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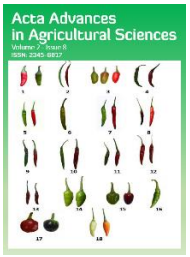


Research Article

# Cytokinins improve shoot regeneration efficiency in two Indian cotton (*Gossypium hirsutum* cv. Narashima and *G. arboreum* cv. AKA-7) cultivars

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ABSTRACT

In the present investigation, regeneration of the two elite Indian cotton cultivars *Gossypium hirsutum* L. cv. Narashima and *Gossypium arboreum* L. cv. AKA-7 is reported using hypocotyl, cotyledon and shoot tip explants. The explants were cultured on MS (Murashige and Skoog, 1962) medium supplemented with twelve different combinations and concentrations of 6-benzyl amino purine (BAP) and kinetin (KIN), and the shoot regeneration was investigated. High shoot regeneration (86 and 90%) as well as shooting efficiency (83.8 and 85%) was obtained with shoot tip explant on medium containing BAP (1 mg/l) and KIN (1 mg/l) in cv. AKA-7 and Narashima, respectively. Generally, almost in all treatments where BAP and KIN were applied alone or in combinations, shoot tip explants gave higher shoot regeneration efficiency percentage than hypocotyl and cotyledon explants. Elongation of multiple shoots was achieved on MS medium supplemented with $GA_3$ (0.1 mg/l). Shoots successfully rooted on MS hormone free medium followed by hardening and acclimatization of regenerated plantlets. Fully developed cotton plants were obtained only after eight weeks of *in vitro* culture. The described protocol is highly efficient, rapid and reproducible and could be used for genetic transformation of cotton plants.

**Abbreviations:** Analysis of Variance (ANOVA), 6-Benzyl amino purin (BAP), Duncan's Multiple Range Test (DMRT), Gibberellic Acid ($GA_3$), Kinetin (KIN), Indole-3-butyric Acid (IBA), Murashige and Skoog (MS), Polyvinyl Pyrrolidone (PVP).



## Introduction

Cotton (*Gossypium hirsutum* L.) is one of the most important agricultural crops in India. Sixty million people including 4.5 million farmers in India are living depend of cotton (Bennett et al., 2004). Genetic improvement of cotton through conventional breeding is limited by several factors such as lack of useful variation and time consuming. Although Genetic transformation is an attractive tool for improving plant traits, this technique considers an effective regeneration system. Unlike other numerous commercial crops, to our knowledge,

there is no efficient regeneration protocol of cotton for regeneration. Davidonis and Hamilton (1983) obtained plantlet regeneration via somatic embryogenesis from callus culture of *G. hirsutum*. However, described regeneration protocol required a long culture period, and results were not reproducible either. Since then, several reports have described time consuming protocols for regeneration of different cotton cultivars (Agrawal et al. 1997; Gupta et al. 1997, Zhang et al. 2000, 2001; Leelavathi et al. 2004; Jin et al. 2006). Genotype analysis in cotton tissue culture showed that only a limited number of varieties can produce somatic embryos and regenerate plants *in vitro*. The best *in vitro* results were obtained with the lines of no longer cultivated Coker varieties (Feng et al. 1998).

Establishment of efficient regeneration protocol is a prerequisite for genetic manipulation of crop plants. So far, the transgenic cotton plants with desirable agronomic traits are restricted to only a few cotton genotypes. Cotton is a warm season (tropical) crop. It can be profitably grown in the regions having rainfall of 850-1100 mm (Perlak et al. 1990). Unfortunately none of the Indian cotton cultivars could be manipulated for producing transgenic plants due to lack of suitable regeneration protocol. Therefore, we developed an efficient *in vitro* shoot regeneration protocol of two Indian cotton cultivars. To the best of our knowledge, there is no report on plant regeneration from shoot tip culture of elite Indian cotton cultivars.

In the present study, we have established a rapid and highly efficient *in vitro* regeneration protocol of cotton cultivars, fully developed plantlets of two Indian cotton cultivars Narashima and AKA-7 obtained after only 8 weeks. This protocol can be very useful in future genetic transformation experiments of different cotton genotypes.

## Materials and Methods

### *Plant material*

Seeds of two elite Indian cotton, *G. hirsutum* cv. Narashima and *G. arboreum* cv. AKA-7 were obtained from Indian Agriculture Research Institute in New Delhi.

### *Seed sterilization and germination*

Seeds of cv. Narashima and AKA-7 were rinsed with sulphuric acid (100 ml/kg), and washed three times with distilled water containing Tween-20 (100 µl/100ml) for 20 min with continuous shaking. The seeds were further rinsed with sterile double distilled water four to five times in order to remove the detergent completely. Surface seed sterilization included 70% ethanol (1 min) followed by washing the seeds with sterile double distilled water (three times). Cotton seeds were then treated with 0.2% solution of mercuric chloride for 2 min. and further washed subsequently at least three times with sterile double distilled water. The sterilized seeds were then transferred to Patri plates containing wet filter paper and kept at 28 ± 2°C. After 2 days, the germinated seeds were transferred in culture bottles (five seeds per bottle) containing MS (Murashige and Skoog 1962) medium without any growth regulator.

