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A simple approach to improve plant regeneration from callus culture of *Sorghum bicolor* for crop improvement

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This study was conducted to establish and optimise a regeneration system for agronomically important Indian sorghum genotypes including two commercial cultivars (NSH27 and K8) of *Sorghum bicolor*. Callus induction and plant regeneration were achieved on transverse thin cell layers (tTCL) of hypocotyls from aseptically germinated seedlings of seven-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 µM 2, 4-Dichlorophenoxy acetic acid (2,4-D), 5.4-21.5 µM Naphthalene acetic acid (NAA), 5.7-22.8 µM Indole acetic acid (IAA) and 4.9-19.7 µM Indole butyric acid (IBA) and combined with 10% (v/v) coconut water (CW) for callus induction. The calli were cultured on MS medium supplemented with 2.2-17.8 µM 6-Benzyl aminopurine (BAP) combined with 5% (v/v) CW and addition of 2.3 µM 2, 4-Dichlorophenoxy acetic acid (2, 4-D) or 2.7 µM Naphthalene acetic acid (NAA). Highly efficient differentiations of multiple shoot buds were initiated within four weeks after inoculation. Root induction was achieved on half strength MS medium containing IAA (2.9-28.5 µM). Rooted plants were successfully acclimatized and with the survival rate reaching almost 80%. These plants grew normally without showing any morphological variation.

Key words: callus induction, coconut water, coleoptile, hardened plant, ms medium, plant growth regulators, regeneration and rooting

Abbreviations: BAP – 6-benzyl amino purine, 2, 4-D – 2, 4-Dichlorophenoxy acetic acid, CW – coconut water, IAA – indole-3-acetic acid, IBA – indole-3-butyric acid, Kin – Kinetin, NAA - α-naphthalene acetic acid.

Introduction

Sorghum is a very important cereal crop 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

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under cultivation. In addition to its principle used as flour, preparation of porridge and unleavened bread, *Sorghum* species are sources of fibre fuel and secondary products and also used in alcohol industry (sweet *Sorghum*) as it contains high amount of starch. Thin Cell Layer (TCL) is a model system and applications in higher plant tissue, organ culture and genetic transformation. Moreover TCL technology is a solution for ornamental and floricultural crop improvement, since it addresses the issue of plant breeding at the first stage of the problem: regeneration. Since the regeneration of specific organs may be effectively manipulated through the use of TCLs, in conjunction with specific 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 and Teixeira da Silva, 2003).

TCLs have been used as a model system to analysis of biochemical and molecular markers of differentiation (Biondi *et al*., 2001). TCL technology has been effectively used in cereals and grasses, including improvement of *Digitaria sanguinalis*, *Oryza sativa* and *Zea mays* (Nhut *et al*., 2003c). The tTCL technology has been a suitable tool for developing many plant species (Pelissier, 1990; Ohki, 1994; Stefaniak, 1994; Hosokawa *et al*., 1996 and Bui *et al*., 1998a).

Generally in sorghum, plant regeneration has been described using various explants (Zhong *et al*., 1988; Thomas *et al*., 1977; Gamborg *et al*., 1977; Ma *et al*., 1987). The greatest advances in increasing regeneration frequency have come from the realization that cereal tissue cultures produce different types of calli (i.e., masses of undifferentiated cells), which may differ in their regenerative potentials. First, 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 and Shimada, 1978; Shimada and Yamada, 1979; Inoue and Maeda, 1980; Nabors *et al*., 1982). Successful culturing of callus has been reported for the following monocotyledonous species: corn (Mascarenhas *et al*., 1965), oats (Carter *et al*., 1967), rice root (Yatazwa *et al*., 1967) and wheat (Troine *et al*., 1968). In sorghum, immature inflorescences were used as potential explants for regeneration (Elkonin *et al*., 1996 and Raghavendra Rao *et al*., 2000). Reports on plant regeneration via callus of *Sorghum bicolor* are limited. Since the efficient and reproducible regeneration protocols are required before somaclonal variation is carried out and any genetic transformation studies, in the present communication, regeneration abilities of *Sorghum bicolor* var. NSH27 and K8 have been examined.

The purpose of the investigation reported here is to provide a simple, reproducible and efficient *in vitro* culture system for sorghum (*Sorghum*

bicolor). Our aim is to standardize protocols for hypocotyl by the tTCL technology for high frequency regeneration, which is useful for somaclonal variation and genetic engineering experiments.

