Efficient regeneration from hypocotyl explants in three cotton cultivars

K. DIVYA, T. SWATHI ANURADHA, S.K. JAMI and P.B. KIRTI*

Department of Plant Sciences, University of Hyderabad, Hyderabad-500046, India

Abstract

A high frequency *in vitro* shoot bud differentiation and multiple shoot production protocol from hypocotyl segments of 8 to 10-d-old seedlings of cotton has been developed. Murashige and Skoog (MS) basal medium with Nitsch and Nitsch vitamins was found to be optimal in shoot regeneration. A combination of 2 mg dm^{-3} thidiazuron and 0.05 mg dm^{-3} naphthaleneacetic acid was the most effective for shoot regeneration (76 %) and an average of 10.6 shoots per responding explant. Combination of the cytokinins benzylaminopurine and kinetin induced better regeneration response than their individual treatments. Supplementation of the culture medium with ethylene inhibitor silver nitrate and activated charcoal showed beneficial effects. Optimal rooting was obtained on half-strength MS medium supplemented with 1 mg dm^{-3} indolebutyric acid and activated charcoal. Scanning electron micrographs of *in vitro* cultured explants revealed that shoot primordia were formed *de novo*.

Additional key words: benzylaminopurine, ethylene inhibitor, hyperhydricity, multiple shoot regeneration, thidiazuron.

Introduction

Cotton is a high value commercial crop and its importance needs no introduction. Genetic engineering in cotton has been very painstaking as only cvs. Coker or Acala were amenable to genetic transformation and regeneration. The development of protocols for regeneration in other cultivars started recently (Ali *et al*. 2004, Aydin *et al*. 2004, Jin *et al*. 2006). The proliferation of pre-existing meristems from cotyledonary nodes, primary and tertiary leaf nodes, *etc*., into elongated multiple shoots *in vitro* has been used (Saeed *et al*. 1997, Agrawal *et al*. 1997, Gupta *et al*. 1997, Hemphill *et al.* 1998, Morre *et al*. 1998, Zapata *et al*. 1999, Hazra *et al*. 2000, Caramori *et al*. 2001, Ali *et al*. 2004). However, the development of shoots *de novo* in a short period of time is a more advantageous approach because of the possibility of obtaining non-chimeric transgenic plants. The hypocotyls sections were used as explants for direct shoot organogenesis earlier but the efficiency of intact plantlet regeneration reported was low (Ouma *et al*. 2004) and in fact there was no clear mention about the frequency of regeneration and rooting in the report.

In the present communication, we describe an efficient, rapid, genotype-independent regeneration protocol for obtaining direct shoot organogenesis from hypocotyl explants, with successful rooting of the regenerated shoots.

Materials and methods

Seed germination: Mature de-linted seed of cultivated cotton (*Gossypium hirsutum* L.) cultivars Bharani, Durga and JKCH-99 (provided by *JK Agrigenetics*, Hyderabad, India) were surface sterilized using 70 % (v/v) ethanol for 2 min. Subsequently, the seeds were sterilized by agitation in 4 % sodium hypochlorite (v/v) (*Qualigens*, Mumbai, India) for 15 min followed by 4 - 5 rinses with sterile double distilled water. Seeds were soaked in sterile water for 4 - 5 h and kept for germination in culture bottles with sterile moist tissue paper or cotton with half-

Received 21 December 2005, *accepted* 20 January 2007.
Abbreviations: AC - activated charcoal, BAP - benzylaminopurine, EM - elongation medium, GA_3 - gibberellic acid, IAA - indoleacetic acid, IBA - indolebutyric acid, KIN - kinetin, LS - Linsmaier and Skoog, MS - Murashige and Skoog, NAA - naphthaleneacetic acid, NN - Nitsch and Nitsch, RIM - rooting medium, SIM - shoot induction medium, TDZ thidiazuron.
Acknowledgements: DK is grateful to the Council of Scientific and Industrial Research (CSIR), Government of India for a Research Fellowship. Facilities established by the different funding agencies at the Department of Plant Sciences are gratefully acknowledged.
* Corresponding author; fax: (+91) 040 23010120, e-mail: pbksl@uohyd.ernet.in

strength MS liquid medium under dark for 48 h. When radicles emerged, seedlings were transferred to 16-h photoperiod (irradiance of 70 µmol m^{-2} s^{-1}) and temperature of 28 ± 1 °C.

Seedlings (8 to 10-d-old) were collected and their cotyledons were aseptically removed. Acropetal dome like apical bud was visible at this time and a proximal cut was made approximately 2.0 mm below the apical meristematic region and 0.5 cm explants of hypocotyls were prepared. These explants were placed horizontally on the medium and maintained in the above-mentioned photoperiod conditions.

