

Full Length Research Paper

Effects of zeatin and activated charcoal in proliferation of shoots and direct regeneration in cotton (*Gossypium hirsutum* L.)

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A multiple shoot induction protocol was optimized for cotton (*Gossypium hirsutum* L.). Three cultivars of cotton (Sahel, Siokra, Hybrid, that is, Sahel × Siokra) were used to study the effects of zeatin and activated charcoal on proliferation of shoots and direct regeneration from shoot tip explant excised from 10 – 15 day-old seedlings cultured *in vitro*. Growth response of different varieties varied. Root and shoot formation was observed in all varieties. The best treatment for multiple shoot induction in cultivars was the treatment containing Murashig and Skoog (MS) basal medium supplemented with zeatin (0.1 mg/l) and activated charcoal (0.5 mg/l), while treatment containing zeatin (0.1 mg/l) and activated charcoal (2 mg/l) was not good medium for regeneration. Culture of every cultivar continued for 7 subcultures and morphological characteristic was evaluated during every subculture. Maximum length of shoots (4.65 cm), the highest percentage of root development (55%) and maximum number of nodes (6.7) was observed in Siokra variety in the second subculture, Hybrid genotype and siokra in the last subculture, respectively. Also the shape of leaves changed in Siokra variety during subcultures.

Key words: *Gossypium hirsutum* L., regeneration, shoot tip culture, zeatin.

INTRODUCTION

Cotton is an excellent natural source of textile fiber and is cultivated in many countries. Both diploid (*Gossypium herbaceum*) and tetraploid (*G. hirsutum*) cultivars are cultivated in different regions of Iran and are considered as important crop plants of the country because of its high economic importance. Considerable attention has been paid to improving cotton plants by conventional plant breeding methods (Agrawal et al., 1997). Although great progress has been made in the field of improvement of cotton with conventional breeding methodology, it is time-consuming and commercialization of new cotton varieties often takes 6 to 10 years (Sheidai et al., 2008). Cotton is considered recalcitrant to *in vitro* proliferation (McCabe and Martinell, 1993). Though somatic embryo-

genesis in cotton has been reported (Davidonis and Hamilton, 1993; Trolinder and Goodin, 1987; Finer, 1988), the response is restricted to only a few cultivars (Trolinder and Xhixian, 1989; Firoozabady and DeBoer, 1993). *In vitro* culture of shoot apical tips has been reported to give single or sporadically, a few shoots (Bajaj and Gill 1989; Gould et al., 1991). Different regeneration protocols for induction of multiple shoots have been developed for cotton using various explants and through manipulation of media composition but still there is scope for improvement (Saeed et al., 1997; Gupta et al., 1997; Ouma et al., 2004; Rauf et al., 2004).

The composition of the culture medium is an important factor in regeneration of shoots *in vitro*. Some problems such as browning, mortality of the cultured explants and rooting deficiencies are major problems for many tissue cultures because of phenolic components that produce in culture medium (DeProft et al., 1985). Cytokinins stimulate shoot proliferation in tissue culture. Zeatin is an

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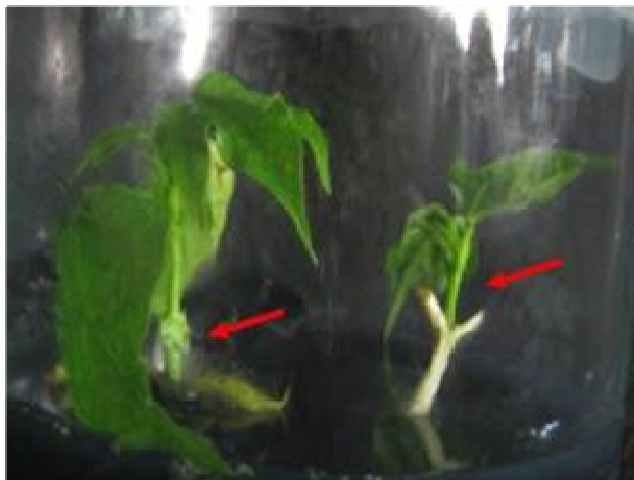


Figure 1. Shoot tip explants development in culture medium.

important cytokinin used in promotion of plant regeneration (Zhang and Wang, 2001; Ikram-ul-Haq, 2005; Lashari et al., 2008). The objective of this study was therefore to optimize the protocol for multiple shoot regeneration based on a modified Murashige and Skoog basal medium and using zeatin as cytokinin and activated charcoal as phenolic-action inhibitor.

