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# Development of an In Vitro Regeneration System in Sorghum [*Sorghum bicolor* (L.) Moench] Using Root Transverse Thin Cell Layers (tTCLs)

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**Abstract:** An efficient and reproducible plant regeneration system was developed from cells or tissues of agronomically important Indian sorghum genotypes including 2 commercial cultivars (NSH27 & K8) of *Sorghum bicolor* (L.) Moench. Callus induction and plant regeneration were achieved on transverse thin cell layers (tTCL) of roots from aseptically germinated 7-day-old seedlings. Callus response depended on the genotype, the concentrations and composition of growth substances and number of in vitro regeneration cycles undergone by the donor plant. Murashige and Skoog (MS) medium supplemented with 4.5-18.1  $\mu\text{M}$  2,4-dichlorophenoxy acetic acid (2,4-D), 5.4-21.5  $\mu\text{M}$  naphthalene acetic acid (NAA), 5.7-22.8  $\mu\text{M}$  indole acetic acid (IAA) and 4.9-19.7  $\mu\text{M}$  indole butyric acid (IBA), and combined with 10% (v/v) coconut water (CW) was used for callus induction. The calli were cultured on MS medium supplemented with 2.2-17.8  $\mu\text{M}$  6-benzylaminopurine (BAP) combined with 2.3  $\mu\text{M}$  2,4-D or 2.7  $\mu\text{M}$  NAA. Highly efficient differentiations of multiple shoot buds were initiated within 4 weeks of culture. Root induction was achieved on half-strength MS medium containing IAA (2.9-28.5  $\mu\text{M}$ ). Rooted plants were successfully acclimatised, with the survival rate reaching almost 80%. These plants grew normally without showing any morphological variation.

**Key Words:** Callus induction, coconut water, hardened plant, MS medium, plant growth regulators, regeneration, rooting

## Introduction

*Sorghum* Moench is an important cereal crop occupying a major place in both food grain and forage production. The crop is well adapted to tropical and subtropical areas throughout the world. In addition to its principle uses as flour, in the preparation of porridge and unleavened bread, *Sorghum* species are sources of fibre, fuel and secondary products and are also used in the alcohol industry (sweet *Sorghum*) as they contain high amounts of starch. In the developing world, improving sorghum through biotechnology is the latest in a long series of technologies that have been applied to this crop (Maqbool et al., 2001). However, transverse thin cell layer (tTCL) is a model system which has applications in higher plant tissue and organ culture, and genetic transformation. Since the regeneration of specific organs may be effectively manipulated through the use of tTCLs, in conjunction with specifically controlled in vitro conditions and exogenously applied plant growth regulators (PGRs), many problems hindering the improvement of in vitro plant systems are

potentially removed (Jaime & Teixeira da Silva, 2003). TCL technology has been effectively used in cereals and grasses, including *Digitaria sanguinalis* (L.) Scop., *Oryza sativa* L. and *Zea mays* L. The transverse tTCL technology has been successful in many plant species (Pelissier, 1990; Ohki, 1994; Stefaniak, 1994; Hosokawa et al., 1996; Bui et al., 1998a; Baskaran and Jayabalan, 2005). Plant regeneration in sorghum has been described using various explants (Gamborg et al., 1977; Thomas et al., 1977; Ma et al., 1987; Zhong et al., 1988; Hagio, 2002; Mishra and Khurana, 2003). Cereal tissue cultures produce different types of calli (i.e. masses of undifferentiated cells), which may differ in their regenerative potentials. Most cereals seem to produce callus tissue with and without green spots, and a positive correlation between the presence of such spots in a callus and its regenerative potential has been observed (Ogura & Shimada, 1978; Shimada & Yamada, 1979; Inoue & Maeda, 1980; Nabors et al., 1982). Successful culturing of callus has been reported for corn (Mascarenhas et al., 1965), oats (Carter et al.,

1967), rice (Yatazwa et al., 1967) and wheat (Troine et al., 1968). In sorghum, immature inflorescence was used as potential explants for regeneration (Elkonin et al., 1996; Raghavendra Rao et al., 2000). Since an efficient and reproducible regeneration protocol is required before any genetic transformation study, in the present communication, the regeneration abilities of *Sorghum bicolor* cvs. NSH27 and K8 were examined. The purpose of the investigation reported here is to provide a simple, reproducible and efficient in vitro culture system for sorghum (*Sorghum bicolor*).