Culture medium: The morphogenic potential of hypocotyl segments of the three cultivars was tested on MS basal medium with 3 % glucose (*Himedia*) and with vitamins according to Murashige and Skoog (1962; MS), Gamborg *et al.* (1968; B_5), Nitsch and Nitsch (1969; NN), Linsmaier and Skoog (1965; LS), or Lloyd and McCown (1980; McCown) and these media were designated as MS_1, MS_2, MS_3, MS_4 and MS_5, respectively. The culture medium was solidified using 2 g dm^{-3} *Phytagel* (*Sigma-Aldrich*, St. Louis, USA). Initial factorial experiments were performed with the above mentioned vitamin combinations with MS basal media, and different concentrations of cytokinins, thidiazuron (TDZ; 0.5 - 3.0 mg dm^{-3}), benzylaminopurine (BAP; 0.5 - 5.0 mg dm^{-3}) and kinetin (KIN; 1.0 - 5.0 mg dm^{-3}) each in combination with naphthalene acetic acid (NAA; 0.01 - 0.1 mg dm^{-3}) were tested. The experiment was performed following a randomized block design. Later synergistic effect of cytokinins TDZ, BAP, KIN at concentrations 0.5 - 3.0 mg dm^{-3} each was examined. In another experiment, varying concentrations of silver nitrate (1 - 9 mg dm^{-3}) were added to MS_3 with TDZ and NAA. Control experiments were performed in parallel with these trials. The third experiment included 0.1 % activated charcoal (AC) in MS_3 with TDZ, NAA and silver nitrate. Different auxin concentrations like indole butyric acid (IBA), NAA and indole acetic acid (IAA) (0.01 - 1.0 mg dm^{-3}) were tried for root induction. Rooting medium comprised half-strength MS basal medium with 3 % sucrose and 1 g dm^{-3} acid washed neutralized AC. The pH of the culture medium was adjusted to 5.8 prior to adding AC and *Phytagel* and sterilized by autoclaving at a pressure of 1.1 kg cm^{-2} and a temperature of 121 ºC for 20 min. Filter sterilized silver nitrate was added to the culture medium before dispensing into the Petri dishes. Further, experiments were conducted by culturing hypocotyls on the shoot induction medium (SIM) comprising optimized concentrations of growth regulators.

Optimization of activated charcoal and silver nitrate: Concentration of activated charcoal was maintained at 1.0 g dm^{-3} in all the experiments and silver nitrate concentration was optimized using various concentrations of 1.0, 3.0, 5.0, 7.0 and 9.0 mg dm^{-3} with MS_3 containing 2.0 mg dm^{-3} TDZ and 0.05 mg dm^{-3} NAA. The experiments were conducted simultaneously with or without AC and silver nitrate to observe the additive effects of the same on the morphogenic potential of explants.

Elongation of shoot buds: Explants showing visible shoot primordia after two subcultures of 10 d each on a culture medium containing TDZ were transferred and maintained on a medium with 2.0 mg dm^{-3} BAP and 0.1 mg dm^{-3} NAA to obtain full growth of individual shoots (SIM). After 3 - 4 subcultures on SIM, shoots that reach 1.0 cm in height were cut at the internodal region and were cultured on elongation medium (EM) fortified with 1.0 mg dm^{-3} BAP and 2.0 mg dm^{-3} GA_3. From each organogenic clump, 3 - 5 shoots were excised and the explants with developing buds were transferred back to SIM. After two subcultures of 10 d duration each, shoot primordia were grown to individual shoots and the shoots on elongation medium were ready (2 - 3 cm) for root induction.

Rooting and transfer to soil: Elongated and well-developed shoots (> 2.0 cm) were excised and transferred to half-strength MS medium (RIM) augmented with different concentrations of NAA, IBA and IAA (0.01, 0.1 or 1.0 mg dm^{-3} each) individually for root initiation. In subsequent experiments, shoots were cultured on MS_3 with 1.0 mg dm^{-3} IBA, which has been observed as the optimal concentration for rooting. Well-rooted plantlets were transferred to small pots containing soil and *Vermiculite* (1:1). Later, these were transferred to bigger pots and maintained under greenhouse conditions.

Statistical analysis: Cultures were observed on visual basis and the shoot primordia were counted microscopically. The regeneration percentage was calculated as the percent of responding explants with a minimum two shoots each out of the total cultured explants. The data were analyzed using *ANOVA* for a completely randomized design and the treatment means were compared using *MSTAT* software and Duncan's multiple range test.

Scanning electron microscope studies: The proximal parts of the hypocotyls were fixed in 2.5 % glutaraldehyde at 4 ºC for 4 h. These tissues were removed, washed with double distilled water and again fixed in 1% OsO_4 for 2 h at 4 ºC. After a few washes in double distilled water, the specimens were dehydrated in graded ethanol solutions. Tissues were mounted on specimen stub Critical point drying was done in *Palaron Jumbo Critical Dryer*. Gold sputter coating was applied on the samples under reduced pressure After coating, the proximal region of the tissues were examined under scanning electron microscope (*FEI XL 30 ESEM*) at University of Hyderabad.

Results

Germination frequencies in cotton seeds was 94, 88, and 72 % in Bharani, Durga, and JKCH-99, respectively. The germination percentage was distinctly higher, if seeds were incubated in culture bottles containing filter papers moistened with half-strength MS liquid medium, when compared to germination on MS agar medium.

Among different explants tried for *de novo* regeneration, hypocotyls gave a better response. Age of the seedlings from which explants were prepared influenced the regeneration frequency considerably. In the beginning, 2 to 20-d-old seedlings were tested and explants from 8 - 10-d-old seedlings were chosen for further analysis because of their better morphogenic ability (data not shown). When hypocotyl sections (0.5 - 1 cm) with apical bud removed were placed horizontally on the medium (Fig. 3*A*), both ends of the hypocotyls bulged and proximal ends differentiated shoot buds by the end of second week of culture. These microscopic buds developed into individual shoots at the proximal region whereas, it was a simple cell division and minor callus formation at the distal end of the explant.