MATERIALS AND METHODS

Three cultivars of cotton *Gossypium hirsutum* L.; Sahel, Siokra and Sahel × Siokra (Source: Central Cotton Research Institute, Golestan, Gorgan); were used in this study. Seeds were delinted using Sulphuric Acid. Lint was removed completely. For sterilization, the seeds were first dipped in 70% ethanol for 1 - 2 min and then in 15% commercial bleach (5% active ingredient) for 60 - 80 min. The MS medium (Murashige and Skoog, 1962), containing 30 g/l sucrose and 6 g/l agar was used for seed germination. The medium was sterilized by autoclaving for 20 min at 121°C. Four to five seeds were placed into glass bottles and maintained at 25 ± 2°C under a 16/8 h light photoperiodic, in a growth chamber.

Cotyledons were removed from 10 - 15 day-old *in vitro* raised seedlings. The shoot tip explants excised and transferred to 3 different media: MS1 containing MS+ 0.1 mg/l (zeatin) Zt., MS2: MS+ 0.1 mg/l Zt. + 0.5 g/l activated charcoal, MS3: MS+ 0.1 mg/l Zt. + 2 g/l activated charcoal (Zhang et al., 2001). All media were supplemented with 30 g/l sucrose and 6 g/l agar and the pH was adjusted to 5.8 before autoclaving. Each culture glass contained 3 explants.

Three explants were placed in each culture and incubated for 30 days at 25 ± 2°C under a light intensity of approximately 3000 lux (provided by cool white fluorescent lamps) with 16/8 h photoperiod. Plant regeneration was studied in all medium used and the best medium was selected. Explants from shoot tip placed in media for 30 days then transferred to fresh media and processed until seventh sub-culture for detection of morphological variation. Morphologic characteristics such as: number of leaves and nodes, length of shoot, shape and color of leaves and rooting were evaluated in each subculture for 3 cultivars. The experiment was repeated for 3 times for each treatment used and morphological data were analyzed by analysis of variance test (ANOVA) followed by The Least Significant Difference tests (LSD).

RESULTS

Seed germination

Germination frequencies in cotton seeds were 90, 80 and 95% in Sahel, Siokra and their Hybrid, respectively. The germination percentage was distinctly higher, if seeds were incubated in culture bottles containing with MS solid medium.

Age of seedlings from which explants were prepared influenced the regeneration frequency considerably. In the beginning, 5 to 15-day-old seedlings were tested and explants from 10 - 15-day-old seedlings were chosen for further analysis because of their better morphogenic ability. This could be due to apical dominance of growing shoots (Agrawal et al., 1997). When shoot tip sections (0.5 - 1 cm) with apical bud were placed on the medium, both ends of shoot tip bulged and proximal ends differentiated shoot buds by the end of third 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 explants (Figure 1).

Induction of multiple shoots

Experiments with MS + 0.1 mg/l Zt. + activated charcoal resulted in good regeneration response. Besides attaining a maximum number of responding explants, the highest number of shoot primordia (5) per responding explant and a maximum number of shoots per explant (3) with higher regeneration percentage up to 90% was attained on 0.1 mg/l Zt. and 0.5 g/l activated charcoal (Figure 2c).

Zeatin induced the formation of higher number of shoot primordia and a many of them were converted into individual shoots, however higher levels of zeatin concentrations produced several shoot primordia which were developed into hyperhydric shoots associated with fasciated water soaked callus (Figure 3).

The shoot elongation was observed on MS2 in all three cotton cultivars studied and the highest value of rooting percentage (55%) was obtained on this medium too (Figures 2c and 4).

Activated charcoal enhanced the recovery of shoot primordia into elongated shoots with good internodes length. Leaves were large in size and dark green in color. Activated charcoal greatly enhanced regeneration capacity and shoot yield with almost similar response in all the three varieties. It was observed that inclusion of activated charcoal 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. In general, survival of rooted shoots transferred to pots was good but



Figure 2. Comparison of three media in multiple shoot formation. a) MS1, b) MS3, and c) MS2.



Figure 3. Effect of zeatin in multiple shoot formation.

Siokra genotype showed relatively more survival rate compared to the other two cultivars studied (Figure 5).

Sahel and its sub-cultures regenerated plants (S1 - S7)

Comparison of the Sahel parental genotype and its sub-cultures regenerated plants (S1 - S7) revealed their morphologic differences showing a significant increase ($p < 0.05$) in length of shoots, number of nodes and leaves in regenerated plants of the latter sub-cultures compared to those of parental genotype. For example significant difference in leaf number was observed between third, sixth and seventh sub-cultures compared to parental plants. Similarly significant increase in the number of nodes occurred between all subcultures and parental genotype; however the stem length differed significantly

in sixth and seventh sub-cultures compared to fifth subculture plants (Table 1)

Length of shoots, number of node and leaf almost was increased during subcultures, the leaf color changed to bright-green in third and fourth subcultures, but the shape of leaves did not changed. Rooting percentage increased in latter sub-cultures reaching up to 45% in the last sub-culture (Figure 6a - d).