## Materials and Methods

### Plant material

Seeds of *Sorghum bicolor* cvs. NSH27 and K8 were obtained from Tamil Nadu Seed Germination Testing Laboratory, Tiruchirappalli, India. The seeds were kept under running tap water for 1 h before being 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 with further 3 to 5 rinsings in sterile distilled water. The seeds were then surface sterilised with 0.2% (w/v) aqueous mercuric chloride solution for 10 min and finally rinsed with sterile distilled water (5 to 7 changes). The seeds were then germinated on autoclaved MS basal medium or in moistened cotton. The root segments derived from 7-day-old aseptic seedlings were used as explants. The root segments (0.3-0.5 mm) were dissected transversely in aseptic conditions.

### Culture conditions

Murashige and Skoog (1962) medium (MS) supplemented with 3% (w/v) sucrose (Himedia, India) was used in all the experiments. The pH of the medium (supplemented with respective growth regulators) was adjusted to 5.8 with 1 N NaOH or 1 N HCl prior to the addition of 0.8% (w/v) agar. In all the experiments, the chemicals used were of analytical grade (Himedia, Qualigens, Merck, Loba Chemie, Fischer and Sigma). The medium was dispensed into culture vessels (Borosil, India) and plugged tightly with non-absorbent cotton and autoclaved at 105 kPa (121 °C) for 15 min. All the cultures were maintained at 25 ± 2 °C under a 16-h photoperiod of 45-50 mmol m<sup>-2</sup>s<sup>-1</sup> irradiance provided by cool white fluorescent tubes (Philips, India) and with 55%-60% relative humidity (RH). All subsequent subcultures were performed at 4-week intervals.

### Callus induction medium

Transverse TCL segments (0.3-0.5 mm) of roots were cultured on MS medium supplemented with 10% (v/v) coconut water (CW), and with different concentrations and combinations of PGRs, including 4.5-18.1 µM 2,4-D, 5.4-21.5 µM NAA, 5.7-22.8 µM IAA and 4.9-19.7 µM IBA.

### Plant regeneration medium

White friable calli (30-days-old) were cultured on MS medium supplemented with 5% coconut water (CW) and different concentrations and combinations of PGRs, including 2.2-17.8 µM BAP and the addition of 2.3 µM 2,4-D.

### Rooting medium

Elongated shoots were excised from each culture passage and transferred to half-strength MS medium (1/2 MS) supplemented with different concentrations of IAA (2.9-28.5 µM).

### Acclimatisation and transfer of plantlets to soil

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

### Statistical analysis

Experiments were set up in a randomised block design (RBD), with 3 replications. Ten to fifteen explants were used per treatment in each replication. Observations were recorded on the percentage of response of callus formation, percentage of response of shoots, number of shoots per callus, shoot length, percentage of response of roots, roots per shoot and root length. The treatment means were compared using Duncan's multiple range test (DMRT) at a 5% probability level according to Gomez and Gomez (1976).

**Results and Discussion**

**Callus Induction**

Cv. NSH27 was more responsive to media for callus induction and shoot regeneration compared with K8 (Figure 1a & b). Callus initiation was observed in NSH27 within 10 days, but in K8 after 15 days of culture. Optimum callus production was observed in NSH27 after 30 days of culture, whereas lower callus growth was observed in K8. Frequency of callus induction, type of callus and regeneration of plantlets were influenced by the genotypes. A similar phenomenon was reported by Patil et al. (1998). The callus cultured for plant regeneration medium after the first subculture was very effective in obtaining high regeneration potential. The second and third subculture cycles resulted in the

browning of calli. Initially, yellowish compact callus was formed directly at tTCL of roots containing MS medium fortified with auxins, but this callus later turned whitish compact after 30 days of culture in both cultivars. However, most of the cultivars formed yellowish compact callus as described by Hagio (1994). Among the 4 auxins, only 2,4-D was effective for callus growth in both cultivars. Similar results were also observed by Arti et al. (1994), Nguyen et al. (1998), Saradamani et al. (2003) and Baskaran and Jayabalan (2005). Callus growth of *S. bicolor* was highly influenced by concentrations of growth regulators and coconut water (CW) added to the culture medium. Addition of CW [10% (v/v)] to MS medium fortified with 2,4-D increased callus growth in cv. NSH27 (Figure 5A). A similar result was observed in sugarcane (Mamun et al., 2004). Among the 4 auxins, 2,4-D (9.0