Among all vitamin combinations added to MS basal medium along with 2.0 mg dm^{-3} TDZ and 0.05 mg dm^{-3} NAA, MS_3 (MS salts with NN vitamins) was most responsive in producing highest regeneration (76 %) at the end of 5th week. Therefore this medium was selected for further studies (Fig. 1). The order of vitamin combinations influencing the regeneration was NN > Mc Cown > MS >LS > B_5 and the least response was recorded in MS_2.

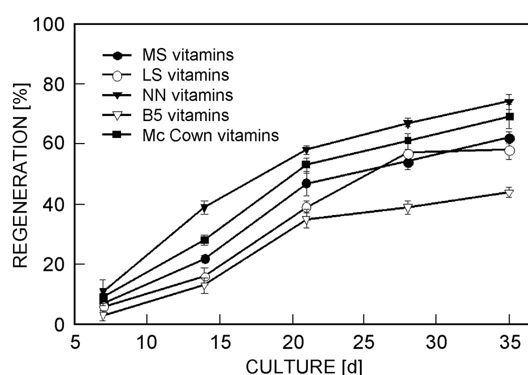

Fig. 1. Regeneration of hypocotyl explants using different vitamin (according to media MS, LS, NN, B5 and Mc Cown) added to MS basal medium comprising 2 mg dm^{-3} TDZ, 0.05 mg dm^{-3} NAA, 5 mg dm^{-3} $AgNO_3$ and 1 g dm^{-3} AC as constant components. Regeneration percentage has been calculated as the percentage of the responding explants out of the total explants cultured. *Vertical bars* represent SE of the means; *n* = 20.

Experiments with TDZ (0.5 - 3.0 mg dm^{-3}), BAP and kinetin (1.0 - 5.0 mg dm^{-3} each) and NAA (0.01 - 0.1 mg dm^{-3}) resulted in good regeneration response. Besides attaining a maximum number of responding explants, the highest number of shoot primordia (19.2 per responding explant) (Table 1) and a maximum number of shoots per explant (10.6) with high regeneration percentage up to 76 % was attained on 2.0 mg dm^{-3} TDZ and 0.05 mg dm^{-3} NAA (Table 2, Fig. 2). This was followed by the combination of BAP and KIN (12.3 shoot buds per responding explant). Lower concentrations of TDZ induced the formation of high number of shoot primordia (Fig. 3*B*) and a many of them were converted into individual shoots, whereas numerous shoot primordia appeared at higher TDZ concentrations developed into hyperhydric shoots associated with fasciated water soaked callus. Hence, explants with shoot primordia from TDZ medium were transferred to 2.0 mg dm^{-3} BAP and 0.1 mg dm^{-3} NAA after two subcultures to obtain optimal growth of shoot buds into individual shoots. Shoots cultured on TDZ medium also showed delayed rooting by more than eight weeks with 1 - 2 roots per shoot only.

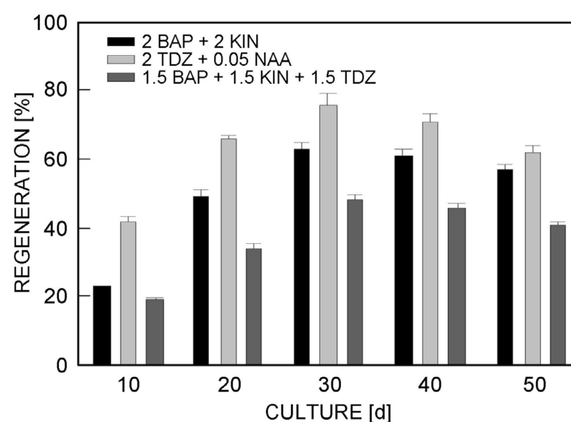

Fig. 2. Regeneration of hypocotyl sections in MS basal medium augmented with NN vitamins, 5 mg dm^{-3} $AgNO_3$ and 1 g dm^{-3} AC with different growth regulator combinations [mg dm^{-3}]. *Vertical bars* represent SE of the means; *n* = 20.

The shoot elongation was observed on EM with 1.0 mg dm^{-3} BAP and 2.0 mg dm^{-3} GA_3 (Fig. 3*C*) From different media tried for rooting of well-developed shoots, highest rooting percentage (86 %) was obtained on medium with 1.0 mg dm^{-3} IBA (Table 3, Fig. 3*D*). Though higher levels up to 10.0 mg dm^{-3} IBA induced rooting on shoots, it was associated with callus at the cut end. The rooting of shoots on RIM with NAA was 75 % but with less number of roots per shoot than on RIM with IBA. Rooting on RIM with IAA was very poor. The survival rate of plantlets rooted on IAA appeared to be less because of the callus formation.

Activated charcoal (AC) enhanced the recovery of shoot primordia into elongated shoots with good internodal length. Leaves were large in size and dark green in colour. Activated charcoal in combination with silver nitrate greatly enhanced regeneration capacity and

Table 1. Effect of various combinations of growth regulators on regeneration in hypocotyl explants of cotton. Data represents means from three replicates ± SE. Means denoted by different letters differ significantly at $P = 0.05$. Number of explants forming shoot primordia (SP) and number of shoots per explant were scored in 4-week-old cultures.