Materials and methods

Plant material

Seeds of *Sorghum bicolor* var. NSH27 and K8 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 three minutes followed by rinsing with distilled water and 70% (v/v) ethanol for one minute 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 ten minutes 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 use of hypocotyl segments of *Sorghum bicolor* enabled us to isolate several hundred homogenous plants at the same time. Seven days old immature hypocotyl segments, 0.4-0.6 mm tTCLs were selected from the *in vitro* raised plants for callus induction.

Culture conditions

In vitro raised hypocotyl segments of *S. bicolor* were cultured on Murashige and Skoog (1962) basal medium (MS) supplemented with 3% (w/v) sucrose (Himedia, India) and 0.8% (w/v) agar for culture initiation. The pH of the medium (supplemented with respective growth regulators) was adjusted to 5.8 with 1 N NaOH or 1 N HCl. 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 autoclaved at 105 kPa (121ºC) for 15 minutes. The tTCL hypocotyls were implanted 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 performed at four week intervals.

Callus induction medium

Hypocotyl (tTCL) segments were cultured on MS medium supplemented with 10% (v/v) coconut water (CW) and different combinations and concentrations of plant growth regulators, 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 were cultured on MS medium supplemented with 5% coconut water (CW) and different combinations and concentrations of plant growth regulators, including 2.2-17.8 µM BAP and addition of 2.3 µM 2,4-D.

Rooting medium

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

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 and sand (2:1:1). All were irrigated with 1/8 MS basal salt solution devoid of sucrose and inositol every 4 days for two 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 and 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 Randomized Block Design (RBD) and each experiment usually had 10 replicates and was repeated three times. 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 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 and discussion

Effect of auxins with coconut water on callus induction

Callus induction were tried using tTCL hypocotyl explants of *S. bicolor* (var. NSH27 and K8) on MS medium containing various concentrations of auxins (2, 4-D, NAA, IAA and IBA) with 10% CW. Pelissier (1990) observed different hypocotyl *Helianthus annuus* tTCL for their callogenic capacities with respect to NAA, BA and coconut water. Gill *et al*. (1992) showed that in tTCL hypocotyls, callus and somatic embryo could form in *Geranium*. *S. bicolor* var. NSH27 and K8 were screened from callus production and regeneration. Variety NSH27 was highly effective for callus induction and regeneration potential compared with K8. Callus initiation was observed in NSH27 within ten days but in K8 after only fifteen days of culture. Optimum callus production was observed in NSH27 after 30 days of culture whereas there was low callus growth in K8. Frequency of callus induction, type of callus and regeneration of plantlets were influenced on genotypes of sorghum. A similar result was reported by Patil *et al*. (1998). The first subculture cycle had been very fast in the production of callus and it had high regeneration potential. The second and third subculture cycle of the callus has been brown in colour. The colour of the callus was pale yellow initially, and also white colour callus was observed after 30 days of inoculation in all auxins and both varieties. Hagio (1994) reported that most of the varieties formed compact yellowish calli. In auxins, 2, 4-D with 10% CW was found to be more callus production in NSH27 but limited in K8. Mamun *et al*., (2004) reported that 2, 4-D and 10% coconut milk produced maximum amount of regenerative callus from leaf sheath in sugarcane. Callus can also be grown on medium without coconut water but at a much slower rate. Similar results were observed by Masteller and Holden (1970) in sorghum.

The high frequency of callus was observed in NSH27 (Fig. 1a). In the second and third subcultures of the callus on MS medium with low concentrations of NAA (5.4-10.7 µM), IAA (5.7-11.4 µM) and IBA (4.9-9.8 µM) with 10% CW, roots were formed as small white bumps on the surface of the cultures in both varieties. Similar results were also observed by Masteller and Holden (1970). In both varieties, callus tissues that were subcultured on MS medium and supplemented with higher concentrations of 2, 4-D (above 13.6 µM) and 10% CW produced some blackened tissue than NAA, IAA and IBA.

