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Rapid production of multiple shoots from cotyledonary node explants of an elite cotton (*Gossypium hirsutum* L.) variety

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***In vitro* sprouting and simultaneous elongation of multiple shoots were achieved by culturing cotyledonary node explants of an elite cotton (*Gossypium hirsutum* L.) variety (NC-601), on Murashige and Skoog basal medium containing N^6-Benzylaminopurine, 6- Furfurylaminopurine and Thidiazuron in combination with 1-Naphthaleneacetic acid. A combination of N^6-Benzylaminopurine (1.5mg/l) and 1-Naphthaleneacetic acid (0.5mg/l) was found most effective for producing maximum number (10.31±0.22) of multiple shoots per explant. Although the explants of different sets of seedlings could produce multiple shoots, the explants derived from the 15-day old seedlings show maximum response compared to 5- and 10-day old nodal explants. Furthermore, maximum frequency (82.6%) of rooting was observed in the elongated shoots when cultured on the half-strength MS medium containing Indole-3-butyric acid. Regenerated plants could survive upto 95 to 100% under the glass house conditions. Following this procedure, plants were obtained within a period of 12 to 15 weeks. Normal flowering and boll formation were observed in regenerated plants similar to that of control plants obtained through seed germination.**

Keywords: Cotton, cotyledonary node, multiple shoots, explant age, simultaneous elongation of shoots

The genus *Gossypium* comprises of 51 species, out of them, 46 species are diploid ($2n=2x=26$) while 5 species are tetraploid ($2n=2x=52$) (Fryxell, 1992). Tetraploid upland cotton (*G. hirsutum*), 2n=52, the world's premier source of natural fiber is cultivated in 80 nations, with an annual global economic impact of $500 billion and provides livelihood to more than 180 million people (Chakravarthy *et al*., 2012). Besides being the backbone of the textile industry, cotton and its byproducts are also part of the livestock food, seed-oil, fertilizers, paper and other consumer products. Extracts of cotton plants have been used medicinally for treating hypertension (Hasrat *et al*., 2004). Gossypol present in the cotton tissues has been found to show anti-cancerous activity, besides its use as a male contraceptive (Coutinho, 2002; Moon *et al*., 2008).

Because of its immense economic importance considerable attention has been paid for the genetic improvement of this species through conventional breeding methods. However, due to the narrow genetic base of *G. hirsutum,* not much success has been achieved to improve its agronomic traits such as resistance to pests and pathogens. It is feasible to overcome these limitations through genetic

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engineering approaches, which are primarily dependent on reliable and reproducible *in vitro* regeneration systems. *In vitro* regeneration of cotton has been a difficult goal to achieve, because morphogenic response is genotype dependent and most of the elite cultivars are recalcitrant. Initial attempts to regenerate cotton using embryos (Beasley, 1940) and protoplasts (Bhojwani *et al*., 1977) proved unsuccessful. Davidonis and Hamilton, (1983) demonstrated first successful plantlet regeneration through somatic embryogenesis using Coker 310 variety. However, regeneration of plants via embryogenesis is restricted to limited number of cultivars and efforts made to increase the range of cultivars that can produce somatic embryos have met with low success rate (Sakhanokho *et al*., 2005). Regeneration of cotton plants from apical meristems, cotyledonary nodes and embryonic axes have been attempted to gain genotype independence (Agrawal *et al*., 1997; Ouma *et al*., 2004a; Morre *et al*., 1998).

Although, several reports pertaining to direct regeneration of shoots have been published, these procedures however necessitated additional sub-culturing steps to achieve elongation of multiple shoots besides low number of shoots per explant. Thus, there is an need for development of a rapid regeneration system, which not only enables the production of high number of multiple shoots but also facilitates simultaneous elongation of shoots in relatively shorter periods of time. The present communication deals with the observations pertaining to the development of an efficient protocol for rapid multiplication and simultaneous elongation of multiple shoots from the cotyledonary node explants of an elite cotton variety.