Growth regulator [mg dm^{-3}]				Bharani		Durga		JKCH-99	
TDZ	BAP	KIN	NAA	SP	[explant^{-1}]	SP	[explant^{-1}]	SP	[explant^{-1}]
0.5	-	-	0.01	3.7 ± 0.0kl	5.7 ± 0.8	3.3 ± 1.2lm	3.9 ± 0.1	3.8 ± 0.2kl	3.0 ± 1.1
1.0	-	-	0.01	5.6 ±.0.6jk	9.8 ± 1.9	4.6 ± 2.0jk	6.9 ± 2.1	4.0 ± 0.4kl	4.8 ± 2.3
1.5	-	-	0.01	11.8 ± 0.6e	12.5 ± 0.5	11.1 ± 2.2de	8.8 ± 1.7	9.2 ± 0.1fg	8.1 ± 2.1
2.0	-	-	0.01	14.2 ± 0.8ab	16.9 ± 0.1	11.6 ± 2.0de	12.1 ± 0.0	10.9 ± 0.0de	9.7 ± 0.6
2.5	-	-	0.01	10.8 ± 1.7de	10.0 ± 0.8	9.3 ± 1.6fg	9.6 ± 0.5	9.1 ± 0.3fg	5.8 ± 0.2
3.0	-	-	0.01	5.7 ± 0.1ij	7.8 ± 1.2	6.2 ± 0.2k	4.2 ± 0.8	5.0 ± 1.6jk	3.8 ± 0.0
0.5	-	-	0.05	4.6 ± 1.0k	7.1 ± 1.4	4.5 ± 0.0k	5.6 ± 1.8	4.0 ± 2.0kl	3.2 ± 2.1
1.0	-	-	0.05	8.4 ± 1.2g	12.5 ± 0.1	8.3 ± 0.2g	8.8 ± 0.1	5.3 ± 1.1jk	5.6 ± 1.2
1.5	-	-	0.05	11.6 ± 0.3cd	14.8 ± 0.4	11.3 ± 0.9cd	10.4 ± 0.8	9.1 ± 1.8f	8.8 ± 0.6
2.0	-	-	0.05	15.5 ± 0.4a	19.2 ± 0.7	12.2 ± 1.3bc	14.7 ± 0.4	12.0 ± 1.0cd	9.8 ± 1.9
2.5	-	-	0.05	13.7 ± 0.5bc	13.0 ± 0.2	11.9 ± 1.1bc	10.2 ± 0.6	9.2 ± 0.2fg	6.7 ± 2.0
3.0	-	-	0.05	8.0 ± 0.1gh	9.6 ± 0.5	7.8 ± 1.9g	6.8 ± 1.3	5.4 ± 0.7k	4.9 ± 1.8
-	1.0	-	0.10	2.8 ± 0.8lm	3.2 ± 0.2	2.8 ± 1.0m	2.9 ± 1.9	2.0 ± 0.1m	2.4 ± 0.4
-	2.0	-	0.10	5.2 ± 1.0k	6.0 ± 1.1	4.5 ± 1.4k	5.1 ± 1.2	3.4 ± 1.2l	4.6 ± 0.5
-	3.0	-	0.10	8.7 ± 0.7fg	9.5 ± 0.4	8.1 ± 0.2gh	8.2 ± 0.4	6.9 ± 1.6ij	7.7 ± 0.8
-	4.0	-	0.10	7.4 ± 1.2ghi	8.0 ± 0.2	6.8 ± 0.8ijk	6.4 ± 0.9	6.3 ± 0.1ij	5.4 ± 0.1
-	5.0	-	0.10	4.6 ± 2.2k	5.2 ± 0.3	3.8 ± 1.7kl	4.7 ± 0.1	3.5 ± 2.0kl	3.2 ± 0.0
-	-	1.0	0.10	0.0 n	-	0.0 n	-	0.0 n	-
-	-	2.0	0.10	1.9 ± 0.7m	3.8 ± 0.6	1.8 ± 0.2m	2.6 ± 0.8	0.0 n	-
-	-	3.0	0.10	2.6 ± 1.9m	6.4 ± 1.3	2.6 ± 0.8m	5.7 ± 1.0	2.2 ± 0.8m	5.3 ± 0.4
-	-	4.0	0.10	1.7 ± 0.4m	4.3 ± 1.4	1.7 ± 1.4m	3.4 ± 0.2	1.6 ± 1.8m	3.0 ± 0.4
-	-	5.0	0.10	1.2 ± 0.4m	1.3 ± 0.9	1.2 ± 1.8m	2.2 ± 0.8	1.3 ± 0.4m	1.8 ± 1.0
-	0.5	0.5	-	4.0 ± 1.8kl	3.2 ± 0.2	3.5 ± 0.8l	2.4 ± 0.5	2.8 ± 0.7m	2.0 ± 2.2
-	1.0	1.0	-	4.9 ± 0.1k	5.7 ± 0.3	3.8 ± 1.5kl	5.1 ± 0.9	3.7 ± 0.4kl	4.4 ± 0.9
-	1.5	1.5	-	7.9 ± 0.5fg	9.8 ± 0.6	7.5 ± 1.4fg	9.6 ± 1.0	7.6 ± 0.1gh	8.8 ± 1.3
-	2.0	2.0	-	12.3 ± 0.8bc	14.5 ± 1.2	11.8 ± 0.0cd	12.6 ± 0.9	9.4 ± 1.6fg	10.8 ± 0.8
-	2.5	2.5	-	9.4 ± 0.8ef	11.4 ± 1.3	8.7 ± 1.8fg	9.9 ± 2.0	8.6 ± 0.4fg	7.1 ± 1.1
-	3.0	3.0	-	5.8 ± 1.4k	7.3 ± 1.0	3.9 ± 0.3kl	7.2 ± 0.4	3.6 ± 1.5kl	6.5 ± 1.2
0.5	0.5	0.5	-	3.0 ± 0.2lm	4.2 ± 0.7	2.3 ± 0.8m	2.9 ± 0.1	2.2 ± 1.2m	2.0 ± 1.2
1.0	1.0	1.0	-	4.8 ± 1.6k	7.7 ± 1.3	4.7 ± 0.8k	6.5 ± 0.7	4.0 ± 1.6kl	6.1 ± 0.3
1.5	1.5	1.5	-	9.4 ± 0.9fg	11.9 ± 1.4	8.4 ± 1.3fg	10.3 ± 0.5	5.5 ±0.2k	8.6 ± 0.2
2.0	2.0	2.0	-	7.1 ± 1.1hij	9.6 ± 0.6	6.4 ± 1.7k	9.2 ± 1.1	4.2 ± 1.5k	7.0 ± 1.8
2.5	2.5	2.5	-	6.6 ± 0.9k	5.4 ± 0.9	3.8 ± 0.1kl	3.6 ± 0.8	3.1 ± 1.0m	3.6 ± 2.0
3.0	3.0	3.0	-	2.4 ± 0.4m	3.9 ± 0.8	2.1 ± 1.2m	3.2 ± 1.8	1.8 ± 1.4m	2.4 ± 0.7