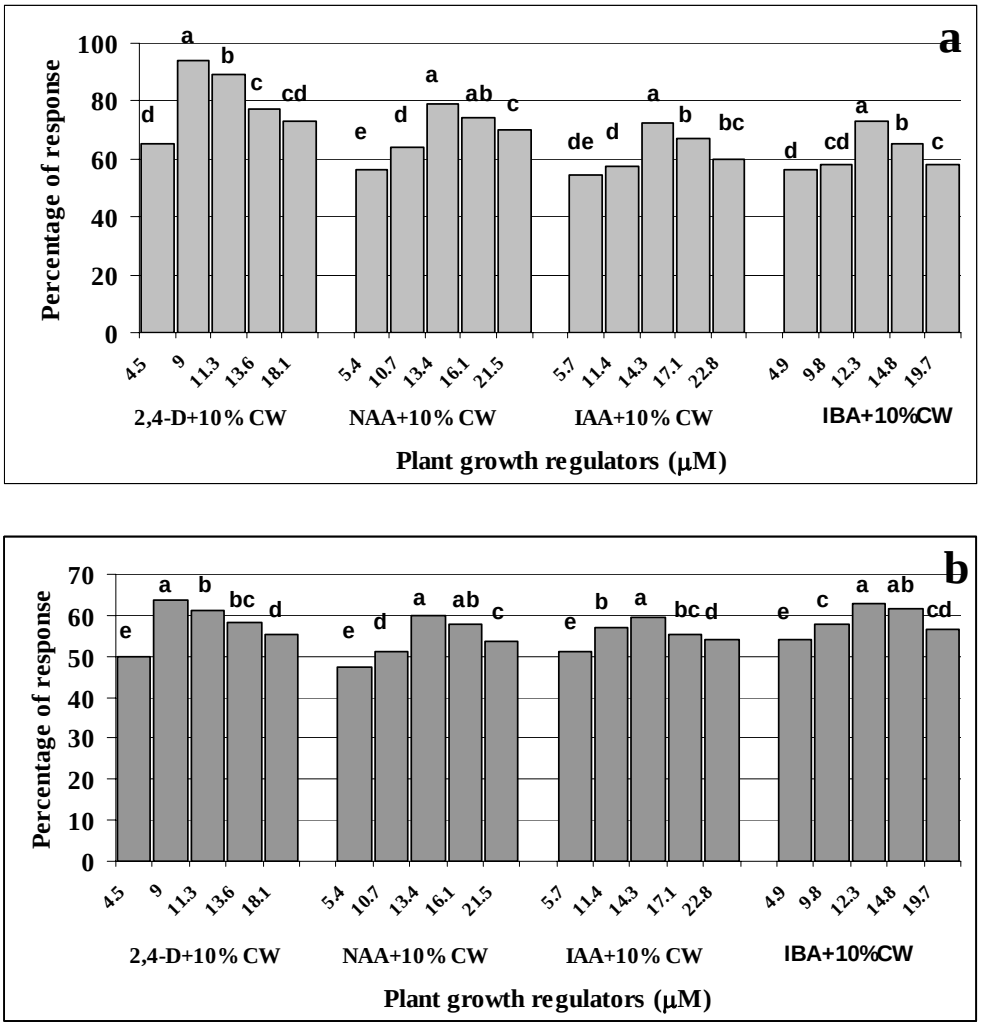

Fig. 1. Effect of different concentrations of auxins with 10% CW on the mean percentage of callus production (a-cultivar NSH27 and b-K8). The bars bearing mean followed by different letters in their top are significantly different from each other ($p<0.05$); comparison by DMRT. Data recorded after 30 days of culture.

The range 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 white friable callus after 30 days of culture (Figs 1a,b). Masteller and Holden (1970) observed, optimum callus was found in 2, 4-D with coconut milk. Abubachker and Murugesan (1999) reported that highest compact callus was induced in

stem explants of sweet sorghum. Among the various concentrations of auxins, the highest percentage of white friable callus was induced in the MS medium containing 9 µM 2, 4-D with 10% CW (Fig. 5A). Murray *et al*., (1983) reported that higher levels of 2, 4-D or 2, 4, 5-T increased callus production. Arti *et al*., (1994), Nguyen *et al*., (1998) and Saradamani *et al*., (2003) reported that callus induction was found be best in 2, 4-D. White compact callus with root formation on the surface of the cultures were observed on MS medium containing 16.1-21.5 µM NAA, 17.1-22.8 µM IAA and 14.8-19.7 µM IBA with 10% CW in both varieties but the vigorous roots production was noted in variety K8 after 45 days of culture.

