In vitro plant regeneration from the callus of shoot tips in cotton (*Gossypium hirsutum* L. cv. SVPR 2)

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Abstract
A high efficiency shoot organogenesis from callus cultures and plant establishment protocol has been developed for cotton (Gossypium hirsutum L. cv SVPR-2) through shoot tip. After 6 weeks of culture, green-compact-nodular organogenic callus induction was observed on the medium fortified with (MS) basal salts, B5 vitamins, 2.0 mgl^{-1} of benzylaminopurine (BAP), 1.0 mgl^{-1} kinetin (KN), 30 gl^{-1} glucose and 8 gl^{-1} agar. The highest rate of shoot proliferation was achieved at 12th week and 8.6 shoots were produced per callus clump. Among the different concentrations tested media fortified with MS salts, B5 vitamins, 2 isopentynyl adenine *(2-iP) (1.5 mgl^{-1}),* gibberellic acid (*GA$_3$) (0.6 mgl^{-1}), glutamine (25 mgl^{-1}) and glucose (30 gl^{-1}) showed best response for multiple shoot proliferation from the induced callus. All the in vitro regenerated shoots were rooted on the medium fortified with MS salts, B5 vitamins, 30 gl^{-1} sucrose, 8 gl^{-1} agar and 1.5 mgl^{-1} of indole butyric acid (IBA). The regenerated plantlets, with tertiary roots are considered as matured plantlets and they were hardened on paper pots containing sand, soil and vermiculite in 1:1:1 ratio. The hardened plants showed 85% survival rate and showed parental phenotypic characters.*
Keywords*: Basal callus; Organogenesis; Rooting; Hardening; Auxins cytokinins*

INTRODUCTION

Plant Biotechnology offers new technologies for sustainable development and utilization of natural resources. Plant cell, tissue culture and genetic engineering of plants have contributed significantly to crop improvement and production of high quality planting material. Among the economically important crops, cotton (*Gossypium hirsutum* L.) is a well-known fiber crop, particularly important in textile industry and also cultivated for its seed-oil. Among the cotton producing countries, India ranks first in cultivation, making 32% of the world's total area followed by USA (23%) and China (20%) and it has been estimated that 180 million people depend on cotton production (Benedict and Altman, 2001). Cotton genetic engineering play an vital role to improve the quality as well as quantity of fiber by means of producing plants resistant to insect, herbicide, fungi, bacteria and nematode through genetic engineering.

Price and Smith (1979) first reported cotton (*Gossypium klotzschianum* Anders.) somatic embryogenesis, but were unable to regenerate from somatic embryos. Later, Davidonis and Hamilton (1983) reported complete plant regeneration from the somatic embryos of *Gossypium hirsutum* L. cv Coker310. To date, only few reports of high frequency regeneration in cotton somatic embryos are available due to a genotype dependent response (Ganesan and Jayabalan, 2004). In the same way cotton shoot organogenesis through callus induction has its own difficulties including the excretion of secondary metabolites from the explants into the medium, browning of callus after a short period of culture, a low frequency of organogenic callus formation, and very slow response for shoot proliferation from the selected organogenic callus cultures. Due to these unavoidable difficulties, cotton plant regeneration through organogenic callus cultures is still a problem. Hence, this experiment was undertaken for the production of a good regenerative and reproducible protocol for cotton organogenesis through shoot tip explants.

MATERIALS AND METHODS

Collection of plant materials and aseptic culture of seedlings: Delinted cotton seeds cv. SVPR2 were col-

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lected from the Cotton Research Institute, Tamil Nadu Agricultural University, Srivilliputhur, Tamil Nadu, India. For surface sterilization, the seeds were wrapped in several layers of cheesecloth and placed in running tap water for 30 min to remove surface particles. The seeds were then surface-sterilized in 70% ethanol for 10 min and kept in 0.1% mercuric chloride for another 20 min under aseptic conditions. The surface-sterilized seeds were inoculated onto seed germination medium. The surface-sterilized seeds were cultured on MS medium (Murashige and Skoog, 1962) containing 0.1-1.0 mgl^{-1} benzylaminopurine (BAP), 0.02-0.4 mgl^{-1} gibberellic acid (GA3), 30 gl^{-1} sucrose and 0.8 gl^{-1} agar (Ganesan and Jayabalan, 2004, 2005). Initially the cultures were maintained in dark condition for 48h at 25 ± 2°C and then under 16h light and 8h dark photoperiod condition with the light intensity of 3000 lux.