shoot yield with almost similar response in all the three varieties. It was observed that inclusion of AC in the culture medium promoted root initiation and elongation that may be due to the immediate adsorption of phenolics at the cut end. Complete plantlets with root system having well developed lateral roots survived upon transfer to pots in the greenhouse and appeared morphologically normal (Fig. 3*E*,*F*).

Silver nitrate had a marked influence on the growth and differentiation from hypocotyl. An optimal response was noticed at 3 - 5 mg dm^{-3} (Fig. 4). The hyperhydricity decreased in the presence of silver nitrate and the quality of shoot primordia as well as elongated shoots improved when compared to shoots regenerated on media without silver nitrate. The effect of silver nitrate was genotype dependent. Increase in concentration of silver nitrate beyond 5.0 mg dm^{-3} disturbed the organization of shoot primordia leading to the formation of friable callus. Visual observations indicated that the shoots appeared healthy and well elongated with inclusion of silver nitrate in the culture medium. Though the number of shoot primordia has not increased significantly by silver nitrate, their conversion rate into individual shoots improved significantly.

Scanning electron micrographs of control explants grown on MS medium without growth regulators did not show any developing shoot buds (Fig. 5*A*), whereas, the proximal parts of *in vitro* cultured hypocotyls showed clusters of shoot primordia obtained *de novo* on SIM (Fig. 5*B*).

Fig. 3. Plant regeneration by organogenesis from hypocotyl segments in cotton (*Gossypium hirsutum* L.). *A* - Hypocotyl explants from 8 to 10-d-old seedlings on SIM; *B* - Shoot initiation from proximal ends of hypocotyls at the end of 3 weeks on SIM; *C* - Multiple shoot production and elongation on EM; *D* - Isolated and elongated shoot with developing roots when cultured on RIM; *E* - Well-rooted plant transferred to greenhouse; *F* - Regenerated plants showing flowers and bolls.

Discussion

An efficient protocol for multiple shoot regeneration in a genotype independent manner is a pre-requisite for genetically manipulation of crop plants. Though there is a large body of literature available on regeneration in cotton cv. Coker through somatic embryogenesis, efforts on the establishment of *de novo* shoot regeneration protocols in commercial cotton cultivars were rare (Ouma *et al*. 2004).

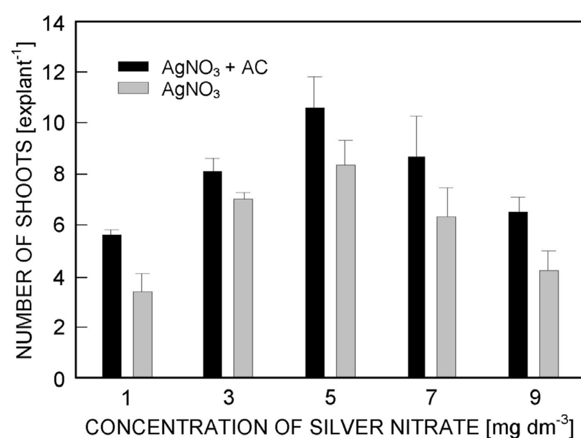

Fig. 4. Effect of different concentrations of $AgNO_3$ with or without AC on multiple shoots induction on TDZ medium. *Vertical bars* represent SE of the means.