Effect of cytokinin, auxins and coconut water on shoot regeneration

These experiments were designed to improve plant regeneration from callus of hypocotyl (tTCL) in *Sorghum bicolor* cultivar NSH27 and K8. However, manipulations of the culture medium components (particularly type, combination and concentration of hormones) and use of these cultivars have resulted in increasing and extending the regeneration of plants. This result was supported by Conger, (1981). Induction of organogenic callus and regeneration occurred on MS medium fortified 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). In our study, tTCL hypocotyl callus had effective shoot regeneration in NSH27. Teixeira da Silva (2003) and Teixeira da Silva and Fukai (2003) reported that tTCLs had effective for shoot regeneration and morphogenesis in *Chrysanthemum*. Alagumanian *et al*. (2004) reported that 10% CW with various concentrations of BAP was effective for callus regeneration in *Solanum*. In this investigation, quick and faster regeneration occurred in cultivar NSH27 than K8. In the present study, higher concentrations of 2, 4-D (above 2.3 µM) and NAA (above 2.7 µM) in the regeneration medium but decreased the frequency of shoot regeneration (data not shown) in both varieties. This work was supported by Bhaskaran *et al*. (1992) and Murray *et al*. (1983). Higher levels of 2, 4-D (above 13.6 µM) 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 µM with 5% CW and addition of 2, 4-D (2.3 µM) was found to be optimum for shoot initiation (Figs 2,3, 5B). The maximum number of shoots and very fast elongation was observed in cultivar NSH27 after four weeks (Figs 2b, 5C). The maximum number of shoots was obtained in MS medium containing BAP (13.3 µM) with