Initiation, proliferation and selection of organogenic callus: In this study shoot tip explants (0.5-0.7 cm in length) were aseptically dissected from 5-7 day old seedlings and placed vertically in callus initiation medium which contained MS salts (Murashige and Skoog, 1962), B5 vitamins (Gamborg *et al*., 1968), BAP (1.0-5.0 mgl^{-1}), KN (0.5 to 2.5 mgl^{-1}), glucose (30 gl^{-1}) and 0.8 % agar (pH 5.8). For callus induction the test tubes (22 × 5 cm Brorosil, India) were used. After media preparation and pH measurement, agar (8 gl^{-1}) was added slowly and the medium was distributed to the test tubes (about 15 ml/test tube) plugged with non-absorbent cotton wrapped in one layer of cheese cloth. After plugging with cotton, the containers were autoclaved at 12°C for 15 min with 15 lb pressure.

After 3 weeks of culture, a mass of calli were formed from the base of the shoot tip explants. From the obtained mass, some callus portions were organogenic in nature. The organogenic nature of the callus was identified by the presence of green colour with compact texture (Rugini and Muganu, 1998). The organogenic portions were isolated and subcultured in the same medium. Whitish green, brown, watery brown and black colored non-organogenic callus were also observed and they were not selected and discarded for further studies due to nil response. The selected organogenic callus was weekly subcultured for four more weeks for the induction of matured green compact organogenic callus. For callus induction maximum of 85 explants were tested and each experiment was repeated three times with 6 replicates.

Adventitious shoot proliferation: After the induction of calli the shoot tip explants were removed and then the calli were allowed for shoot proliferation studies. Fresh organogenic callus (250 mg) were transferred to 200 ml narrow bottles (Borosil, India) containing 25 ml of shoot initiation medium. Then the cultures were subcultured for another 2 months for the initiation of shoots. During each subculture dead and dark brown cells were removed. To avoid the whole callus tissues become necrotic and become dead. The plant growth regulators like, BAP (0.2-2.0 mgl^{-1}) and 2 isopentynyl adenine (2-iP, 0.2-2.0 mgl^{-1}) were tested individually and the combined effect of BAP + GA_3 (0.2-1.0 mgl^{-1}) and 2-iP + GA_3 (0.2-1.0 mgl^{-1}) were also tested for enhanced proliferation of shoots along with the medium consisting of MS salts, B5 vitamins, glucose 30 gl^{-1}, and 0.8 % agar (pH 5.75). The influence of different amino acids such as, proline, serine, glutamine and alanine in varying concentrations (5-50 mgl^{-1}) were tested for the enhanced production of shoots from the obtained organogenic callus. During shoot proliferation from callus cultures maximum of 80 callus cultures were tested and each experiment was repeated for 3 times with 6 replicates.

Effect of carbon source and amino acids on shoot proliferation frequency: In this study, the effect of widely used 4 different carbon sources with varying concentrations (10-40 mgl^{-1}) of sucrose, glucose, fructose and maltose were tested for organogenic callus induction and multiple shoot proliferation from the induced callus. Likewise, the influence of different amino acids like alanine, glutamine, proline and serine were tested in different concentrations (5-50 mgl^{-1}) for increased multiple shoot production from the obtained callus. The impacts of carbon source as well as amino acids were measured on each step of cotton organogenesis. Here also during shoot proliferation from callus cultures maximum of 80 callus cultures were tested and each experiment was repeated for 3 times with 6 replicates.

Root induction and hardening: Root induction from the elongated shoots were obtained from the medium fortified with MS salts (full strength), B5 vitamins, indole butyric acid (IBA) (0.1-3.0 mgl^{-1}), glucose 30 gl^{-1} and agar (0.8 %). After 4 weeks maintenance, the regenerated plantlets with tertiary roots (35-40 days after root induction) the regenerated plants were transferred to plastic pots containing sand, soil and vermiculate in 1:1:1 ratio for hardening in the green house condition. The hardened plants were completely covered with plastic bags for 2 weeks to maintain the humidity and it was progressively removed to adapt

normal environmental condition. The survival percentage of all the hardened plants was recorded regularly. The selected regenerated plants, adapted to normal environmental condition, were transferred to earthen pots for further growth and development.

Statistical analysis: Means and standard errors were used throughout the study and the values were assessed using a parametric Moods median test (Snedecor and Cochran, 1989). The data were analyzed for variance by Duncan's multiple range test (DMRT) using the SAS programme (SAS Institute, Cary, N.C.).