Our studies showed that TDZ induced high frequency production of shoot primordia from hypocotyls sections. TDZ has been shown to regulate plant morphogenesis by possessing a high cytokinin like activity (Murthy *et al*. 1998). Lower concentrations of TDZ yielded more shoot primordia compared to higher concentrations, and resulted in hyperhydricity of shoots associated with water soaked callus (Huetteman and Preece 1993, Ouma *et al*. 2004). Ouma *et al*. (2004) reported that TDZ concentrations higher than 0.5 mg dm^{-3} resulted in callusing in contrast to our observations where TDZ concentrations up to 2 mg dm^{-3} appeared to be optimal and did not induce significant amounts of callus. This was probably due to the different genotypes used. TDZ played a vital role in enhancing the *de novo* regeneration potential in pea and lentil (Malik and Saxena 1992), chickpea (Murthy *et al*. 1996) and many other species. However, shoot elongation was greatly hindered when

the cultures were maintained for a long period on TDZ medium as reported in pepper (Hyde and Phillips 1996) and cotton (Caramori *et al.* 2001). Similarly, TDZ inhibited shoot elongation in our cultures. The induction of roots on shoots obtained from TDZ containing media was delayed and they did not elongate further as reported by (Nielson *et al.* 1993, Ouma *et al.* 2004). To avoid these negative effects of TDZ, subculture of explants was carried out on a medium containing 2.0 mg dm^{-3} BAP and 0.1 mg dm^{-3} NAA after shoot primordia formed. Even though shoot bud differentiation was effective in all the tested vitamin combinations with MS basal medium, better response was achieved in MS_3 medium along with TDZ (upto 76 % regeneration frequency with a maximum of 10.6 shoots per explant) followed by 62 % obtained on 2.0 mg dm^{-3} each of BAP and KIN. The regeneration response and shoot yield per explant decreased considerably after 30 d of culture because of the excessive production of callus. To avoid excessive callusing on SIM, cultures were transferred to a medium with lower cytokinin concentration. Addition of GA_3 in the EM promoted shoot elongation but only one or two shoots per cluster elongated at a time and the clumps with shoot buds were again cultured on SIM for further elongation of shoots.

Ethylene, a gaseous hormone released by tissues was reported to accumulate in the intercellular spaces forming aerenchyma thus blocking the differentiating ability of tissues (Topa and McLeod 1988, Biddington 1992, Kong and Yeung 1994). To counteract this, silver nitrate, an anti-ethylene compound, was used to enhance regeneration in many recalcitrant species including cotton (Ouma *et al.* 2004). Silver nitrate incorporation was beneficial in increasing regeneration response by lowering hyperhydricity, as reported in sunflower (Mayor *et al.* 2003) and potato (Turhan 2004) and resulted in better quality shoots.

Table 2. Effect of growth regulators [mg dm^{-3}] on number of regenerated shoots from responding explant and the shoot regeneration response [%]. Means from three replicates ± SE. Data were scored after 4 weeks of culture.

TDZ	BAP	KIN	NAA	Number of shoots [$explant^{-1}$]	Regeneration frequency [%]
0.5			0.05	4.5 ± 0.5	59.0
1.0			0.05	7.2 ± 1.4	65.2
1.5			0.05	10.1 ± 0.2	73.3
2.0			0.05	10.6 ± 0.3	76.7
2.5			0.05	9.2 ± 1.0	69.5
3.0			0.05	6.0 ± 1.2	66.0
	1.0		0.1	2.2 ± 1.1	28.0
	2.0		0.1	4.1 ± 0.3	40.0
	3.0		0.1	5.6 ± 0.5	55.1
	4.0		0.1	5.0 ± 1.0	53.0
	5.0		0.1	3.0 ± 0.2	42.4
		1.0	0.1	0.0 ± 0.0	0.0
		2.0	0.1	1.8 ± 0.2	21.6
		3.0	0.1	3.4 ± 1.6	32.8
		4.0	0.1	2.8 ± 0.8	26.5
		5.0	0.1	2.2 ± 0.8	25.4
	0.5	0.5		3.0 ± 1.6	44.0
	1.0	1.0		6.8 ± 2.0	50.5
	1.5	1.5		8.2 ± 0.8	62.4
	2.0	2.0		8.8 ± 0.6	70.5
	2.5	2.5		7.5 ± 1.0	68.0
	3.0	3.0		5.2 ± 1.1	55.5
0.5	0.5	0.5		3.0 ± 0.5	28.5
1.0	1.0	1.0		3.2 ± 0.5	41.0
1.5	1.5	1.5		6.2 ± 0.1	58.0
2.0	2.0	2.0		5.8 ± 1.0	48.8
2.5	2.5	2.5		3.6 ± 0.8	39.0
3.0	3.0	3.0		1.2 ± 1.4	16.5

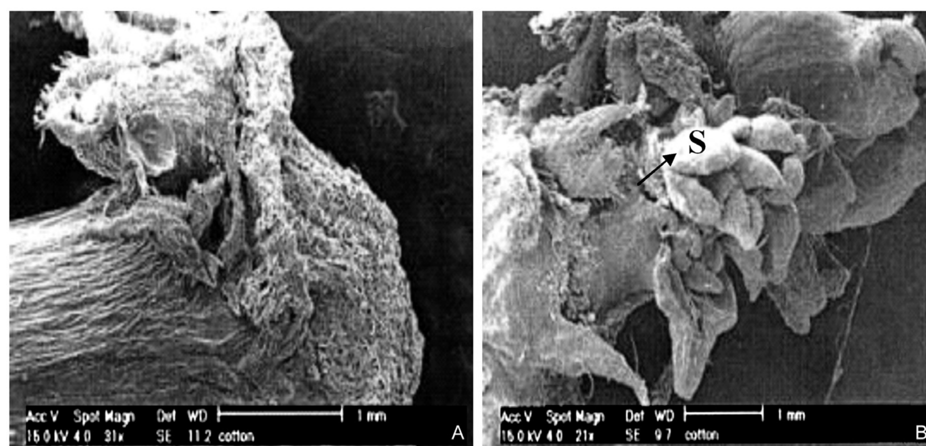

Fig. 5. SEM studies of the *in vitro* cultured explants. *A* - Control explant grown on MS medium without growth regulators, $AgNO_3$ and AC showing callus formation; *B* - Acropetal end of hypocotyl with clusters of shoot primordia when cultured on MS medium supplemented with growth regulators and AC; S - shoot bud