Materials and methods

Plant material

Seeds of cotton variety (NC-601) obtained from Swarna Bharathi Biotech Pvt., Ltd., Hyderabad, were delinted with concentrated sulphuric acid and thoroughly rinsed under running tap water for about 20 min and kept for drying. Later, the dried seeds were mixed with bavestein and stored for further use. For germination, seeds were surface sterilized with 0.1 % (w/v) aqueous mercuric chloride solution for 10 to 12 min, followed by 3 rinses of 1 min duration each with sterile distilled water. The sterilized seeds were then allowed to imbibe in sterile distilled water in dark for 4h at 30±2^{0}C prior to inoculation into petridishes containing half-strength Murashige and Skoog (MS) (Murashige and Skoog, 1962) basal medium. Later, seeds with emerging radicals were transferred to the culture tubes containing the similar medium and allowed for seedling development. All the cultures were maintained in the culture room at 25±2°C under cool white fluorescent light intensity of 3000 lux.

Media preparation

Media used for seed germination contained half-strength MS salt formulation with 1.5% maltose. The media used for multiple shoot induction (MMS0-MMS15) was prepared by adding full-strength MS salt formulation with 3% maltose and the media used for root induction contained half-strength MS salt formulation with 2% maltose. The pH of the media was adjusted to ~5.8 with 0.1N NaOH before adding 0.8% agar and sterilized by autoclaving at a pressure of 1.1 kg cm^{-2} at 121^{o}C for 20 min. All plant growth regulators used in this study were added prior to autoclaving except Thidiazuron (TDZ).

Explant preparation and optimization of culture conditions

Cotyledonary node explants were excised from 5,10 and 15 days old *in vitro* raised seedlings and where kept on MS basal medium augmented with different concentrations of cytokinins. A total of 15 different media formulations were used for induction of multiple shoots and were designated as MMS1 to MMS5 containing N^6-Benzylaminopurine (BAP), MMS6 to MMS10 containing 6-Furfurylaminopurine

(KN) and MMS11 to MMS15 containing TDZ (1-3mg/l). In all the media combinations (MMS1 to MMS15), 0.5mg/l 1-Naphthaleneacetic acid (NAA) was included. A control experiment without any plant growth regulator (MMS0) was also conducted parallelly. Experiments were replicated thrice using 50 explants each and data were recorded after four-weeks.

Rooting of *in vitro* shoots and acclimatization

To identify the optimal auxin concentration for induction of early and healthy root system, shoots (4 to 5 cm long) regenerated from cotyledonary node explants were excised and individually transferred to half-strength MS medium containing 2% maltose and different concentrations of Indole-3-butyric acid (IBA), NAA and Indole-3-acetic acid (IAA) (0.25, 0.50, 1.0 and 1.5 mg/l). A control experiment without any plant growth regulator (MS0) was also conducted parallelly. Experiments were replicated thrice using 25 explants each and data were recorded after four-weeks. Plantlets with well developed root and shoot system were carefully removed from the culture tubes and washed with tap water to remove the adhering medium and transferred to the plastic pots containing sand, soil and vermiculite (1:1:1) for acclimatization. After two weeks, plants were transferred to pots containing the black soil and maintained in the glass house.

Statistical analyses

Latin square experimental design was followed for the experiments. Statistical analyses of data such as mean, standard error, univariate analyses of variance (one-way ANOVA) and multiple comparisons were performed using SPSS version 16.0 (USA) statistical packages.