RESULTS

Induction of organogenic callus: The surface sterilized seeds exhibited 95 % of germination after 10 days of inoculation on the medium supplemented with MS basal salts, B5 vitamins BAP (0.5 mgl^{-1}) and KN (0.2 mgl^{-1}). Explants were collected from 5 to 10 day old *in vitro* grown seedlings. Among them, 7-day-old explants showed good response because they produced young shoot apices with two to three leaf primordia favoring *in vitro* callus induction of multiple shoot apices. More number of leaf primordia and more length of explants also affect the callus initiation and regeneration potential. Five days after inoculation, callusing response of explants was observed besides the hormonal effects on callus induction. From the different concentrations of BAP, KN and combined effect of BAP and KN tested, combination of BAP (2.0 mgl^{-1}) and KN (1.0 mgl^{-1}) showed maximum response and it also produced maximum amount of organogenic callus (nodular green compact callus) on the medium fortified with MS salts, B5 vitamins, 30 gl^{-1} glucose and 8 gl^{-1} agar (Fig. 1a) (Table 1). Individual treatment of BAP and KN in all the concentrations showed poor response for organogenic callus induction. Instead of organogenic callus, formation of callus with variation in colour and texture was noticed. During individual supplementation of BAP and KN, yellowish friable, yellowish green compact, yellowish green friable and

Figure 1. Plant regeneration from the shoot tip derived callus cultures of Cotton (*Gossypium hirsutum* L.). a) 4 weeks old shoot tip derived organogenic callus *Bar = 1 cm*; b) Induction of multiple shoots from 12 weeks old callus *Bar = 1 cm*; c) Elongated multiple shoots on multiple shoot initiation medium *Bar = 1.5 cm*; d) Root induction from the elongated shoots after 20 (*bar = 1 cm*); e) 40 days old fully regenerated plantlet with well developed shoot and root system *Bar = 2.5 cm*; f) 20 days old hardened plants grown in plastic pots *Bar = 6 cm*; g) 35 days old plantlet on earthen pot before flowering *Bar = 10 cm.*

Table 1. Individual and combined effect of BAP and KN on organogenic callus of shoot tip explants on MS medium supplemented with B5 medium vitamins.

Growth Regulators (mgl^{-1})		Organogenic callus Formation (%)	Type and nature of callus	Abnormalities
BAP	KN			
0.5	--	34.5 ef	YF	--
1.0	--	39.2 de	YGF	--
2.0	--	41.6 d	YGC	+
3.0	--	33.2 e	YGF	+
4.0	--	29.2 f	YC	++
5.0	--	24.5fg	BYC	++
--	0.5	13.7 i	GF	++
--	1.0	17.5 h	GYF	+
--	1.5	22.5 g	GF	+
--	2.0	19.5 gh	GF	+
--	2.5	15.5 hi	GF	+
2.0	0.5	72.5 bc	GC	--
2.0	1.0	81.2 a	GCN	--
2.0	1.5	74.3 b	GC	--
2.0	2.0	70.1 c	GC	--

YF: Yellowish Friable, YGF: Yellowish Green Friable, YGC: yellowish Green compact, YC: Yellowish Compact, BYC: Brownish Yellow Compact, GF: Greenish Friable, GYF: Greenish Yellow Friable, GC: Green Compact, GCN: Green Compact Nodular. Number of explants tested: 85, Values are mean ± SE of three repeated experiments. Each treatment consisted of 6 replicates. Means within a row followed by the same letters are not significant at P=0.05 according to DMRT.

yellow compact calli were induced from the explants. Further experiments on the above calli, we observed that they are non organogenic in nature.

Shoot proliferation from the callus: High percentage of shoot proliferation was achieved by the weekly subculture of organogenic callus on the medium fortified with MS salts, B5 vitamins, 2-iP (1.5 mgl^{-1}), GA_3 (0.6 mgl^{-1}), glucose 30 gl^{-1} and agar (8.0 gl^{-1}). After one month, maximum of 4.2 shoots were successfully regenerated from the callus (Fig. 1b). In the case of BAP and GA_3 combination, the percentage of response and number of shoots produced per callus cultures are very low (2.8 ± 0.21 shoots/callus) when compared with 2-iP and GA_3 combination (Table 2). In this present investigation, we confirmed that addition of 2-iP also favors shoot organogenesis from the callus cultures of cotton. During multiple shoot proliferation, effects of different concentrations of carbon sources were tested with varying concentrations. Among them glucose 30 g/l showed best response and maximum of 4.2 shoots were initiated in this concentrations. In the other concentrations and forms of carbon sources tested reduced percentage of multiple shoot induction frequency was observed (Fig. 2). At the same time, severe browning of callus was noticed in other concentration of sucrose, fructose and maltose tested. The influence of various amino acids like alanine, glutamine, proline and serine were also evaluated for the multiple shoot initiation from the obtained callus. All the four amino acids showed enhanced activity on multiple shoot induction. Among the four amino acids tested, 25 mgl^{-1} glutamine showed best response for multiple shoot proliferation and maximum of 8.6 shoots/callus clump were regenerated (Fig. 3). This results proved that addition of amino acids particularly glutamine (25.0 mgl^{-1}) is necessary for the enhanced multiple shoot induction frequency of cotton (Fig. 1c).