Table 3. Root induction in *in vitro* regenerated shoots of cotton by different auxin concentrations. Means from three replicates ± SE; $n = 30$. Means denoted by different letters differ significantly at $P = 0.05$. Data were scored after 4 weeks.

Growth regulators	Conc. [mg dm^{-3}]	Bharani number of responding shoots	number of roots [shoot^{-1}]	rooting [%]	Durga number of responding shoots	number of roots [shoot^{-1}]	rooting [%]
IBA	0.01	20.2 ± 2.2cd	3.1 ± 0.9	70	18.9 ± 1.8d	2.2 ± 1.2	60
	0.10	22.9 ± 3.0ab	5.0 ± 0.5	83	22.5 ± 2.4bc	4.4 ± 0.8	73
	1.00	26.0 ± 3.2a	6.4 ± 0.6	86	24.2 ± 3.0ab	5.5 ± 0.2	80
NAA	0.01	16.9 ± 3.1e	2.8 ± 1.2	53	13.7 ± 2.6ef	1.9 ± 1.0	43
	0.10	19.3 ± 1.8d	4.4 ± 1.4	63	17.0 ± 2.4e	3.4 ± 0.4	56
	1.00	23.7 ± 1.8bc	5.3 ± 0.8	75	20.1 ± 1.6cd	4.3 ± 0.7	66
IAA	0.01	9.5 ± 1.6g	1.2 ± 0.2	30	6.1 ± 2.0g	2.4 ± 1.5	20
	0.10	11.2 ± 2.4f	3.8 ± 0.6	36	10.2 ± 1.2fg	3.1 ± 0.9	33
	1.00	15.8 ± 2.2e	4.2 ± 0.8	50	14.3 ± 2.2e	3.8 ± 1.2	46

Activated charcoal has been used in *in vitro* tissue culture for adsorbing inhibitory compounds such as phenolics, excessive growth hormones and release of growth promoting substances (Van Winkle *et al*. 2003). It was reported that there would be an equilibrium established with adsorbed and desorbed molecules between explant and AC, thereby making growth hormones available to the explant (Pan and Van Staden 1998). Usage of tissue culture grade AC was effective in reducing browning and subsequent necrosis of the explant due to excessive production of phenolic compounds and promoting regeneration response of hypocotyl explants in our experiments. The addition of AC to the RIM enhanced number of robust roots and establishment of tissue culture raised plants (Dumas and Monteuuis 1995, Pan and Van Staden 1998).

In summary, we have established a fast and genotype-independent efficient regeneration system for *de novo* shoot regeneration from hypocotyl explants of three commercial cotton cultivars including their *ex vitro* transfer. The time taken from explanting to the establishment of plants in the greenhouse was about 16 - 20 weeks compared to the longer periods as reported in published regeneration protocols using somatic embryogenesis. Regenerated plants were phenotypically normal and flowered well. Present experiments are aimed at utilizing this simple regeneration protocol in the future development of transgenic cotton.

References

Agrawal, D.C., Banerjee, A.K., Kolala, R.R., Dhage, A.B., Kulkarni, W.V., Nalawade, S.M., Hazra, S., Krishnamurthy, K.V.: *In vitro* induction of multiple shoots and plant regeneration in cotton (*Gossypium hirsutum* L.). - Plant Cell Rep. **16**: 647-652, 1997.

Ali, R.M., Husnain, T., Hussain, S.S., Mahmood, N., Riazuddin, S.: Multiple shoot regeneration response of recalcitrant cotton (*Gossypium hirsutum* L.) cultivar CIM-443. - Pak. J. biol. Sci. **7**: 1371-1375, 2004.

Aydin, Y., Ipekci, Z., Talas-Ogras, T., Zehir, A., Bajrovic, K., Gozukirmizi, N.: High frequency somatic embryogenesis in cotton. - Biol. Plant. **48**: 491-495, 2004.

Biddington, N.L.: The influence of ethylene in plant tissue culture. - Plant Growth Regul. **11**: 173-187, 1992.

Caramori, L.P.C., Favaro, S., Vieira, L.G.E.: Thidiazuron as a promoter of multiple shoots in cotton explants (*Gossypium hirsutum* L.). - Acta Sci. **23**: 1195-1197, 2001.

Dumas, E., Monteuuis, O.: *In vitro* rooting of micropropagated shoots from juvenile and mature *Pinus pinaster* explants – influence of activated charcoal. - Plant Cell Tissue Organ Cult. **40**: 231-235, 1995.

Gamborg, O., Miller, R.A., Ojima, K.: Nutrient requirements of suspension cultures of soyabean root cells. - Exp. Cell. Res. **50**: 151-158, 1968.

Gupta, S.K., Srivastava, A.K., Singh, P.K. Tuli, R.: *In vitro* proliferation of shoots and regeneration of cotton. - Plant Cell Tissue Organ Cult. **51**: 149-152, 1997.