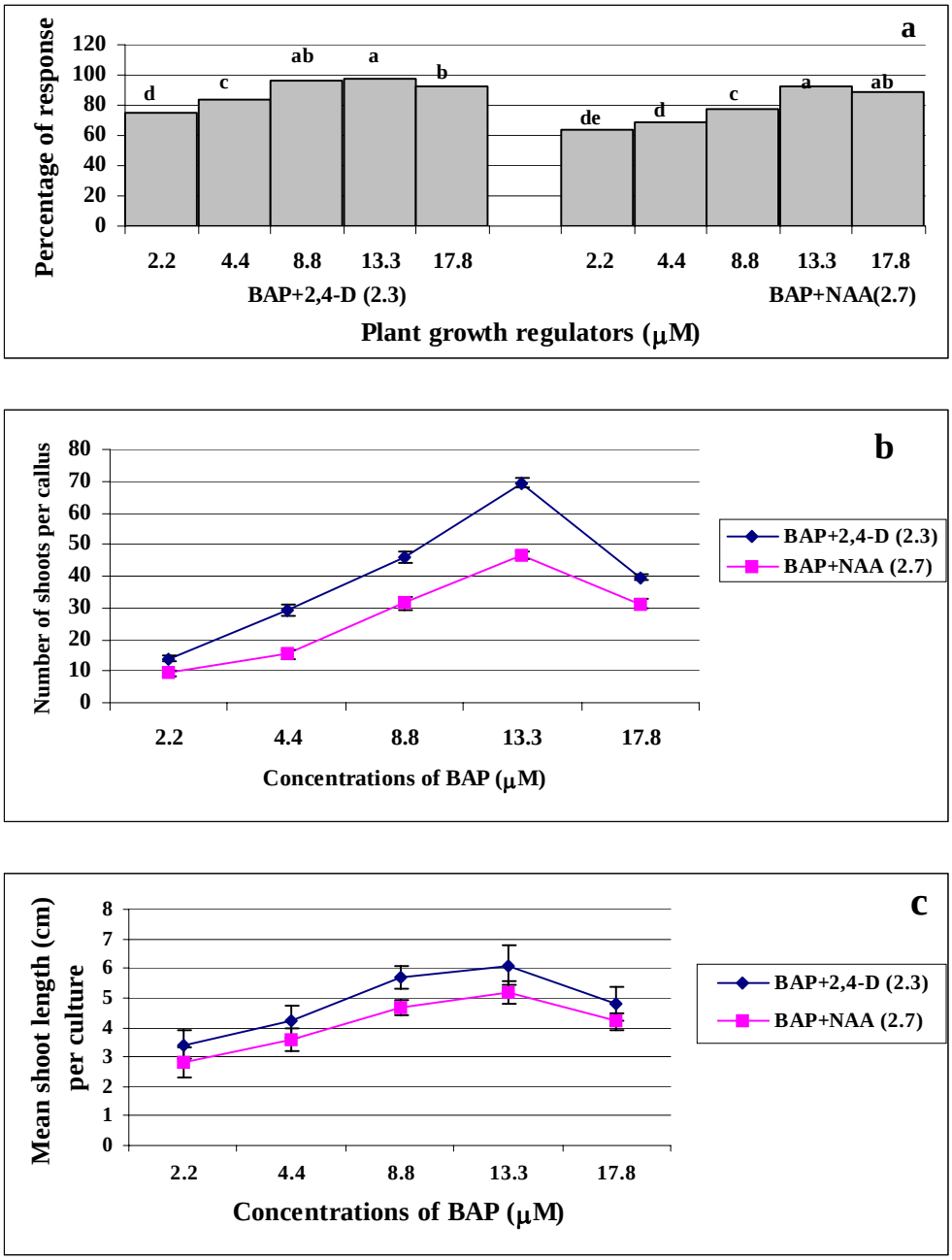

Fig. 2. Effect of different concentrations of BAP with 5% CW and addition of auxins on the mean percentage of shoots response (a), the mean shoot number (b), the mean shoot length (c). The bars bearing mean followed by different letters in their top are significantly different from each other ($p<0.05$); comparison by DMRT. Error bars represent the standard error. Data recorded after 8 weeks culture of *S. bicolor* (var. NSH27).