Root induction and hardening: Root induction was achieved within 7 days of culture. IBA alone was effective for induction of roots. In the present study IBA (1.5 mgl^{-1}) was effective for induction of roots. In this concentration, the roots reached maximum length of 24.5 ± 0.95 cm within three weeks of culture (Fig. 1d and e, Table 3). To our knowledge, this is the first report for indirect organogenesis of cotton through shoot apices. The increased root length leads to increase in the survival percentage of hardened and field grown plants. For hardening process sand, soil and vermiculated soil were used in 1:1:1 ratio (Fig. 1f). After proper acclimatization the hardened plants were transferred to earthen pots for boll yielding and bolls were produced after 2 months of proper irrigation (Fig. 1g).

Table 2. Effect of BAP and 2-iP and combination of BAP and 2-iP with GA_3 on regeneration of shoot buds from immature leaf derived organogenic callus cultured on the medium fortified with MS salts and B5 vitamins.

Growth regulators (mgl^{-1})	Response (%)	Number of shoots
BAP		
0.2	22.5 ± 1.24 d	1.3 ± 0.20 d
0.4	25.6 ± 1.54 c	1.6 ± 0.15 bc
0.8	38.2 ± 1.22 ab	1.8 ± 0.12 ab
1.0	42.6 ± 1.11 a	1.9 ± 0.14 a
1.2	31.5 ± 0.85 b	1.7 ± 0.12 b
1.5	25.3 ± 1.85 cd	1.7 ± 0.15 b
1.8	14.5 ± 0.96 e	1.5 ± 0.18 c
2.0	7.5 ± 0.90 f	1.5 ± 0.21 c
2-Ip		
0.2	21.5 ± 1.85 h	1.4 ± 0.25 fg
0.4	25.3 ± 1.96 g	1.9 ± 0.56 e
0.8	35.8 ± 1.75 f	2.1 ± 0.32 d
1.0	49.5 ± 3.15 e	2.2 ± 0.25 c
1.2	67.8 ± 2.85 c	2.5 ± 0.28 b
1.5	82.5 ± 3.92 a	2.9 ± 0.34 a
1.8	70.5 ± 2.45 b	2.2 ± 0.24 c
2.0	55.6 ± 2.24 d	1.5 ± 0.22 f
BAP + GA_3		
1.0 + 0.2	42.5 ± 3.25 d	2.1 ± 0.25 d
1.0 + 0.4	48.5 ± 2.85 c	2.3 ± 0.21 c
1.0 + 0.6	53.5 ± 3.14 a	2.8 ± 0.21 a
1.0 + 0.8	52.2 ± 2.18 b	2.4 ± 0.15 b
1.0 + 1.0	40.2 ± 3.45 de	0.9 ± 0.11 e
2-iP + GA_3		
1.5 + 0.2	72.5 ± 3.65 d	3.0 ± 0.15 c
1.5 + 0.4	79.5 ± 4.25 bc	3.4 ± 0.25 bc
1.5 + 0.6	86.4 ± 3.80 a	4.2 ± 0.86 a
1.5 + 0.8	80.2 ± 2.85 b	3.6 ± 0.55 b
1.5 + 1.0	75.2 ± 2.50 c	2.7 ± 0.45 d

Number of callus cultures tested-85. Values are mean SE of three repeated experiments. Each treatment consisted of 6 replicates. Means within a row followed by the same letters are not significant at P=0.05 according to DMRT.