Siokra and its sub-cultures regenerated plants (Sk1 - Sk7)

Similar study in Siokra and its tissue culture regenerated plants showed a significant increase ($p < 0.05$) in the number of nodes and rooting percentage (52%) in regenerated plants of latter sub-cultures compared to those of parental genotype. For example significant difference in leaf number was observed in first and fifth sub-cultures compared to third sub-culture. Similarly significant increase in the number of nodes occurred between all subcultures and parental genotype; significant difference in stem length was observed in first, fourth, fifth, sixth and seventh sub-cultures compared to second sub-culture plants (Table 1).

The highest length of shoot and number of leaf was observed in the second and third subcultures respectively. Shape of leaves changed. Color of leaves was green as parental genotype (Figure 6a - d).

Hybrid (Sahel × Siokra) and its sub-cultures regenerated plants (H1 - H7)

The hybrid genotype Sahel X Siokra also showed a significant increase ($p < 0.05$) of the number of nodes and rooting percentage in regenerated plants of the latter sub-cultures compared to those of parental genotype. For example, significant difference in leaf number occurred between all subcultures and parental genotype. Also significant difference in stem length was observed in first and fifth sub-cultures compared to second sub-culture.



Figure 4. Multiple shoots induction and root formation in three cultivars. a) Sahel genotype, b) Siokra genotype, and c) Hybrid genotype.



Figure 5. Regenerated plants transferred to soil.

Similarity significant difference in stem length was observed in third, sixth and seventh sub-cultures compared to fourth subculture plants (Table 1).

The highest length of shoot was evaluated in the second subculture, number of node increased during subcultures, while the most number of leaves observed in

the third subculture and shape of leaves did not change. The leaf color was dark-green. Rooting percentage increased during subcultures and observed 55% in the last subculture (Figure 6a - d).

All of results were significant statistically in three varieties.

Table 1. Representative mean difference test (LSD) for morphological characters among genotypes and their sub-cultures.

(I) leaf number	(J) leaf number	Mean Difference (I-J)	Sig.
s1	s3	-2.03571(*)	0.011
	s6	-2.64286(*)	0.001
	s7	-2.86905(*)	0.001
(I) node number	(J) node number	Mean Difference (I-J)	Sig.
s1	s2	-1.39862(*)	0.042
	s3	-1.69196(*)	0.014
	s4	-1.61330(*)	0.021
	s5	-1.72689(*)	0.011
	s6	-3.00000(*)	0.000
	s7	-3.30423(*)	0.000
(I) stem length	(J) stem length	Mean Difference (I-J)	Sig.
S5	s6	-0.71429(*)	0.026
	s7	-0.90741(*)	0.005
(I) Leaf number	(J) Leaf number	Mean Difference (I-J)	Sig.
sk3	sk1	1.54091(*)	0.040
	sk5	1.23377(*)	0.046
(I) node number	(J) node number	Mean Difference (I-J)	Sig.
sk1	sk2	-2.31957(*)	0.001
	sk3	-2.84394(*)	0.000
	sk4	-1.90946(*)	0.004
	sk5	-2.37857(*)	0.000
	sk6	-2.79211(*)	0.000
	sk7	-3.16053(*)	0.000
(I) stem length	(J) stem length	Mean Difference (I-J)	Sig.
sk2	sk1	1.72717(*)	0.000
	sk4	.93596(*)	0.024
	sk5	1.71170(*)	0.000
	sk6	1.07323(*)	0.009
	sk7	.94165(*)	0.023
(I) leaf number	(J) leaf number	Mean Difference (I-J)	Sig.
H1	H2	-1.90942(*)	0.010
	H3	-3.00333(*)	0.000
	H6	-1.71970(*)	0.022
	H7	-1.81061(*)	0.016
(I) node number	(J) leaf number	Mean Difference (I-J)	Sig.
H4	H3	-1.80000(*)	0.004
	H6	-2.11818(*)	0.001
	H7	-2.16364(*)	0.001
(I) stem length	(J) stem length	Mean Difference (I-J)	Sig.
H2	H1	0.91486(*)	0.020
	H5	0.97652(*)	0.012

S = Sahel genotype, SK = Siokra genotype, and H = Hybrid genotype.