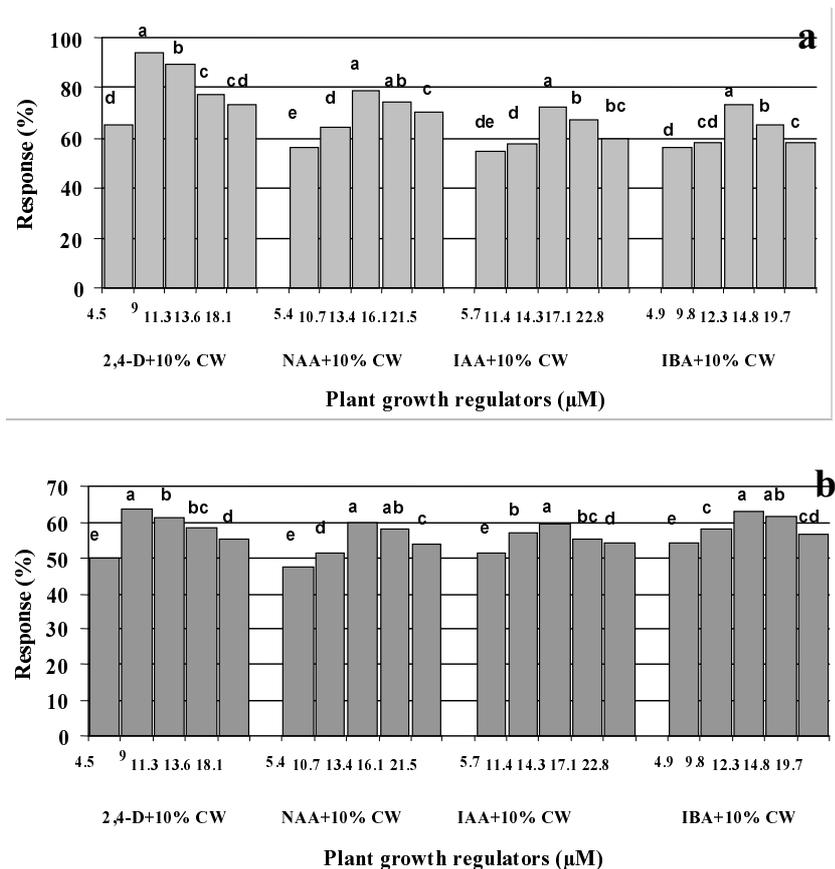


Figure 1. Effect of different concentrations of auxins with 10% CW on the mean percentage of callus production (a – cv NSH27 and b – K8). The bars bearing a mean followed by different letters on top are significantly different from each other (P < 0.05); comparison by DMRT.

Data recorded after 30 days of culture.

µM) with 10% CW proved to be better for callus growth than the other PGRs. A similar result has been reported for the tTCL hypocotyl explant in *S. bicolor* (Baskaran and Jayabalan, 2005). On the other hand, callus can also be grown on medium without CW but at a much slower rate in both cultivars (data not shown) as in line with Vance and David (1970). The ranges of 4.5-11.3 µM 2,4-D, 10.7-13.4 µM NAA, 11.4-14.3 µM IAA and 9.8-12.3 µM IBA with 10% CW were found to be optimal for obtaining white friable callus after 30 days of culture (Figure 1a & b). However, the medium containing 16.1-21.5 µM NAA or 17.1-22.8 µM IAA or 14.8-19.7 µM IBA and 10% CW produced roots on surface of the white compact callus.

This occurred vigorously in cv. K8 after 45 days of culture.