DISCUSSION

Induction of contamination free seedlings is an important process in plant tissue culture, because throughout the study, the surface sterilized seeds and seedlings derived plant parts were used as explants. To enable seeds to germinate, moistened cotton or filter papers were used either in test tubes or in petri plates (Davidonis and Hamilton, 1983; Trolinder and Goodin, 1998) besides MS medium without phytohormones (Agrawal *et al.*, 1997) was also used for seed germination. Regarding callus induction, similar to our results, in *Melia azedarach*, organogenic callus cultures were initiated by using combination of BAP (4.4 μM) and KN (0.46 μM) and successful regeneration of plantlets was obtained by using the callus cultures (Vila *et al*., 2003). Usually a combination of high amount auxin [2, 4-Dichlorophenoxyacetic (2,4-D) or Naphthaleneacetic acid (NAA)] with low amount cytokinin (BAP or KN) was widely used for the initiation of organogenic callus (Caboni *et al*., 2000; Rugini and Muganu, 1998) and some times cytokinins alone (BAP or KN) was also used for the induction of organogenic callus (Yam *et al*., 1990). Our experiments proved that combined effect of BAP and KN showed best response for organogenic callus induction. During organogenic callus formation, different types of calli with variation in colour and texture were noticed and among them, the compact green calli responded well for the induction of shoots. Individual effect of different concentrations of BAP and KN were also tested for the induction of organogenic callus, unfortunately, in these concentrations, abnormalities like, induction of roots, bulging of explants and necro-

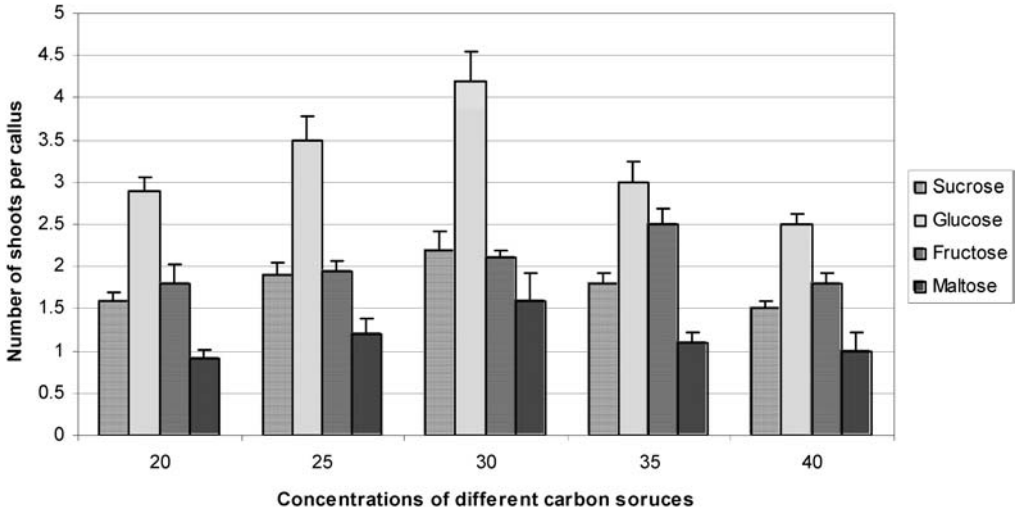

Figure 2. Effect of different carbon sources on shoot proliferation from the shoot tip derived callus of cotton.