Results and Discussion

Effect of explant age and media composition on shoot regeneration

For germination of seeds, sterilized preimbibed (sterile water for 4h at 30±2^{0}C) cotton seeds were inoculated on half-strength MS basal medium and kept overnight in dark at 25±2^{0}C. Later, the seeds with emerging radicals were transferred into culture tubes containing the same medium for seedling development. This procedure resulted in significant increase (90 to 95%) in the seed germination in comparison with the poor (25 to 30%) germination observed in the unimbibed seeds. The cotyledonary nodal explants, cultured for multiple shoot induction on MS basal medium supplemented with BAP/KN/TDZ and with NAA, showed a cluster of elongated and healthy shoots after four weeks of culture (Fig 1A,B,C). One way ANOVA for interactions of main effects (age of explants, different growth regulators and concentrations of growth regulators), two factor interactions (age of explants / different growth regulators, age of explants / concentrations of growth regulators, and different growth regulators / concentrations of growth regulators) and three factor interactions (age of explants / different growth regulators / concentrations of growth regulators) indicated that the axillary bud proliferating ability of the explant varied depending on the type and concentration of plant growth regulator and age of the explant (Table 1). Although, cotyledonary nodal explants cultured on the MS basal medium devoid of plant growth regulators produced an average of 1 to 2 shoots, a further proliferation of these shoots was not observed. This may be perhaps due to the ability of cytokinins to promote cell division. These results are in agreement with the findings made using nodal explants of Brahmi, where the presence of cytokinins induced a higher number of multiple shoots than the medium without phytohormone treatments (Asha *et al*., 2013).

BAP at a concentration of 1.5 mg/l along with 0.5 mg/l NAA showed 100% response and produced maximum number of multiple shoots ranging from 7.76±0.15 to 10.31±0.22 shoots/explant for 5,10 and 15-day old explants tested (Table 2).

Table 1. Univariate analyses of variance (one way ANOVA) for effect of age of explant, type of growth regulator and concentration of growth regulators on multiple shoot induction from cotyledonary nodes of cotton

Source	Sum of squares	*df*	Mean square	*F*
Age*(days)	25.593	2	12.797	196.156
Treatment* (growth regulators)	335.065	2	167.533	2568.061
Concentration* (mg/l)	395.437	5	79.087	1212.308
Age X Treatment**	9.113	4	2.278	34.922
Age X Concentration**	6.813	10	0.681	10.444
Treatment X Concentration**	96.180	10	9.618	147.432
Age X Treatment X Concentration***	11.155	20	0.558	8.550

All interactions are significant at 0.05% level; *response due to main effects, ** response due to two factor interaction, *** response due to three factor interaction, X: interaction between

Table 2. Effect of BAP, KN, TDZ, NAA and age of explants on regeneration from the cotyledonary nodes of cotton

Medium code	Growth regulators (mg/l)				Seedling age					
					5 days old		10 days old		15 days old	
	BAP	KN	TDZ	NAA	Frequency of explant response (%)	No. of multiple shoots/ explant (Mean ± SE)	Frequency of explant response (%)	No. of multiple shoots/explant (Mean ± SE)	Frequency of explant response (%)	No. of multiple shoots/explant (Mean ± SE)
MMS0	--	--	--	--	62.6	0.90 ± 0.08	65.3	0.94 ± 0.12	72.0	1.03 ± 0.12
MMS1	1.0	--	--	0.5	85.3	4.38 ± 0.24	87.3	5.36 ± 0.22	95.3	7.14 ± 0.32
MMS2	1.5	--	--	0.5	100.0	7.76 ± 0.15	100.0	9.52 ± 0.24	100.0	10.31± 0.22
MMS3	2.0	--	--	0.5	90.6	6.62 ± 0.22	99.3	7.22 ± 0.12	99.3	8.02 ± 0.30
MMS4	2.5	--	--	0.5	87.3	5.82 ± 0.15	96.6	6.49 ± 0.27	97.3	6.90 ± 0.26
MMS5	3.0	--	--	0.5	87.0	4.56 ± 0.28	92.0	5.69 ± 0.21	96.0	6.40 ± 0.25
MMS6	--	1.0	--	0.5	61.6	1.42 ± 0.14	69.3	1.92 ± 0.11	61.3	2.14 ± 0.24
MMS7	--	1.5	--	0.5	66.6	1.94 ± 0.11	67.3	3.13 ± 0.18	68.0	4.00 ± 0.24
MMS8	--	2.0	--	0.5	75.0	3.55 ± 0.15	68.0	3.28 ± 0.20	76.0	5.05 ± 0.31
MMS9	--	2.5	--	0.5	67.3	2.50 ± 0.11	64.0	2.18 ± 0.16	62.6	3.54 ± 0.27
MMS10	--	3.0	--	0.5	57.3	2.14 ± 0.14	57.3	1.74 ± 0.17	58.6	2.28 ± 0.17
MMS11	--	--	1.0	0.5	65.3	2.72 ± 0.20	59.3	2.57 ± 0.27	66.6	3.18 ± 0.18
MMS12	--	--	1.5	0.5	68.0	3.72 ± 0.17	74.0	4.04 ± 0.28	74.6	4.32 ± 0.23
MMS13	--	--	2.0	0.5	81.3	5.10 ± 0.22	78.0	5.27 ± 0.11	89.3	6.02 ± 0.16
MMS14	--	--	2.5	0.5	77.3	4.63 ± 0.34	75.3	4.28 ± 0.27	76.0	3.74 ± 0.24
MMS15	--	--	3.0	0.5	62.0	2.78 ± 0.15	68.0	2.74 ± 0.18	69.3	3.40 ± 0.21