### *Experimental design and culture condition*

From 8-10 days old seedlings of both cultivars cotyledon, shoot tip and hypocotyls explants were isolated. Initially, all the explants were cultured on MS medium (Murashige and Skoog 1962) supplemented with activated charcoal (1g/l). After 5-7 days the explants were transferred on MS media supplemented with different concentrations of 6-Benzyl amino purin (BAP) and kinetin (KIN) either singly or in combination ranging from 0.5 to 2.0 mg/l. All the media were supplemented with sucrose (3%), agar (0.8%), PVP (750 mg/l), ascorbic acid (750 mg/l) and citric acid (750 mg/l). The pH of the media was adjusted to 5.8

before autoclaving. All *in vitro* cultures were maintained in an automated culture room at temperature 28 ± 2 ºC and at 16 hours light/dark photoperiod and 3000 lux with cool white fluorescent lamps. Different cotton explants were sub cultured weekly into fresh medium in order to avoid browning due to phenolic compounds.

***Shoot elongation and rooting***

Two-weeks-old regenerated shoots were removed from explants, and further subcultured on the shoot elongation MS medium supplemented with 0.1 mg/l gibberllic acid ($GA_3$). Shoots were elongated in two weeks following culturing on shoot elongation medium. After two weeks these shoots were rooted on MS medium supplemented with or not with 0.5 mg/l IBA).

***Acclimatization***

After six weeks, rooted shoots were carefully removed from the medium and washed with distilled water. The shoots were potted in sterilized agropeat, sand and vermiculite (1:1:1) and watered using 100 ml Hoagland medium (Hoagland and Arnon, 1938) at alternate days. The plants were then covered with polythene bags (size 15 X 23 cm.) and placed in growth chamber during one week. After 2-3 days, holes were made in the polythene bags in order to stepwise expose plantlets to the external environment. After ten days plants were transferred to larger pots containing sand and soil (1:1) in glasshouse conditions.

***Statistical analysis***

Statistical analysis was performed using SPSS Statistics, Version 22.0.0 (IBM, USA). The data were subjected to one-way analysis of variance (ANOVA) and comparisions between the mean values of 25 replicates were made by Duncan's Multiple Range Test (DMRT) at $p \leq 0.05$.

**Results and Discussion**

In India, an area of about nine million hectares is under cotton cultivation. Almost 90% of the area is covered by *G. hirsutum* (Khan et al. 2010). The major cotton cultivars grown in India are MCU 5, MCU 7, Khandwa 2, Bikaneri Nerma and F 846. The Indian cotton genotypes are generally considered as recalcitrant to *in vitro* regeneration (Trolinder and woodin 1987; Firoozabady and DeBoer 1993; Kumar and Pental 1998).

*Regeneration efficiency*. In this study, we report a highly efficient, rapid and reproducible regeneration method of *G. hirsutum* cv. Narashima and *G. arboreum* cv. AKA-7. Also, high shooting efficiency of two Indian cotton cultivars is reported for the first time. The regeneration protocol is rapid and it takes only eight weeks to have regenerated cotton plants. The regeneration efficiency was investigated on different type of explants. Shoot tip explants showed the best regeneration efficiency compared to other explants. The best regeneration efficiency, obtained on MS medium supplemented with 1.0 mg/l KIN and 1.0 mg/l BAP, was 90% for Narashima and AKA-7, respectively. The shoot regeneration efficiency in the treatments where BAP and KIN applied separately was ranged between 14 and 66%, irrespective of explants type. However, different combinations of both cytokinins stimulated shoot regeneration so the regeneration efficiency approached to 40-90%. Initially, explants showed swelling at the cut ends followed by slight callus formation. Small shoot primordia were developed on the slight callusing swollen explants which soon gave rise to the shoots (Table 1, Fig. 1). Cytokinins play a major role in multiple shoot induction in various plants like *Arachis hypogaea* (Venkatachala et al. 1999), *Allium cepa* (Zhang et al. 1999) and cotton (Chowdhury et al. 2011). Our results showed that combination of BAP and KIN have positive effects on cotton shooting efficiency compared to medium supplemented

only with BAP. We selected MS medium supplemented with both cytokinins in order to investigate the optimum combination that stimulate shoot primodial formation. Increased regeneration frequency was observed when applied concentration of both cytokinins was equal (1mg/l). The shooting efficiency was also increased on the same medium. The overall regeneration frequency was significantly higher in shoot-tip explants compared to cotyledon and hypocotyl explants in both analyzed cotton cultivars Narashima and AKA-7 (Table 1). It is known that different explants have different regeneration capacity (Yang et al. 2010). In present work, shoot tips showed the best regeneration efficiency. This data correspond with previous reporters (Gould et al. 1991; Zapata et al. 1999). There is one meristematic region present in the shoot tip explants between the two cotyledons which rapidly divide producing undifferentiated cells which may eventually differentiate to form the tissue and cell types. When shoot tip was isolated and cultured on regeneration medium, multiple shoots were developed from all the sides of shoot tip explants with slight callus formation.