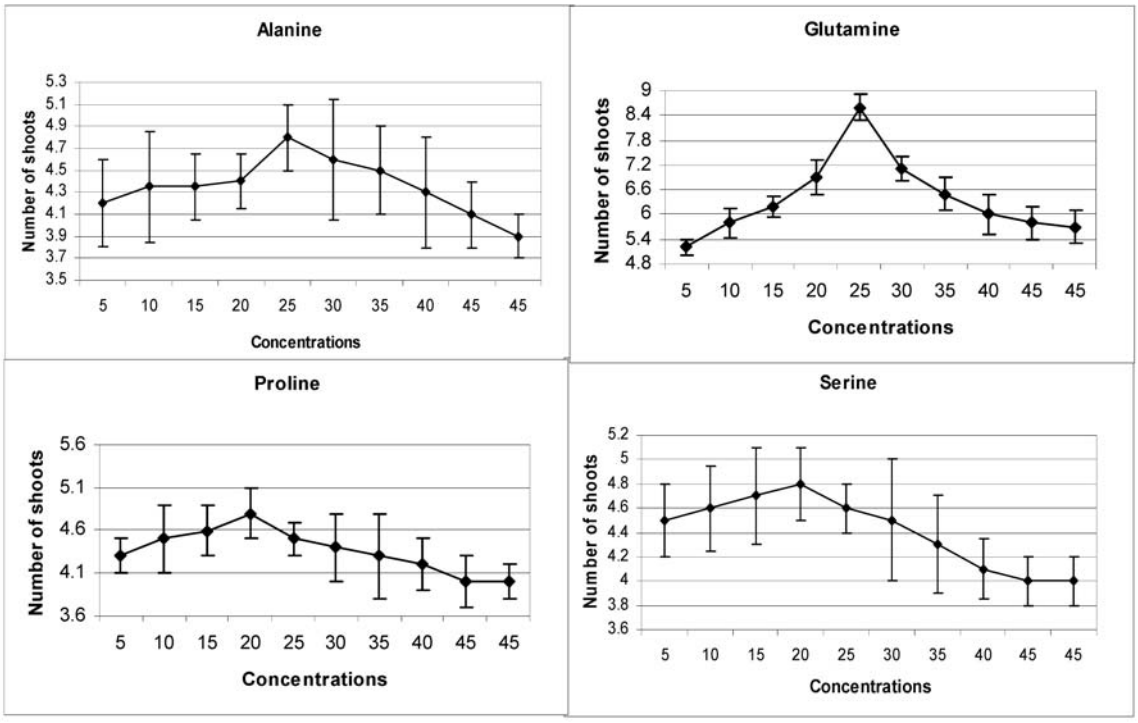

Figure 3. Effect of various concentration of alanine, glutamine, proline and serine (mgl^{-1}) on number of shoots induction per callus clump cultured on the medium fortified with MS salts, B5 vitamins, 2-iP (1.5 mgl^{-1}) and GA_3 (0.6 mgl^{-1}).

sis of tissues were clearly observed. It is also observed that BAP or KN applied alone, induced waste brownish friable callus from shoot tip explants. Recently in *Holostemma adakodien*, basal callus obtained from shoot tip and nodal explants was used for the indirect plant regeneration and maximum of 15 shoots were regenerated through basal callus by using BAP (2.0 mgl^{-1}) on MS medium (Martin, 2002). In the same way organogenic calli and plantlet were obtained from the shoot tip of apple (*Malus domestica*) (Caboni *et al*., 2000) by the combination of BA and NAA. In contradiction to our results, Sudha *et al*. (1998) observed very low percentage of callus induction and shoot initiation from shoot tips and nodal explants of *Holostemma indica*. The excretion of phenolic compounds from explants to the medium was strictly avoided by regular sub-culturing of callus without using any additives.

In our present investigation, 2-iP showed enhanced plantlet proliferation from the callus cultures. Our previous experiments proved that somatic embryogenesis of cotton was accelerated by the addition of 2-iP as one of PGR (Ganesan and Jayabalan, 2004, 2005). In this present investigation, we confirmed that addition of 2-iP also favors shoot organogenesis from the callus cultures of cotton. Similar to this result, shoot multiplication and regeneration was efficiently achieved by 2-iP (0.17 μM) from the callus derived rhizomes of *Cymbidium ensifolium* (Chang and Chang, 2000). In *Fraxinus angustifolia* and *Carica papaya* also 2-iP showed a vital role in the shoot organogenesis from different explant derived callus (Tonon *et al*., 2001; Khatoon and Sultana, 1994). Usually, BAP or KN was widely used for multiple shoot initiation from the callus cultures (Martin, 2002). During multiple shoot proliferation, addition of sucrose (carbon sources) showed enhanced response for multiple shoot proliferation. Similar to our report, in *Coyylus avellana* glucose-mediated shoot multiplication was effectively achieved and maximum of 3-4 shoots were regenerated (Yu and Reed, 1993). Apart from the different carbon sources, addition of glutamine showed superior response for multiple shoot induction from the organogenic callus cultures. Generally, glutamine has been used for the induction of embryogenic callus and direct and indirect induction of somatic embryos (Kim *et al*., 1997; Ritala *et al*., 2001; Ipekci and Gozukirmizi, 2002). But in our work, multiple shoot initiation was effectively accelerated by the addition of glutamine (25 ml^{-l}) as one of the media component.

Usually, addition of GA_3 with shoot proliferation medium was used for the elongation of shoots (Caboni

Table 3. Effect of different concentrations of IBA on root induction of elongated shoots of cotton *Gossypium hirsutum* cv SVPR 2.