Data represents means from the replicates ± SE; Number of shoots per explant and percentage response was scored after four-week of culture; BAP: N^6-Benzylaminopurine, KN: 6-Furfurylaminopurine, TDZ: Thidiazuron, NAA: 1- Naphthalene acetic acid, SE: Standard error

The shoots produced on BAP-containing medium showed smaller leaves with larger internodes (Fig 1A). An increase in the BAP concentration upto 3.0 mg/l in the culture medium resulted in decreased mean number of multiple shoots (Table 2), besides abnormal morphology and vitrification of the tissues. Similar observations were reported with high concentrations of BAP from the cotyledonary node explants of *Pisum sativum* (Jackson and Hobbs, 1990). In the present study, BAP proved superior to TDZ and KN in inducing maximum number of shoots as well as frequency of explant response (Tables 2&3). The superior effect of BAP on multiple shoot formation from cotyledonary nodes indicates its greater specificity towards cytokinin receptors present in the explants. Similar observations were also made in cotton (Agrawal *et al*., 1997), Brahmi (Asha *et al*., 2013).

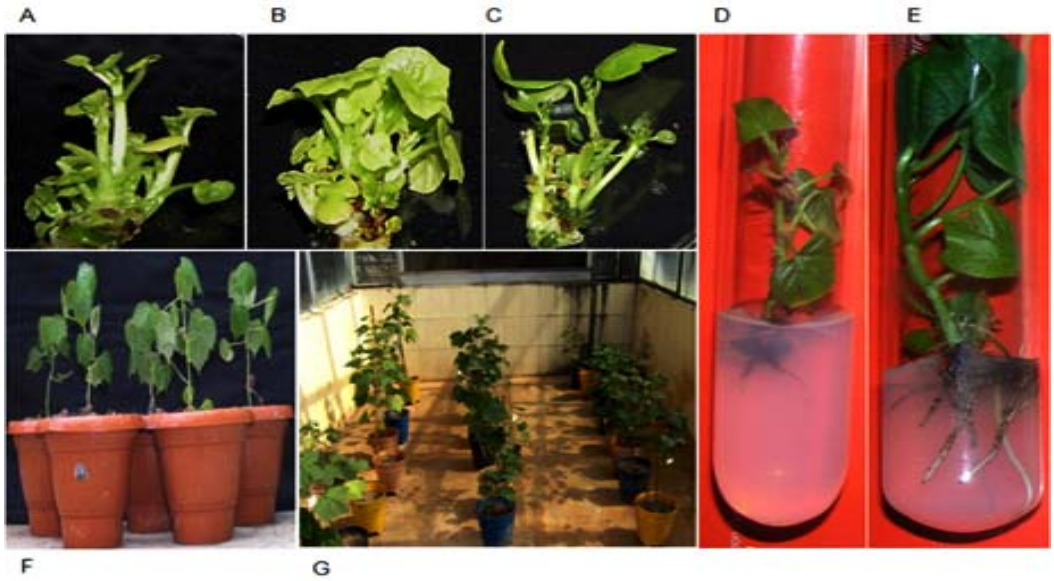

Figure: 1. Plant regeneration from cotyledonary node explants of cotton (*G. hirsutum*) NC-601 : A Multiple shoots produced on medium containing BAP-1.5 mg/l and NAA 0.5 mg/l; B Multiple shoots produced on medium containing TDZ 2.0 mg/l and NAA 0.5 mg/l; C Multiple shoots produced on medium containing KN 2.0 mg/l and NAA 0.5 mg/l; D Root formation in shoot inoculated on growth regulator free half-strength MS medium; E Plantlet with well developed root system on half-strength MS medium containing IBA 1.5mg/l; F Hardening of regenerants; G Tissue culture raised plants at maturity in glasshouse.

Table 3. Multiple comparisons for the effects of BAP, KN and TDZ on induction of multiple shoots from cotyledonary nodes of cotton

Treatment (A)	Treatment (B)	Mean difference (A-B)
BAP	KN	3.4189*
BAP	TDZ	2.4448*
KN	BAP	-3.4189*
KN	TDZ	-0.9741*
TDZ	BAP	-2.4448*
TDZ	KN	0.9741*

The mean difference is significant at 0.05 % level*; BAP: N^6-Benzylaminopurine, KN: 6- Furfurylamino-purine, TDZ: Thidiazuron

Cotyledonary node explants cultured on TDZ (2 mg/l) containing medium produced a mean number of 6.02±0.16 shoots / explant from 15-day old seedlings, whereas explants from 5 and 10 days old seedlings produced 5.10±0.22 and 5.27±0.11 shoots, respectively. The shoots produced on TDZ-containing medium were closely arranged with rosette like appearance and leaves of the shoots were similar to those of multiple shoots produced on BAP-containing medium (Fig 1B). Although TDZ played a vital role in shoot regeneration, higher concentrations of TDZ often resulted in the decreased number of shoots per explant and percentage response, due to the formation of watery callus at the base of the explants.