Hazra, S., Kulkarni, A.V., Nalawade, S.M., Banerjee, A.K., Agrawal, D.C., Krishnamurthy, K.V.: Influence of explants, genotypes and culture vessels on sprouting and proliferation of pre-existing meristems of cotton (*Gossypium hirsutum* L.). - *In Vitro* cell. dev. Biol. Plant **36**: 505-510, 2000.

Hemphill, J.K., Maier, C.G.A., Chapman, K.D.: Rapid *in vitro* plant regeneration of cotton (*Gossypium hirsutum* L.). - Plant Cell Rep. **17**: 273-278, 1998.

Huetteman, C.A., Preece, J.E.: Thidiazuron: a potent cytokinin for woody plant tissue culture. - Plant Cell Tissue Organ Cult. **33**: 105-119, 1993.

Hyde, C., Phillips, G.: Silver nitrate promotes shoot development and plant regeneration of chilli pepper (*Capsicum annum* L.) via organogenesis. - *In Vitro* cell. dev. Biol. Plant **32**: 72-80, 1996.

Jin, S., Zhang, X., Nie, Y., Guo, X., Liang, S., Zhu, H.: Identification of a novel elite genotype for *in vitro* culture and genetic transformation of cotton. - Biol. Plant. **50**: 519-524, 2006.

Kong, L., Yeung, E.C.: Effects of ethylene and ethylene inhibitors on white spruce somatic embryo maturation. - Plant Sci. **104**: 71-80, 1994.

Linsmaier, E.M., Skoog, F.: Organic growth factor requirements

of tobacco tissue-cultures. - Physiol. Plant. **18**: 100-127, 1965.
Lloyd, G., McCown, B.: Commercially feasible micropropagation of mountain laurel, *Kalmia latifolia*, by use of shoot-tip culture. - Proc. Int. Propag. Soc. **30**: 421-427, 1980.
Malik, K.A., Saxena, P.K.: Thidiazuron induces high frequency shoot regeneration in intact seedlings of pea (*Pisum sativum*) and lentil (*Lens culinaris*). - Aust. J. Plant Physiol. **19**: 731-740, 1992.
Mayor, M.L., Nestares, G., Zorzoli, R., Picardi, L.A.: Reduction of hyperhydricity in sunflower tissue culture. - Plant Cell Tissue Organ Cult. **72**: 99-103, 2003.
Morre, J.L., Permingeat, H.R., Romagnoli, M.V., Heisterborg, C.M., Vallejos, H.R.: Multiple shoot induction and plant regeneration from embryonic axes of cotton. - Plant Cell Tissue Organ Cult. **54**: 131-136, 1998.
Murashige, T., Skoog, F.: A revised medium for rapid growth and bioassays with tobacco tissue cultures. - Physiol. Plant. **15**: 473-497, 1962.
Murthy, B.N.S., Victor, J., Singh, R.P.S., Fletcher, R.A., Saxena, P.K.: *In vitro* regeneration of chick pea (*Cicer arietinum* L.) stimulation of direct organogenesis and somatic embryogenesis by thiadiazuron. - Plant Growth Regul. **19**: 233-240, 1996.
Murthy, B.N.S., Murch, S.J., Saxena, P.K.: Thidiazuron: a potent regulator of *in vitro* plant morphogenesis. - *In Vitro* cell. dev. Biol. Plant **34**: 267-275, 1998.
Nielson, J.M., Brandt, K., Hansen, J.: Long-term effects of thidiazuron are intermediate between benzyladenine, kinetin or isopentenyladenine in *Miscanthus sinensis*. - Plant Cell Tissue Organ Cult. **35**: 173-179, 1993.
Nitsch, J.P., Nitsch, C.: Haploid plants from pollen grains. - Science **169**: 85, 1969.
Ouma, J.P., Young, M.M., Reichert, N.A.: Optimization of *in vitro* regeneration of multiple shoots from hypocotyls sections of cotton (*Gossypium hirsutum* L.). - Afr. J. Biotechnol. **3**: 169-173, 2004.
Pan, M.J., Van Staden, J.: The use of charcoal in *in vitro* culture – a review. - Plant Growth Regul. **26**: 155-163, 1998.
Saeed, N.A., Zafar, Y., Malik, K.A.: A simple procedure of *Gossypium meristem* shoot tip culture. - Plant Cell Tissue Organ Cult. **51**: 201-207, 1997.
Topa, M.A., McLeod, K.W.: Promotion of aerenchyma formation in *Pinus serotina* seedlings by ethylene. - Can. J. Forest. Res. **18**: 276-280, 1988.
Turhan, H.: The effect of silver nitrate (ethylene inhibitor) on *in vitro* shoot development in potato (*Solanum tuberosum* L.). - Biotechnology **3**: 72-74, 2004.
Van Winkle, S., Johnson, S., Pullman, G.S.: The impact of Gelrite and activated carbon on the elemental composition of two conifer embryogenic tissue initiation media. - Plant Cell Rep. **21**: 1175-1182, 2003
Zapata, C., Srivatanakul, M., Park, S.H., Lee, B.M., Salas, M.G., Smith, R.H.: Improvements in shoot apex regeneration of two fiber crops: cotton and kenaf. - Plant Cell Tissue Organ Cult. **12**: 43-50, 1999.