Sample No.	IBA (mgl^{-1})	Number of roots / shoot	Response (%)	Root length (cm)	Basal callus formation
1	0.0	0.5 ± 0.01 d	84.5	1.4 ± 0.20	-
2	0.5	2.9 ±0.12 bcd	94.0	20.4 ± 0.15	-
3	1.0	3.2 ± 0.19 ab	91.0	21.8 ± 0.72	-
4	1.5	4.0 ± 0.15 a	95.5	24.5 ± 0.95	+
5	2.0	3.2 ± 0.15 ab	92.5	19.2 ± 0.65	++
6	2.5	3.0 ± 0.03 b	91.0	15.2 ± 1.10	+
7	3.0	2.7 ± 0.15 cd	89.3	11.2 ± 0.85	+

Number of explants tested: 85, Values are mean ± SE of three repeated experiments. Each treatment consisted of 6 replicates. Means within a column followed by the same letters are not significant at P=0.05 according to DMRT.

et al., 2000). But in our study, regular weekly sub-culturing was done on the same medium for complete elongation and it was achieved after total of 12-14 weeks. Wilting of leaves was also controlled by weekly interval subculture. Sometimes supplementation of minimum quantity of BAP was also used for the shoot proliferation from the callus (Pretto and Santarem, 1997; Reddy *et al*., 2002). During multiple shoot proliferation, as per Pretto and Santarem (1997), we maintained the callus cultures in dark room condition also. Unfortunately, there is no significant increase or decrease in number of shoots was observed in the callus cultures.

During root induction, IBA treatment alone showed superior response for induction of roots. Usually root induction in cotton was difficult when compared to other plants. In such cases, rootone was used to induce the roots from elongated shoots in sterilized soil (Gould *et al.*, 1997). Similar to our reports, influence of IBA in root induction has been reported for several plants including *Hemidesmus indicus* (Sreekumar *et al*., 2000) and *Cunila galiodes* (Fracaro and Echeverrigary, 2001). Regarding cotton regeneration, few whole plant regeneration protocols are available for Indian cotton cultivars (Agrawal *et al.*, 1997; Sathyavathi *et al.*, 2002). Compared with the above reports, this protocol leads to active growth and multiplication of cotton plants, because delay in root induction and phenolic excretion from the explants was controlled.

There are several standardized protocols are existing for cotton micropropagation and somatic embryogenesis (Wilkins *et al*., 2000; 2004). Unfortunately, organogenic callus induction and plant regeneration from the callus cultures of cotton was not reported. Our results proved that organogenic callus induction and direct shoot regeneration was possible through callus culture. In conclusion, an efficient and simple protocol for *in vitro* adventitious shoot multiplication from callus cultures, and whole plant regeneration has been described. The protocol was optimized by manipulations of different PGRs, amino acids and carbohydrates for enhanced multiplication. Protocol explained in this research paper provides a rapid plant regeneration system which could be used for the somaclonal variation induction, and producing transgenic plants in cotton through *Agrobacterium* and biolistic methods.

References

Agrawal DC, Banerjee AK, Kolala RR, Dhage AB, Kulkarni V, Nalawade M, Hazra S, Krishnamurthy KV (1997). *In vitro* induction of multiple shoots and plant regeneration in cotton (*Gossypium hirsutum* L.). *Plant Cell Rep*. 16: 647-652.

Benedict JH, Altman DW (2001). Commercialization of transgenic cotton expressing insecticidal crystal protein. In: Jenkins JN, Saha S (eds) *Genetic improvement of cotton*. USDA-ARS, Oxford & IBH, New Delhi, 136-201.

Caboni E, Lauri P, Angeli SD (2000). *In vitro* plant regeneration from callus of shoot apices in apple shoot culture. *Plant Cell Rep*. 19: 755-760.

Chang C, Chang WC (2000). Micropropagation of *Cymbidium ensifolium* var. Misericors through callus-derived rhizomes. *In vitro Cell Dev Biol Plant*. 36: 517-520.

Davidonis GH, Hamilton RH (1983). Plant regeneration from callus tissue of *Gossypium hirsutum* L. *Plant Sci Lett*. 32: 89-93.

Fracaro F, Echeverrigaray S (2001). Micropropagation of *Cunila galioides*, a popular medicinal plant of south brazil. *Plant Cell Tiss Org*. 64: 1-4.

Ganesan M, Jayabalan N (2005). Carbon source dependent somatic embryogenesis and plant regeneration in cotton, *Gossypium hirsutum* L. cv. SVPR2 through suspension cultures. *Indian J*

Exp Biol. 43: 921-925.