### Shoot Regeneration

Experiments were designed to improve plant regeneration from callus derived from roots (tTCL) in *Sorghum bicolor* (cultivar NSH27 and K8). Induction of organogenic callus and shoot regeneration occurred on MS medium supplemented with 5% CW and different concentrations of BAP (2.2-17.8 µM), and combined with 2,4-D (2.3 µM) or NAA (2.7 µM). tTCL root callus produced shoot regeneration in NSH27 (Figure 2). Teixeira da Silva (2003) and Teixeira da Silva and Fukai

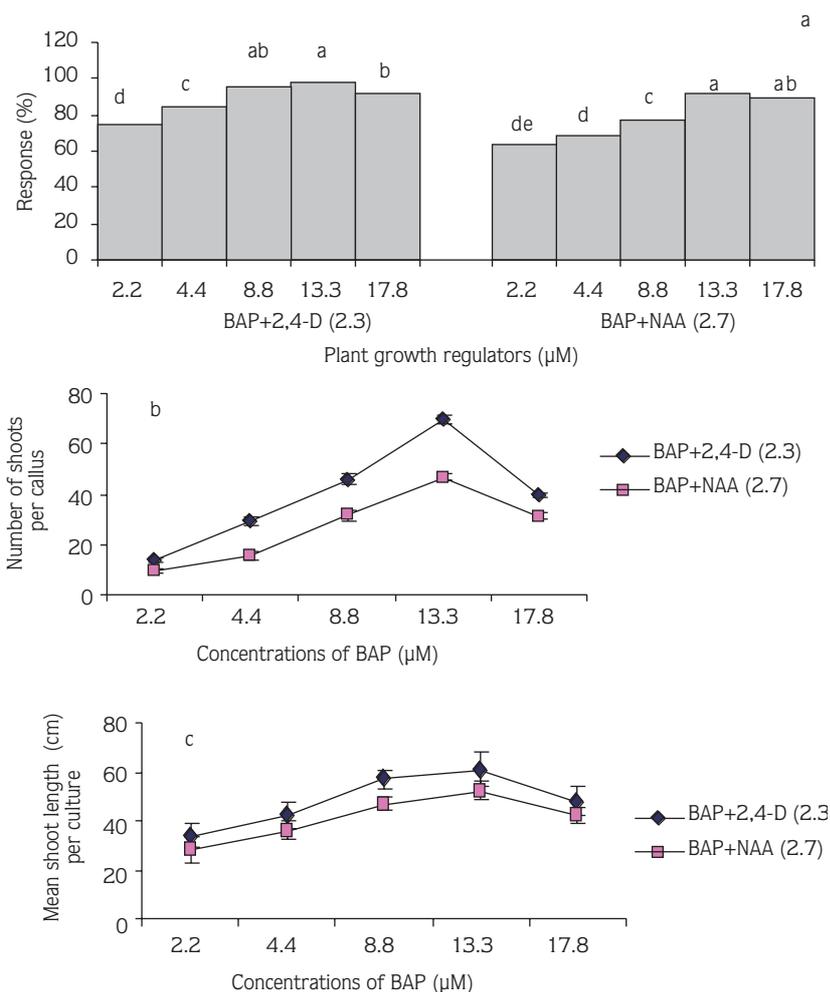


Figure 2. Effect of different concentrations of BAP with 5% CW and addition of auxins on the mean percentage of shoots response (a), the mean shoot number (b), the mean shoot length (c). The bars bearing a mean followed by different letters on top are significantly different from each other (P < 0.05); comparison by DMRT. Error bars represent the standard error.

Data recorded after 8 weeks culture of *S. bicolor* (cv. NSH27).

(2003) reported that tTCLs were effective in shoot regeneration and morphogenesis in *Chrysanthemum* L. However, higher concentrations of 2,4-D (above 2.3  $\mu\text{M}$ ) and NAA (above 2.7  $\mu\text{M}$ ) in the regeneration medium resulted in decreased frequencies of shoot regeneration (data not shown) in both cultivars. These results were in agreement with Bhaskaran et al. (1992) and Murray et al. (1983). Higher levels of 2,4-D (above 13.6  $\mu\text{M}$ ) in callus production medium slowed down the subsequent plant formation on regeneration medium. A similar phenomenon was observed by Murray et al. (1983). The synergistic effect of MS medium containing BAP at 4.4-8.8 and 17.8  $\mu\text{M}$ , 2,4-D (2.3  $\mu\text{M}$ ) and 5% CW was found