DISCUSSION

An efficient protocol for multiple shoot regeneration in a

genotype independent manner is a pre-requisite for genetically manipulation of crop plants (Divya et al., 2008). Though there is a large body of literature available

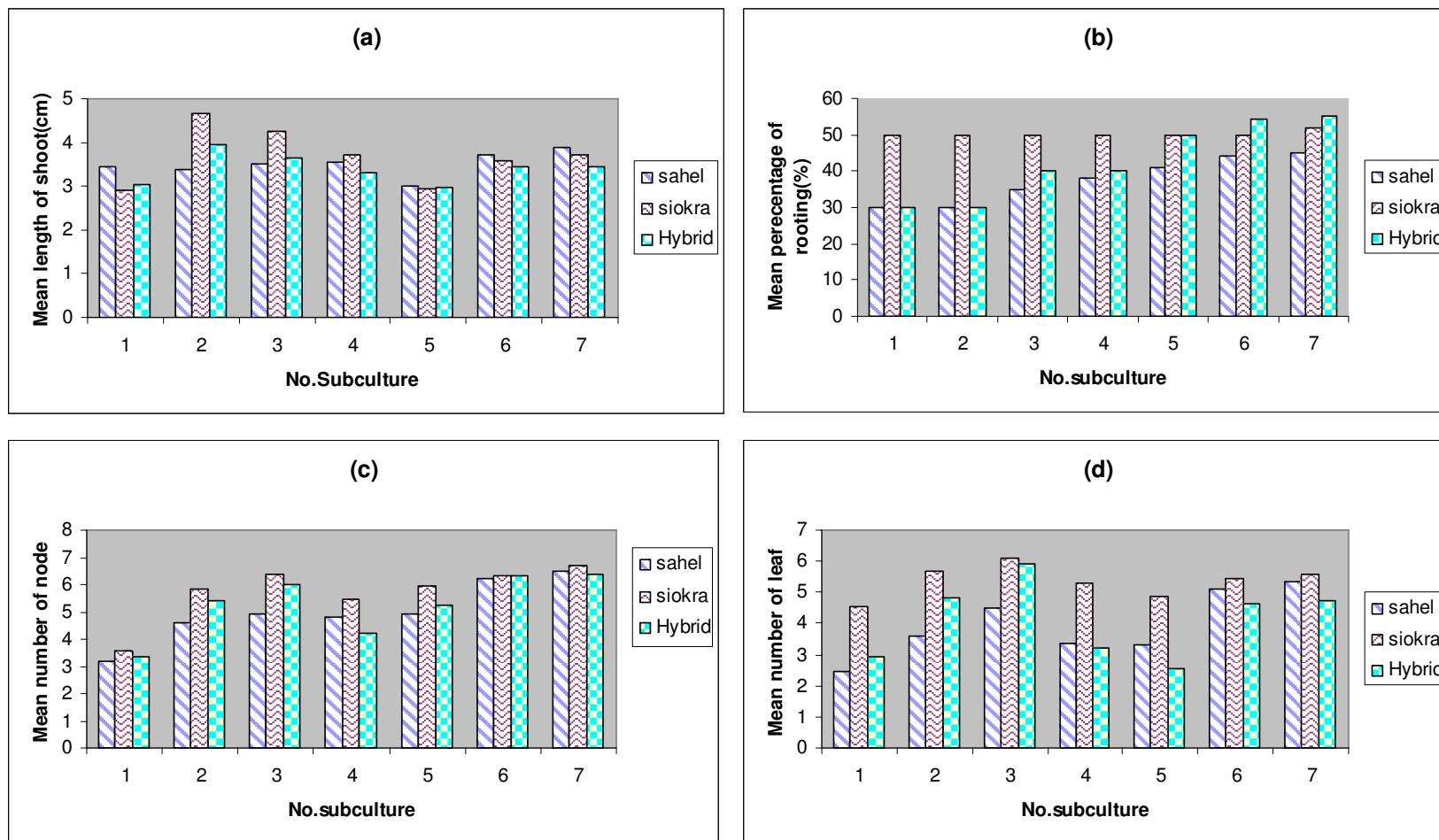


Figure 6. a) Mean length of shoots during subcultures in three varieties; b) Mean percentage of rooting during subcultures in three varieties; c) Mean number of node during subcultures in three varieties; d) Mean number of leaf during subcultures in three varieties.

on regeneration in cotton through somatic embryogenesis, efforts on the establishment of *de novo* shoot regeneration protocols in commercial cotton cultivars are rare.

