mathematisch-Naturwissenschaftlichen Classe der Kaiserlichen. Akademie der Wissenschsften 111: 69-92.

Hagio, T. (1994). Varietal difference of plant regeneration from callus of sorghum mature seed. Breeding Science 44: 121-126.

Heng, Z., Wenling W. and Mariam, S. (1998) *In vitro* morphogenesis of *Sorghum bicolor* (L.) Moench. Efficient plant regeneration from shoot apices. Journal of Plant Physiology 153: 719-726.

Inoue, M. and Maeda, E. (1980). Effects of auxins and cytokinins on the occurrence of green regions in rice callus cultures. Japanese Journal of Plant Science 49: 167-174.

Komalavalli, N. and Rao, M.V. (2000). *In vitro* micropropagation of *Gymnema sylvestre* - A multipurpose medicinal plant. Plant Cell Tissue and Organ Culture 61: 97-105.

Kumar, U.K. (1999). *Methods in Plant Tissue Culture*. Agro Botanica, Bikanes, India.

Latha, R., Ajith, A., Srinivasa, R.C., Eganathan, P. and Balakrishna, P. (1998). *In vitro* propagation of salt-tolerant wild rice relative, *Porteresia coarctata* Tateoka. Journal of Plant Growth Regulation 17: 231-235.

Ma, H., Gu, M. and Liang, G.H. (1987). Plant regeneration from cultured immature embryos of *Sorghum bicolor* (L.) Moench. Theory of Applied Genetics 73: 389-394.

Mukhopadhyay, M.J., Mukhopadhyay, S., Sen, S. (2002). *In vitro* propagation of *Iphigenia indica*, an alternative source of colchicines. Plant Cell Tissue and Organ Culture 69: 101-104.

Medina, R., Faloci, M., Marassi, M.A. and Mroginski, L.A. (2004). Genetic stability in rice micropropagation. Biocell 28: 13-20.

Murashige, T. and Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiology. Planta 15: 473-479.

Mythili, P.K., Madhavi, A., Reddy, V.D. and Seetharama, N. (2001). Efficient regeneration of pearl millet (*Pennisetum glaucum* (L.) R. Br.) from shoot tip cultures. Indian Journal of Experimental Biology 39: 1274-1279.

Nabors, M.W., Kroskey, C.S. and Mchugh, D.M. (1982). Green spots are predictors of high callus growth rates and shoot formation in normal and in salt stressed tissue cultures of oat (Avena-sativa L.) Zeitschrift fur Pflanzenphysiologie 105: 341-349.

Ogura, H. and Shimada, T. (1978). Preliminary report on shoot redifferentiation from wheat callus. Wheat Information Service 45: 26-28.

Oldach, K.H., Morgenstern, A., Rother, S., Girgi, M., Kennedy, O.M. and Lorz, H. (2001). Efficient *in vitro* plant regeneration from immature zygotic embryos of pearl millet (*Pennisetum glaucum* (L.) R.Br.) and *Sorghum bicolor* (L.) *Moench*. Plant Cell Reporter 20: 416-421.

Patil, V.M. and Kuruvinashetti, M.S. (1998). Plant regeneration from leaf sheath cultures of some rabi sorghum cultivars. South African Journal of Botany 64: 217-219.

Raghavendra Rao, R.V., Pavan Kumar, G., Subba Rao, M.V. and Manga, V. (2000). Differential *in vitro* response of genotypes of Sorghum. Phytomorph 50: 253-260.

Reghunath, B.R. and Bajaj, Y.PS. (1992). Micropropagation of cardamom (*Elettaria cardamomum* Maton). In: *Biotechnology in Agriculture and Forestry - High Tech and Micropropagation* (ed. Y.P.S. Bajaj). Springer-Verlag, Berlin: 175-198.

Sairam, R.V., Parani, M., Franklin, G., Lifeng, Z., Smith, B., MacDougall, J., Wilber, C., Sheikhi, H., Kashikar, N., Meeker, K., Al-Abed, D., Berry, K., Vierling, R. and Goldman, S.L. (2003) Shoot meristem: an ideal explant for *Zea mays* L. transformation. Genome 46: 323-329.

Sajina, A., Mini, P.M., John, C.Z., Babu, K.N., Ravindran, P.N. and Peter, K.V. (1997) Micropropagation of large cardamom (*Amomum subulatum* Roxb.). Journal of Spices and Aromatic Crops 6: 145-148.

Saradamani, N., Muralimohan, S., Sudhakar, R. and Pola, D. (2003). *In vitro* morphogenesis in cultivated varieties of *Sorghum bicolor* (L.) Moench. Plant Cell Biotechnology and Molecular Biology 4: 43-48.

Sarma, K.S. and Rogers, S.M.D. (1998). Plant regeneration and multiplication of the emergent wetland monocot *Juncus accuminatus*. Plant Cell Reporter 17: 656-660.

Seetharama, N., Sairam, R.V. and Rani, T.S. (2000). Regeneration of sorghum from shoot tip cultures and field performance of the progeny. Plant Cell Tissue and Organ Culture 61: 169-173.

Sharma, V.K., Hansch, R., Mendel, R.R. and Schulze, J. (2004). A highly efficient plant regeneration from commercial cultivars of barley (*Hordeum vulgare* L.) using meristematic shoot segments excised from germinated mature embryos. Plant Cell Reporter 23: 9-16.

Shimada, T. and Yamada, Y. (1979). Wheat plants regenerated from embryo cell cultures. Japanese Journal of Genetics 54: 379-385.

Skirvin, R.M., Chu, M.C. and Rukan, H. (1980). An improved medium for the *in vitro* rooting of Harbrite Peach. Proceedings of the III State Horticultural Society 113: 30-38.

Smith, J. (2000). *Micropropagation of the Gymea lily - A report for the Rural Industries Research and Development Corporation*. RIRDC Publication, Kingston, Australia: 18-19.

Syamala, D. and Devi, P. (2003). Efficient regeneration of sorghum, *Sorghum bicolor* (L.) Moench, from shoot-tip explant. Indian Journal of Experimental Biology 41: 1482-1486.

Thomas, E., King, P.J. and Portykus, I. (1977) Shoot and embryo-like structure formation from cultured tissues of *Sorghum bicolor*. Naturwissenschaften 64: 587.

Vasil, V. and Vasil, I.K. (1981). Somatic embryogenesis and plant regeneration from tissue cultures of *Pennisetum americanum* and *P. americanum* x *P. purpureum* hybrid. American Journal of Botany 68: 864-872.

Vila, S.K., Gonzalez, A.M., Rey, H.Y. and Mroginski, L.A. (2003). *In vitro* plant regeneration of *Melia azedarach* L. shoot organogenesis from leaf explants. Biologia Plantarum 47: 13-19.

Wernicke, W. and Brettell, R. (1980). Somatic embryogenesis from *Sorghum bicolor* leaves. Nature 287: 138-139.

Wondyifraw, T. and Surawit, W. (2004). Micropropagation of Krawan (*Amomum krervanh* Pierre ex Gagnep). Science Asia 30: 9-150.

Zhong, H., Wang, W. and Sticklen, M. (1998). *In vitro* regeneration of *Sorghum bicolor* (L.) Moench: efficient plant regeneration from shoot apices. Journal of Plant Physiology 153: 719-726.

(Received 29 October 2005; accepted 12 December 2005)