to be optimum for shoot initiation (Figure 5B). The maximum number of shoots was observed in NSH27 after 4 weeks (Figure 2b; Figure 5C). In both cultivars, the maximum number of shoots was obtained in MS medium containing BAP (13.3  $\mu\text{M}$ ), 2,4-D (2.3  $\mu\text{M}$ ) and 5% CW but shoot lengths varied after 8 weeks (Figure 2c; Figure 5D). Higher concentrations of BAP (above 13.3  $\mu\text{M}$ ) reduced the percentage of response, number of shoots and shoot lengths. Culturing the calli on the medium supplemented with BAP (2.2-17.8  $\mu\text{M}$ ), NAA (2.7  $\mu\text{M}$ ) and 5% CW resulted in shoot induction in both cultivars. A marked response as well as shoots regeneration was obtained in medium containing BAP

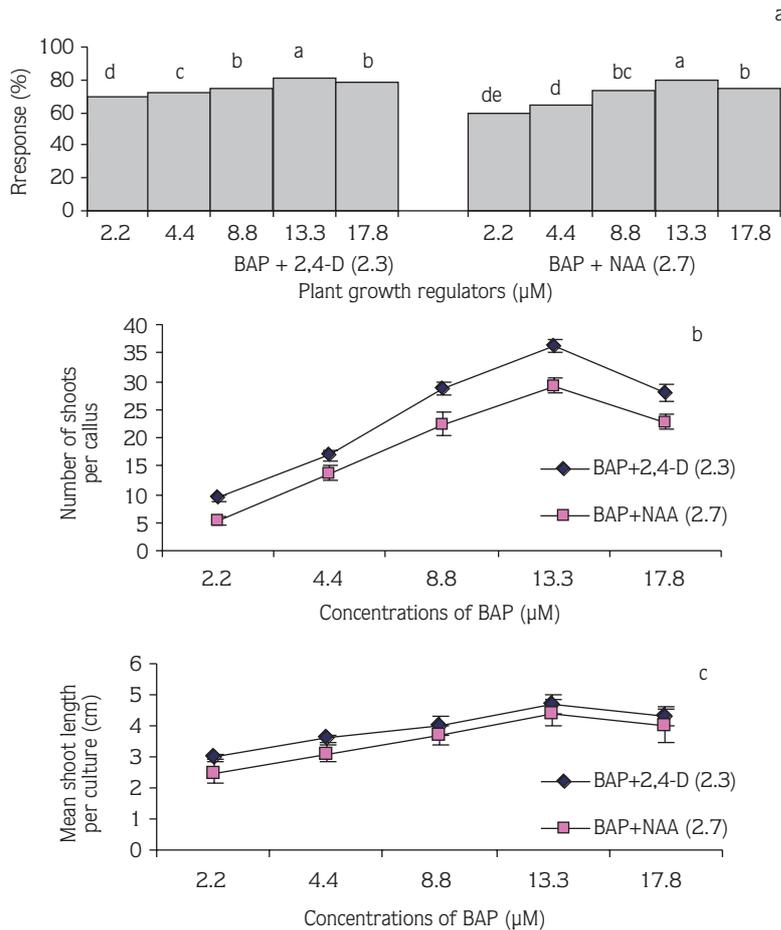


Figure 3. Effect of different concentrations of BAP with 5% CW and addition of auxins on the mean percentage of shoots response (a), the mean shoot number (b), the mean shoot length (c). The bars bearing a mean followed by different letters on top are significantly different from each other ( $P < 0.05$ ); comparison by DMRT. Error bars represent the standard error.

Data recorded after 8 weeks culture of *S. bicolor* (K8).