The frequency of explants responding to KN was found to be moderate at all the concentrations tested (Table 2). However, KN at a concentration of 2.0 mg/l gave maximum number of 3.55±0.15 and 5.05±0.31 shoots from the explants of 5 and 15-day old seedlings, respectively. Multiple shoots produced on KN-containing medium were thick with long internodes and larger leaves, besides secondary shoot bud formation from internodal regions (Fig 1C). However, increased concentrations of kinetin resulted in the suppression of shoot sprouting and produced wrinkled leaves. A similar response was observed in earlier studies

made on *Dianthus caryophyllus* (Saher *et al*., 2004). Although explants derived from 5,10 and 15-day old seedlings could produce multiple shoots with the three cytokinins tested, multiple comparison analysis revealed that explants from 15-day old seedlings proved to be more responsive than others (Table 4).

Table 4. Multiple comparisons for the effects of age of explants on induction of multiple shoots from cotyledonary nodes of cotton

Days (A)	Days (B)	Mean difference (A-B)
5	10	-0.2970*
5	15	-0.9515*
10	5	0.2970*
10	15	-0.6544*
15	5	0.9515*
15	10	0.6544*

*The mean difference is significant at 0.05 % level

In this study, the presence of NAA (0.5 mg/l) along with BAP/KN/TDZ in the medium caused simultaneous elongation of multiple shoots without further sub-culturing for shoot elongation (Fig 1A, B, C). Recently, Sanghera (Sanghera *et al*., 2012) reported production of multiple shoots from the shoot tip explants of cotton, but this procedure required additional four-week subculturing step to achieve shoot elongation. Moreover, the number of responding explants and multiple shoots produced were low in comparison to the present study. The repeated subculturing steps adopted in this study to achieve shoot elongation considerably increased the time required to regenerate complete plants. The decrease in the time span observed in the present study may be attributed to the synergistic effects of auxin and cytokinins used. Similar results were reported earlier in *Pterocarpus marsupium* (Chand and Singh, 2004), where the incorporation of NAA along with BAP in the regeneration medium promoted the induction of healthy and vigorously growing shoots. By adopting this method, cotton plants could be established in the net house within 12 to 15 weeks as compared to 16 to 20 weeks reported in earlier studies (Abdelletef and Khalafalla, 2007; Ali *et al*., 2004).