In cotton regeneration, browning is a major problem resulting in tissue necrosis (ÖzyiğitII and Gozukirmizi 2008). The browning occurs due to the exudation of the phenolic compounds that produce brown coloured growth inhibitory substances following oxidation. However, it can be reduced by adding antioxidants like AC, PVP, ascorbic and citric acid to the medium (Aydin et al. 2004). The AC induces breakdown of sucrose during sterilization and the absorption of medium components, phytohormones etc (Van Winkle and Pullman 1995). In our experiments, we first cultured explants for five to seven days on the medium containing AC followed by explants transfer to a medium containing PVP, ascorbic acid, citric acid but no AC. This indeed helped in the prevention of browning caused by phenolic compound.

***Shooting efficiency***

The best shooting efficiency (85%) was obtained with equal applied concentration of both cytokinins (1.0 mg/l) in cv. Narashima using shoot tip explants. When BAP and KIN were added separately in the medium, the shooting efficiency was decreased compared to media supplemented with different combinations of both plant growth regulators. On MS supplemented with lower concentration of either BAP or KIN (0.5 mg/l), the shooting efficiency reached 36 and 43%, respectively (Table 1) in the cv. AKA-7 using shoot tip explants. At the highest concentration of either BAP or KIN (2 mg/l), the shooting efficiency was found to be maximum i.e. 44 and 47%, respectively, regarding both cultivars in case of BAP using cotyledon explants and in case of KIN using hypocotyls. Notably, at higher concentration of BAP and KIN (1.0 to 2.0 mg/l), the shooting efficiency of both cotton cultivars was significantly higher irrespective of explants type (Table 1, Fig. 1A and B).

The elongation of regenerated cotton shoot was obtained on MS medium supplemented with concentration of 0.1mg/l $GA_3$ (Fig. 1C and D) Lower and higher concentration of $GA_3$ had no effect on shoot elongation (data not shown).

The rooting of regenerated cotton shoots was obtained on basal MS hormone free medium. The rooting of shoots was also tested on MS medium supplemented with different concentration of IBA but with no significant affect on root regeneration (data not shown).

***Hardening and acclimatization***

Initially, plants were kept in Hoagland solution for one week. This helped hardening of roots and adaptation of plantlets to grow without sucrose. Following hardening, plantlets were transferred to pots in greenhouse under controlled conditions.