Ganesan M, Jayabalan N (2004). Evaluation of haemoglobin (erythrogen): for improved somatic embryogenesis and plant regeneration in cotton (*Gossypium hirsutum* L. cv. SVPR 2) *Plant Cell Rep*. 23: 181-187.

Gould J, Zhou Y, Shen Y, Magallanes-Cedeno M, Luo J (1997). Shoot apex transformation of cotton using *Agrobacterium*. *Beltwide Cotton Production Conferences, National Cotton Council, Memphis TN*. pp. 432-434.

Ipekci Z, Gozukirmizi N (2002). Indirect somatic embryogenesis and plant regeneration from leaf and internode explants of *Paulownia elongate*. *Plant Cell Tiss Organ Cul.* 79: 341-345.

Khatoon K, Sultana R (1994). Plant regeneration from *Carica papaya* cv. Malir. grown in tissue culture. *Pak J Bot*. 26: 191-195.

Kim YW, Youn Y, Noh ER, Kim JC (1997). Somatic embryogenesis and plant regeneration from immature embryos of five families of *Quercus acutissima*. *Plant Cell Rep*. 16: 869-873.

Martin KP (2002). Rapid propagation of *Holostemma ada-kodien* Schult., a rare medicinal plant, through axillary bud multiplication and indirect organogenesis. *Plant Cell Rep*. 21: 112-117.

Murashige T, Skoog F (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol Plantarum.* 15: 473-497.

Pretto FR, Santarem ER (1997). Callus formation and plant regeneration from *Hypercium perforatum* leaves. *Plant Cell Tiss Org*. 62: 107-113.

Price HJ, Smith RH (1979). Somatic embryogenesis in suspension cultures of *Gossypium klotzschianum* Anders. *Planta,* 145: 305-307.

Reddy PS, Rodrigues R, Rajasekharan R (2002). Shoot organogenesis and mass propagation of *Coleus forskohlii* from leaf derived callus. *Plant Cell Tiss Org*. 66 : 183-188.

Ritala A, Mannonen L, Oksman-Caldentey KM (2001). Factors affecting the regeneration capacity of isolated barley microspores (*Hordeum vulgare* L.) *Plant Cell Rep.* 20 : 403-407.

Rugini E, Muganu M (1998). A novel strategy for the induction and maintenance of shoot regeneration from callus derived from established shoots of apple (*Malus domestica* Borkh. cv Goldern Delicious. *Plant Cell Rep*. 17: 581-585.

Sathyavathi VV, Prasad V, Gita Lakshmi B, Lakshmi Sita G (2002). High efficiency transformation protocol for three Indian cotton varieties *via Agrobacterium tumifaciens. Plant Sci.* 162: 215-223.

Sreekumar S, Seeni S, Pushpangadan P (2000). Micropropagation of *Hemidesmus indicus* for cultivation and production of 2-hydroxy 4-methyl benzaldehyde. *Plant Cell Tiss Org*. 62: 211-218.

Sudha CG, Krishnan PN, Pushpangadan P (1998). *In vitro* propagation of *Holostemma annulare* (Roxb.) K. Schum. a rare medicinal plant. *In vitro Cell Dev Biol Plant.* 33: 57-63.

Tonon G, Capuana M, Di Marco A (2001). Plant regeneration of *Fraxinus angustifolia* by *in vitro* shoot organogenesis. *Sci Horti.* 87: 291-01.

Trolinder NL, Goodin JR (1998). Somatic embryogenesis in cotton (*Gossypium*) 1-effect of source of explant and hormone regime. *Plant Cell Tiss Org*. 12: 31-42.

Vila SK, Gonzalez AM, Rey HY, Mroginski LA (2003). *In vitro* Plant Regeneration of *Melia azedarach* L.: Shoot Organogenesis from Leaf Explants. *Biologia Plantarum*. 47: 13-19.

Wilkins TA, Mishra R, Trolinder NL (2004). Agrobacterium-mediated transformation and regeneration of cotton. *Food Agri Environ.* l2: 179-187.

Wilkins TA, Rajasekaran K, Anderson DM (2000). Cotton Biotechnology. *Crit Rev Plant Sci.* 19: 511-550.

Yam TW, Webb EL, Arditti J (1990). Callus formation and plantlet development from axillary buds of taro. *Planta.* 180: 458-460.

Yu X, Reed BM (1993). Improved shoot multiplication of mature hazelnut (*Corylus avellana* L.) *in vitro* using glucose as a carbon source. *Plant Cell Rep*. 12: 256-259.