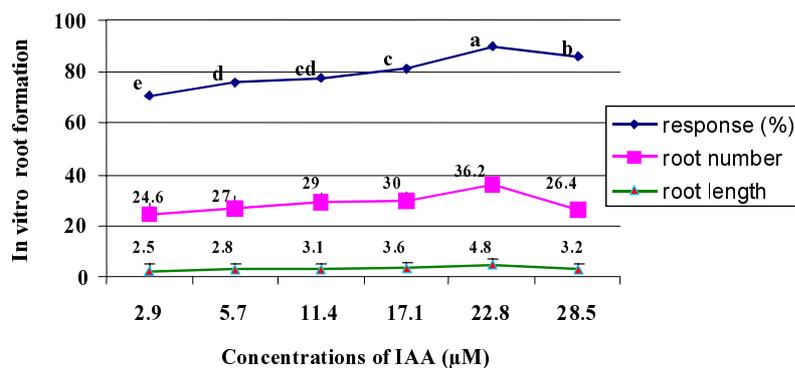


Figure 4. Effect of half-strength MS medium with different concentrations of IAA on the Mean percentage of roots response (●), the mean root number (■), the mean root length (cm) (▲). The line bearing mean followed by different letters in their top are significantly different from each other ( $P < 0.05$ ); comparison by DMRT. Error bars represent the standard error.

Data recorded after 30 days culture of *S. bicolor* (NSH27).

(13.3  $\mu\text{M}$ ), NAA (2.7  $\mu\text{M}$ ) and 5% CW in NSH27 but with variable shoot lengths (Figure 2b & c). Abubachker and Murugesan (1999) have reported the concentration of BAP (6.6  $\mu\text{M}$ ) and NAA (2.7  $\mu\text{M}$ ) to be the most congenial for shoot regeneration in young stem explants of *S. bicolor*. However, Nirwan and Kothari (2004) have reported BAP (8.8  $\mu\text{M}$ ) and IAA (2.9  $\mu\text{M}$ ) to be the effective for shoot bud regeneration in apical meristem explants in *S. bicolor*. On the other hand, shoot regeneration occurred on MS medium containing kinetin (9.2  $\mu\text{M}$ ) and IAA (2.85  $\mu\text{M}$ ) in mature embryo callus (Nirwan and Kothari, 2003). In this study, we observed the culture response as well as shoot number and shoot length subordinate in K8 (Figure 3a, b & c).

#### Rooting of Shoots

Excised shoots from both cultivars of *Sorghum bicolor* were rooted on half-strength MS medium with IAA (2.9-28.5  $\mu\text{M}$ ). No significant differences were observed between the cultivars with respect to rooting (data not shown). 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). Half-strength MS medium supplemented with all concentrations of IAA induced roots from shoots within 15 days of culture. Among the IAA concentrations, the percentage of response, number of roots and root length varied (Figure 4). Half-strength MS medium supplemented with IAA (22.8  $\mu\text{M}$ ) was the

most effective for root induction (Figure 4; Figure 5E). Sarada Mani et al. (2003) reported that MS medium supplemented with IAA produced roots. However, Nirwan and Kothari (2004) reported half-strength MS medium containing 10.7  $\mu\text{M}$  NAA and 2% (w/v) sucrose to be the most congenial for root induction.

#### Acclimatisation and Field Establishment

Rooted plantlets were successfully acclimatised without the need for a growth chamber facility. One hundred percent of the plantlet survival was seen after hardening on garden soil, farmyard soil and sand (2:1:1) for 3 weeks. Hardened plantlets were successfully transferred to a botanical evaluation garden and kept under shade in a net house for further growth and development after 3 weeks (Figure 5F). However, the survival rate decreased from 100% to 80% after 10 weeks of acclimatisation. There was no variation among the acclimatised plants comparable to in vivo plants with respect to morphological, growth characters and yield. All the in vitro derived plantlets were free from external defects.

#### Conclusion

The purpose of this study was to develop an in vitro propagation method on tTCL technology of root segments in *S. bicolor*, an economically important crop plant. Various plant growth regulators and coconut water