***Table 1:*** *The effects of two different cytokinins on shoot regeneration in two different cotton cultivars*

| S. No. | Medium composition | AKA7 | | | | | | Narashima | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Shooting efficiency (%) | | | Regeneration (%) | | | Shooting efficiency (%) | | | Regeneration (%) | | |
| | | H* | C | St | H | C | St | H | C | St | H | C | St |
| 1. | MS**+ 0.5 BAP | $16^{l}$ | $16.6^{j}$ | $36.4^{j}$ | $14^{m}$ | $14^{i}$ | $24^{j}$ | $20.2^{j}$ | $17.2^{l}$ | $31.0^{k}$ | $18^{k}$ | $14^{i}$ | $24^{j}$ |
| 2. | MS+ 1.0 BAP | $29.8^{j}$ | $27.4^{i}$ | $53.6^{d}$ | $34^{i}$ | $26^{g}$ | $34^{i}$ | $33.6^{h}$ | $25.4^{j}$ | $56.8^{d}$ | $34^{h}$ | $26^{g}$ | $34^{i}$ |
| 3. | MS+ 1.5 BAP | $40.0^{g}$ | $31.8^{h}$ | $47.8^{e}$ | $38^{f}$ | $34^{f}$ | $40^{g}$ | $41.4^{e}$ | $32.0^{i}$ | $49.6^{e}$ | $38^{g}$ | $34^{f}$ | $40^{g}$ |
| 4. | MS+ 2.0 BAP | $32.4^{i}$ | $44.0^{e}$ | $39.8^{i}$ | $52^{d}$ | $40^{e}$ | $38^{h}$ | $35.6^{g}$ | $43.8^{e}$ | $42.0^{h}$ | $44^{f}$ | $40^{e}$ | $38^{h}$ |
| 5. | MS+ 0.5 BAP + 0.5 KIN | $43.8^{e}$ | $38.6^{g}$ | $45.6^{f}$ | $48^{e}$ | $46^{d}$ | $50^{f}$ | $43.2^{d}$ | $40.2^{g}$ | $46.2^{f}$ | $50^{d}$ | $46^{d}$ | $50^{f}$ |
| 6. | MS+ 1.0 BAP + 1.0 KIN | $77.2^{a}$ | $64.4^{a}$ | $83.8^{a}$ | $78^{a}$ | $78^{a}$ | $86^{a}$ | $78.8^{a}$ | $64.8^{a}$ | $85.0^{a}$ | $82^{a}$ | $78^{a}$ | $90^{a}$ |
| 7. | MS+ 1.5 BAP + 1.5 KIN | $69.0^{b}$ | $57.0^{b}$ | $78.4^{b}$ | $68^{b}$ | $70^{b}$ | $76^{b}$ | $71.2^{b}$ | $59.4^{b}$ | $80.6^{b}$ | $66^{b}$ | $70^{b}$ | $76^{b}$ |
| 8. | MS+ 2.0 BAP + 2.0 KIN | $57.4^{c}$ | $53.0^{c}$ | $64.2^{c}$ | $60^{c}$ | $60^{c}$ | $68^{c}$ | $47.6^{c}$ | $53.0^{c}$ | $63.6^{c}$ | $64^{c}$ | $60^{c}$ | $68^{c}$ |
| 9. | MS+ 0.5 KIN | $28.8^{k}$ | $21.0^{i}$ | $43.0^{g}$ | $16^{l}$ | $20^{h}$ | $34^{i}$ | $28.0^{i}$ | $19.2^{k}$ | $41.8^{i}$ | $20^{j}$ | $20^{h}$ | $34^{i}$ |
| 10. | MS+ 1.0 KIN | $34.8^{h}$ | $31.2^{h}$ | $43.4^{g}$ | $28^{j}$ | $34^{f}$ | $38^{h}$ | $37.0^{f}$ | $34.0^{h}$ | $42.2^{h}$ | $30^{i}$ | $34^{f}$ | $38^{h}$ |
| 11. | MS+ 1.5 KIN | $41.4^{f}$ | $40.8^{f}$ | $44.8^{g}$ | $26^{k}$ | $34^{f}$ | $54^{e}$ | $41.4^{e}$ | $42.4^{f}$ | $45.6^{g}$ | $30^{i}$ | $34^{f}$ | $54^{e}$ |
| 12. | MS+ 2.0 KIN | $47.2^{d}$ | $45.2^{d}$ | $40.2^{h}$ | $48^{e}$ | $40^{e}$ | $66^{d}$ | $47.4^{c}$ | $44.8^{d}$ | $38.6^{j}$ | $48^{e}$ | $40^{e}$ | $66^{d}$ |

*H = Hypocotyl, C = Cotyledon, St = Shoot tip explants

**MS = Murashige and Skoog medium, BAP = 6 Benzyl aminopurine, KIN= Kinetin

***The values represent means of 25 independent replicates. mean markedwith the same letter differ significantly from controls according to DMRT (Duncan Multiple Range Test ($p \leq 0.05$).

**Fig 1:** Regeneration of cotton plants *in vitro* in cotton cultivar Narashima (a) and AKA-7 (b) shoot regeneration of shoot tip explants on MS medium supplemented with 1.0 mg/l BAP and 1.0 mg/l KIN; (c and d) Elongation of regenerated cotton shoots (e and f) hardening and acclimatization of cotton plantlets.

Cotton plants were irrigated with Hoagland nutrients and covered with plastic bags for one week to maintain high humidity (Fig. 1E). Fully developed plants were transferred to soil after hardening and acclimatization (Fig. 1F). The survival rate of the plants was 85% in both of the cultivars regenerated through shoot tip explants. Similarly, it was 60-70% when regenerated through hypocotyls and cotyledon explants in both cultivars. Gradual reduction of humidity coupled with high temperature (28-30$^{o}$C), showed better growth and percent survival of plants. Since *G. hirsutum* is a tropical crop, at low temperature it remains under stress (Stewart and Guinn, 1969).

## Conclusion

The present study resulted in the establishment of regeneration systems derived from different seedling explants such as cotyledon, hypocotyl and shoot tips. Critical factors affecting regeneration such as different explants and growth regulators were studied in order to optimize the micropropagation protocol providing future application in genetic transformation. The combination of BAP and KIN at a concentration of 1 mg/l was the most suitable for the shoot regeneration from hypocotyl, cotyledon and shoot tips. The regeneration system was optimized from shoot-bud induction to rooting of shootlets, and their hardening and establishment in the nursery. These systems could be used for clonal propagation and transformation of desirable genes for generating cotton transgenic plants having high agronomic traits.

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