Induction of rooting, hardening and establishment of regenerated cotton plants

Shoots cultured on the growth-regulator free half-strength MS medium showed weak root system (Fig 1D). These roots, however, failed to proliferate and the plantlets formed on this media were stunted with few leaves. Whereas, the shoots cultured on half-strength MS medium supplemented with auxins (IBA /NAA /IAA) have shown well developed root system, and the plantlets produced were healthy with expanded leaves and elongated stems (Fig 1E). The vigorous growth of plantlets could be due to optimum utilization of nutrients present in the medium by the well established root system. The frequency of rooted shoots varied from 25.3 to 49.3% in IAA, 37.3 to 62.6% in NAA and 49.3 to 82.6% in IBA containing medium (Fig 2). The variation observed in the effectiveness of different auxins may be attributed to their differential affinity to the auxin receptors involved in the rhizogenesis of the shoots. Earlier it was reported that, efficiency of the auxin to trigger root formation is dependent on several factors such as affinity for the auxin receptor protein involved in rooting, the concentration of free auxin that reaches target competent cells, the amount of endogenous auxin, besides differences in uptake and metabolism (Fogac and Neto, 2005). The superiority of IBA over other auxin sources for *in vitro* root formation, observed in the present investigation, is consistent with the earlier findings in cotton (Ouma *et al*., 2004b) and banana (Govindaraju *et al*., 2012). Plantlets with well developed root system were transferred to plastic pots containing sand, soil and vermiculite (1:1:1). After two weeks of acclimatization, the plants were transferred to the pots containing black soil and shifted to the glass house for further growth (Fig 1G).

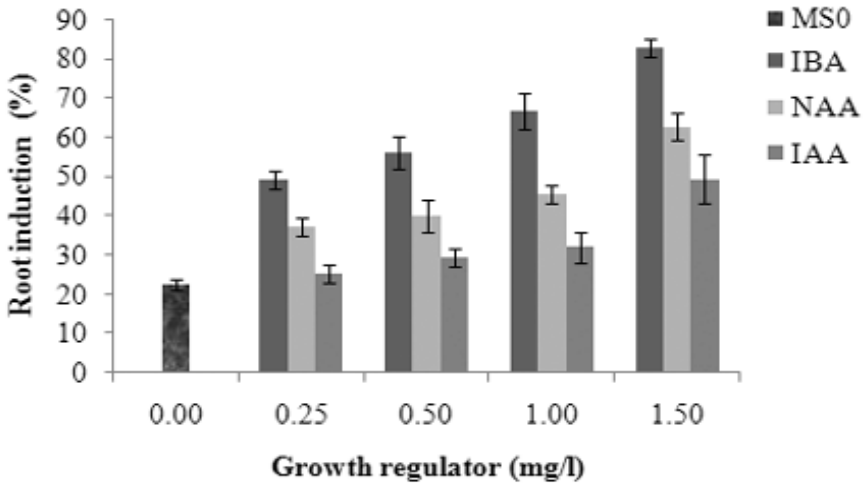

Figure: 2. Effect of different concentrations of IBA, NAA and IAA on root formation from the *in vitro* derived shoots of cotton (*G. hirsutum*) NC-601

Conclusions

The present study reports the development of a rapid and reproducible regeneration system for induction and simultaneous elongation of multiple shoots using cotyledonary node explants of elite cotton variety NC-601. Elimination of subculturing steps involved in the elongation of shoots has considerably shortened the time (4 to 5 weeks) taken from explanting to the establishment of plants in the glasshouse. This procedure will facilitate the application of genetic engineering to incorporate potent candidate genes into elite cotton varieties.

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References

Abdelletef E and Khalafalla MM. 2007. Adventitious shoot and plantlet formation in medium staple cotton cultivar (*Gossypium hirsutum* L. cv Barac [67] B). *Int. J. Agric. Biol*. 6:913-916.