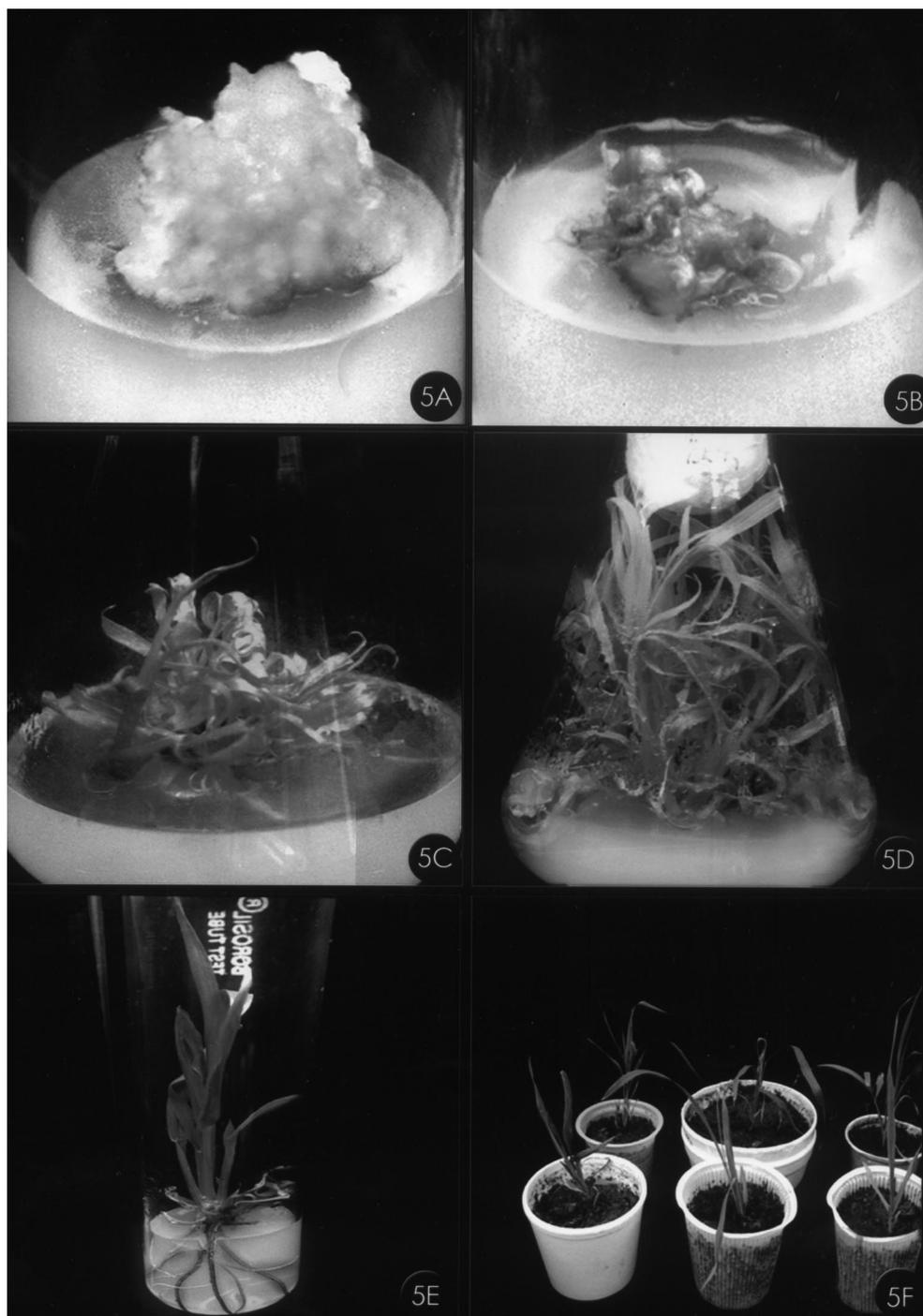


Figure 5. A-F. Organogenesis from roots (tTCL) of *Sorghum bicolor* (cv. NSH27).

- (A) Induction of white friable callus from tTCL (root).
- (B) Shoot bud initiation on MS medium with  $13.3 \mu\text{M}$  BAP and  $2.3 \mu\text{M}$  2,4-D.
- (C) Regenerated shoots after 30 days of culture.
- (D) Elongated multiple shoots after 50 days of culture.
- (E) Rooted plantlet of *S. bicolor*.
- (F) Acclimatized plantlets of *S. bicolor*.

were tested for a rapid and reproducible method of shoot proliferation using tTCL from root segments, which was followed by successive establishment of regenerated plants in soil. The protocol reported here could be used for large-scale propagation of this valuable crop plant. The tTCL in vitro system described here provides an efficient regeneration protocol for *Sorghum bicolor*.

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