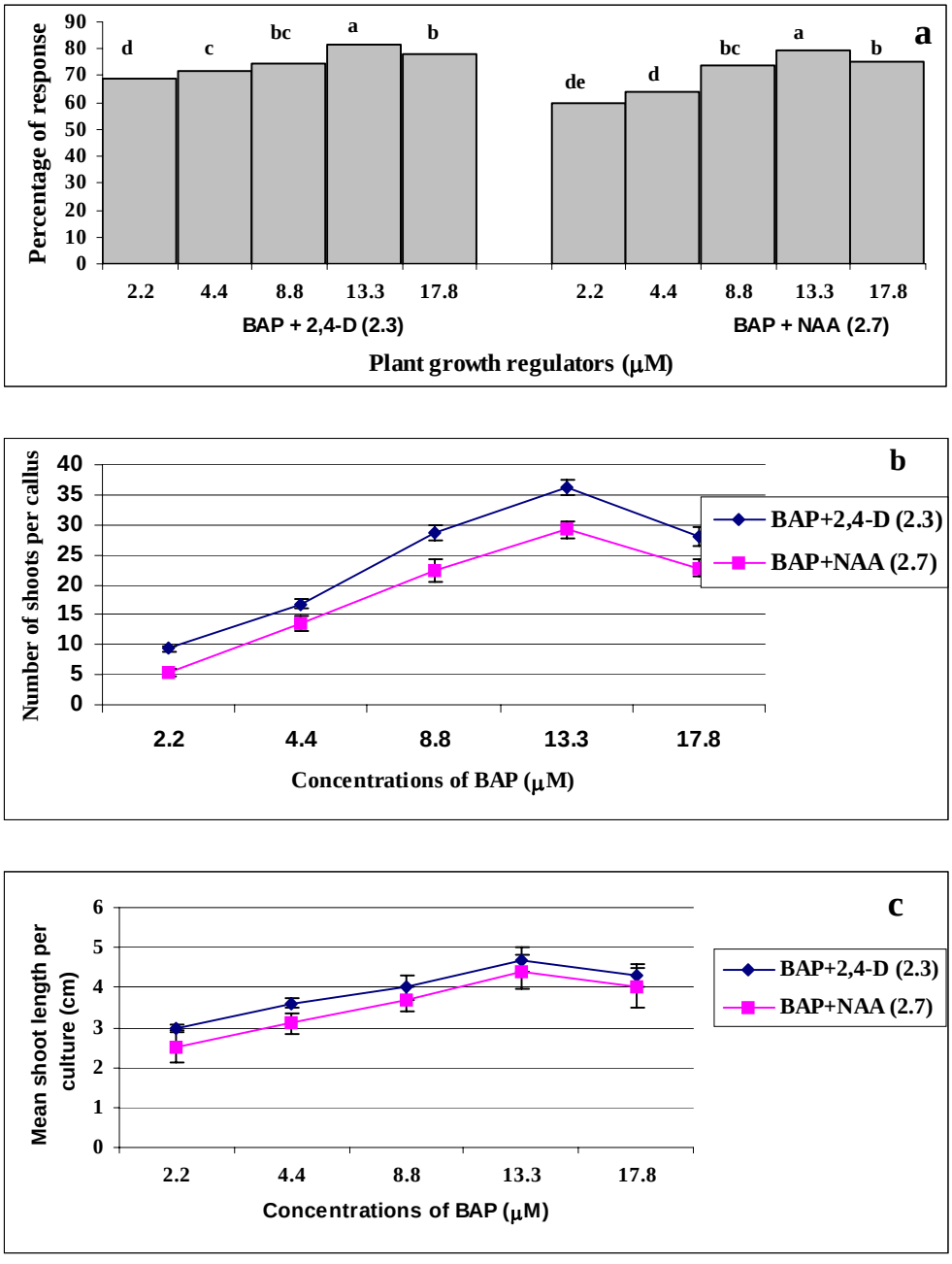

Fig. 3. Effect of different concentrations of BAP with 5% CW and addition of auxins on the mean percentage of shoots response (a), the mean shoot number (b), the mean shoot length (c). The bars bearing mean followed by different letters in their top are significantly different from each other ($p<0.05$); comparison by DMRT. Error bars represent the standard error. Data recorded after 8 weeks culture of *S. bicolor* (K8).

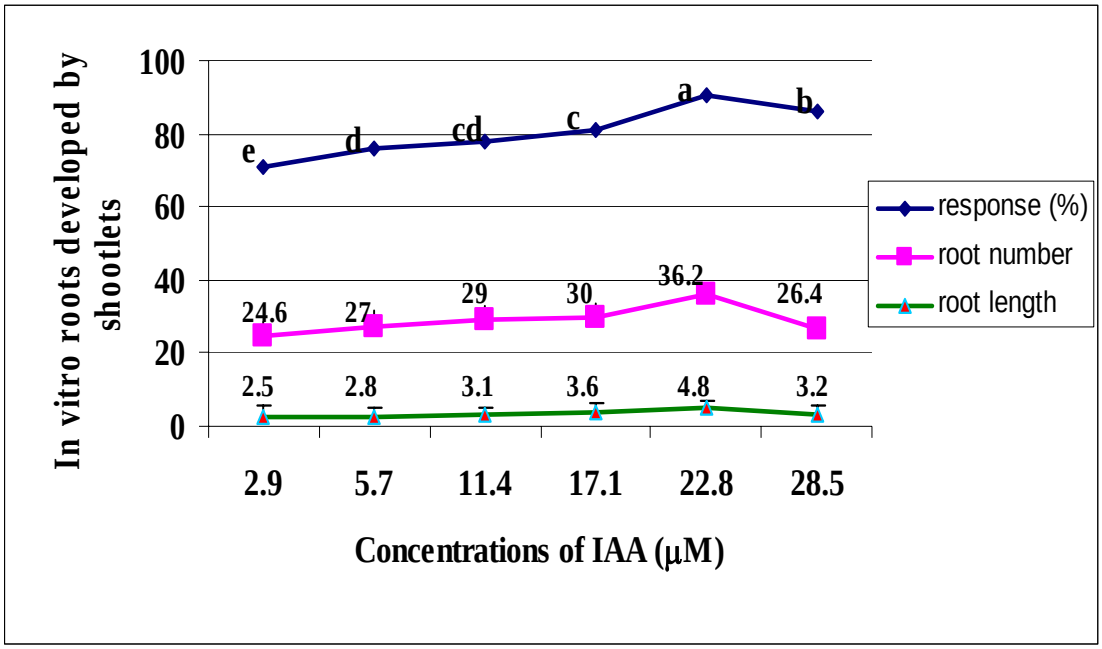

Fig. 4. Effect of half strength MS medium with different concentrations of IAA on the Mean percentage of roots response (♦), the mean root number (■), the mean root length (cm) (▲). The line bearing mean followed by different letters in their top are significantly different from each other ($p<0.05$); comparison by DMRT. Error bars represent the standard error. Data recorded after 30 days culture of *S. bicolor* (NSH27).