Agrawal DC, Banerjee AK, Kolala RR, Dhage AB, Kulkarni WV, Nalawade SM, 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.

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

Asha KI, Devi AI, Dwivedi NK, Nair RA. 2013. *In vitro* regeneration of Brahmi (*Bacopa monnieri* (Linn.) Pennell)-an important medicinal herb through nodal segment culture. *Res. Plant Biol*. 3: 01-07.

Beasley JO. 1940. The production of polyploids in *Gossypium*. *J. Hered*. 31:39-48.

Bhojwani SS, Power JB, Cocking EC. 1977. Isolation, culture and division of cotton callus protoplasts. *Plant Sci. Lett*. 8:85-89.

Chakravarthy VSK, Reddy TP, Reddy VD, Rao KV. 2012. Current status of genetic engineering in cotton (*Gossypium hirsutum* L): an assessment. *Crit. Rev. Biotechnol*. DOI: 10.3109/ 07388551.2012.743502.

Chand S and Singh AK. 2004. *In vitro* shoot regeneration from cotyledonary node

explants of a multipurpose leguminous tree, *Pterocarpus marsupium* Roxb. *In Vitro Cell Dev. Biol.-Plant*, 40:167-170.

Coutinho EM. 2002. Gossypol: A contraceptive for men. *Contraception*, 65:259-63.

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

Fogac CM and Neto AG. 2005. Role of auxin and its modulators in the adventitious rooting of Eucalyptus species differing in recalcitrance. *Plant Growth Regul*. 45:1-10.

Fryxell PA. 1992. A revised taxonomic interpretation of *Gossypium* L. (Malvaceae). *Rheedea*. 2:108-165.

Hasrat JA, Pieters L, Vlietinck AJ. 2004. Medicinal plants in suriname: hypotensive effect of *Gossypium barbadense*. *J. Pharma. Pharmacol*. 56:381-387.

Jackson JA and Hobbs SLA. 1990. Rapid multiple shoot production from cotyledonary node explants of Pea (*Pisum sativum*). *In vitro Cell Dev. Biol.-Plant*, 26:835-838.

Govindaraju S, Saravanan J, Jayanthi B, Nancy D, Arulselvi IP. 2012. *In vitro* propagation of Banana (*Musa sp*-Rasthali variety) from sword suckers for its commercial production. Res. Plant Biol. 2:01-06.

Moon DO, Kim MO, Lee JD, Kim GY. 2008. Gossypol suppresses NF-kB activity and NF-kB-related gene expression in human leukemia U937 cells. *Cancer Lett*. 264:192-200.

Morre JL, Permingeat HR, Romagnoli MV, Heisterborg CM, Vallejos RH. 1998. Multiple shoot induction and plant regeneration from embryonic axes of cotton. *Plant Cell Tiss. Organ Cult*. 54:131-136.

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

Ouma JP, Young MM, Reichert NA. 2004a. Optimization of *in vitro* regeneration of multiple shoots from hypocotyls sections of cotton (*Gossypium hirsutum* L.). *Afr. J. Biotechnol*. 3:169-173.

Ouma JP, Young MM, Reichert NA. 2004b. Rooting of *in vitro* regenerated cotton (*Gossypium hirsutum* L.) is influenced by genotype, medium composition, explant type and age. *Afr. J. Biotechnol*. 3:313-318.

Saher S, Piqueras A, Hellin E, Olmos E. 2004. Hyperhydricity in micropropagated carnation shoots: the role of oxidative stress. *Physiol. Plant*. 120:152-161.

Sakhanokho HF, Ozias PA, May OL, Chee PW. 2005. Putrescine enhances somatic embryogenesis and plant regeneration in upland cotton. *Plant Cell Tiss. Organ Cult*. 81:91-95.

Sanghera GS, Gill MS, Singh G, Gosal SS. 2012. *In Vitro* plant regeneration through multiple shoot induction in cotton (*Gossypium* spp.). *Elixir Appl. Botany*, 43:6870-6876.