In this study, the shoot tip culture procedure was

used for induction of multiple shoots and direct regeneration in three genotypes of cotton, because the cotton is recalcitrant and has proved difficult to manipulate in tissue culture (McCabe and Martinell, 1993). Cotton meristem tissues did

not undergo any form of malformation. Meri-stems are highly organized tissues and showed low variation in phenotypic characters (Saeed et al., 1997). Age and size of explant is a very important factor in this research. Shoot tip from less than 5-

day-old seedlings are difficult to isolate, due to small size and tenderness (Rashid et al., 2004). In the present study, explants from 10-15-day-old seedling showed best response for shoot and root formation on MS2 medium, can due to thickness of shoots explants which reduced burning and death of explants.

In this work, normal shoots and roots were developed, only a small fraction of meristems developed into abnormal shoots. Overall there was no difficulty in raising plants from the meristem tips of cotton in all genotype. Varietal differences were also negligible.

Our studies showed that zeatin induced high frequency production of shoot primordia from seedlings. The available information indicates that zeatin replacement by single synthetic cytokines, such as BAP or kinetin were not very successful as they did not prove to allow good proliferation rates and usually they induced explants hyperhydricity (Lashari et al., 2008). On the other hand effects of hormones like IAA, NAA, BAP, kinetin have been reported more than zeatin in induction of multiple shoots and regeneration of cotton (Zhang et al., 2001; Ikram-ul-Haq 2005; Larish et al., 2008).

In explant preparation stage, when explants are cut, the contents of the cytoplasm and vacuoles mix and exit from the explants and oxidation of phenolic compounds can occur by air. Studies show that oxidized phenolic compounds inhibit enzyme activity and some problems like darkening of the culture medium and subsequent mortality of explants and rooting deficiencies is observed in culture procedure (Arnaldos et al., 2001; Ozyigit et al., 2007). In this case, activated charcoal was used for reducing effect of phenolic compounds and growth improvement in media. Results showed that growth of explants improved in MS2, MS3 compared to MS1 (without activated charcoal). Death of explants, burning, deficiencies in rooting and very little shoot growth were detected in MS1 medium. Explant response for induction of multiple shoots and high level of growth in MS3 was lower than MS2 (Figures 2a, 2b and 2c). It seems that high concentration of activated charcoal (2 g/l) has inhibitory effect on zeatin in rooting and regeneration of these cultivars (Figure 2b). MS2 was selected as the best medium and some parameters like shoots length, percentage of rooting and number of leaf and nod during 7 sub-cultures was evaluated for three varieties. On an average, shoots elongated up to 3.5 - 4 cm with in 30 days in MS2 medium for all genotypes. This improved growth of shoots can be due to composition of MS2 medium. Highest shoot length between three genotypes was obtained for Siokra variety (Figure 6a). Considering that the propose of this research was to achieve a full suitable protocol for regeneration of the cotton cultivars Sahel, Siokra and Hybrid (Sahel × Siokra) and not only the objective of zeatin replacement at the regeneration culture stage, rooting trials were also carried-out. In the trials, rooting rates over 55% (for hybrid variety) were achieved using the pulse technique and the supple-

mentation of culture media with activated charcoal.

It is remarkable that in every genotype, root forming occurred without any auxin hormone (Figure 4a - c). Percentage of rooting during 7 sub-cultures in hybrid and Sahel variety increased but in Siokra it did not very change (Figure 6b). Rooting formation in Siokra from the first sub-culture was good and more than the other genotypes. Highest percentage of rooting was in Hybrid in the last sub-culture; also increase in number of nodes was obtained during every sub-culture for all cultivars and this finding supports the effect of zeatin in proliferation of nodes and shoots.

Plant tissue culture leading to somaclonal variation has been considered as one of the possible sources of inducing genetic variability in crop plants to be used in breeding programs. Somaclonal variation is used to describe the occurrence of genetic variants derived from *in vitro* procedures (Isabel et al., 1993). This variability often arises in tissue culture as a manifestation of epigenetic influence or changes in the genome of differentiating vegetative cells induced by tissue culture and are expected to generate stable plants carrying interesting heritable traits (Soniya et al., 2001).

Significant morphological differences obtained among the regenerated plants of different sub-cultures and also within each sub-culture may indicate that the molecular/genetic variation obtained is partly responsible for morphological variations and also show the possible use of tissue culture in inducing new morphological (possibly new agronomic) characters in the cotton which may be used for breeding purposes. The results obtained in the present study will be used for detection of somaclonal variation by using molecular markers in the near future.

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