5% CW and addition of 2,4-D (2.3 µM) but shoot lengths varied after eight weeks (Figs 2c, 3c, 5D). Higher concentrations of BAP (above 13.3 µM) were reduced the percentage of response, number of shoots and shoot lengths. In both varieties, induction of multiple shoot buds from organogenic callus of tTCL hypocotyl explants occurred on MS medium fortified with BAP (2.2-17.8 µM), 5% CW and addition of NAA (2.7 µM). Comparing the cultivars and different concentrations of BAP with NAA, the maximum percentage of response was observed in BAP (13.3 µM) with 5% CW and addition of NAA (2.7 µM) (Figure 2a). In cultivar NSH27, the maximum number of shoots was observed in BAP (13.3 µM) with 5% CW and addition of NAA (2.7 µM) and shoot lengths varied (Figs 2b,c). Abubachker and Murugesan (1999) reported that the highest organogenesis was observed on MS medium supplemented with 1.5 mg/l BA, 0.5 mg/l NAA young stem explant-derived callus in *Sorghum bicolor*.

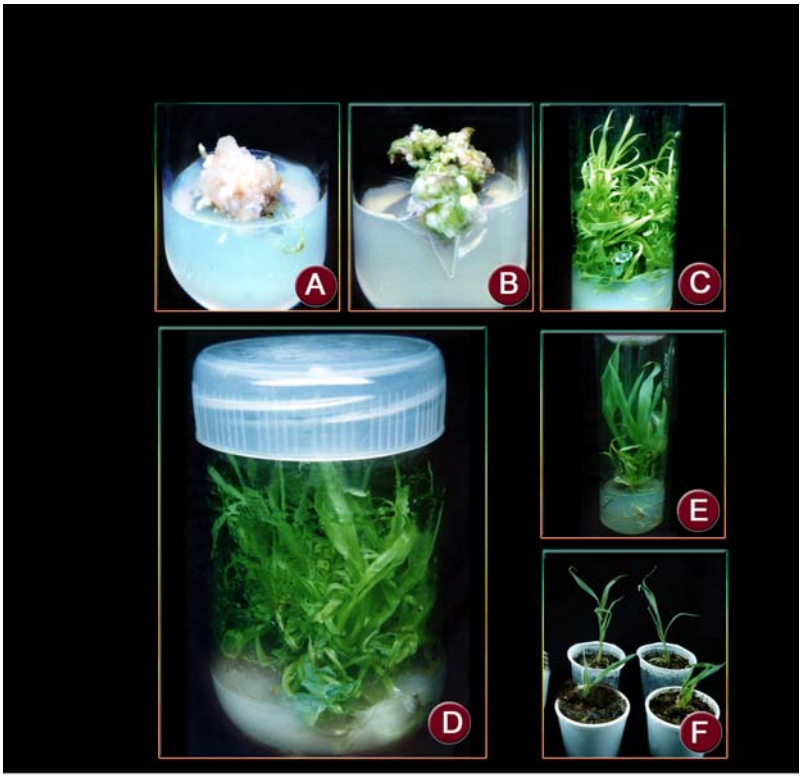

Fig. 5. Different stages in the development of shoot regeneration via callus in *S. bicolor* (Var NSH27) (A) White friable callus from tTCL hypocotyl on MS + 2, 4 - D (9.0 µM) + 10% CW. (B) Callus with regenerated shoot buds (C) Shoot regeneration after 4 weeks culture (D) Elongated multiple shoots after 8 weeks culture (E) Rooted plantlet. (F) Hardened plants in botanical garden in net house conditions.

Effect of auxins on rooting of shoots

Excised shoots from both cultivars of *Sorghum bicolor* were rooted on half strength MS medium with IAA (2.9-28.5 μM). In both the cultivars no more differences were observed in rooting of shoots (cultivar K8-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). Half strength MS medium supplemented with all concentrations of IAA induced roots from shoots within 15 days of culture. Among the IAA concentration, the percentage of response, number of roots and root length varied (Fig. 4). Half strength MS medium supplemented with IAA (22.8 μM) was most effective for root induction (Figs 4, 5E). Sarada Mani *et al*. (2003) reported that MS medium containing 1 mg/l IAA produced roots.

Hardening of regenerated plants and examination of morphological characters and yield

Plantlets were successfully acclimatized without growth chamber facility. 100% of the plantlet survival was seen after hardening on garden soil, farmyard and sand (2:1:1) for three weeks. Hardened plantlets were successfully transferred to botanical evaluation garden and kept under shade in a net house for further growth and development after three weeks (Fig. 5F). However, the survival rate decreased from 100 to 80%, respectively after ten weeks of acclimatization. There was no variation among the acclimatized 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.

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