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IN VITRO MULTIPLE SHOOT INDUCTION AND PLANT REGENERATION IN ELITE SUDANESE COTTON (*Gossypium hirsutum* L.) CULTIVAR (BARAC - B - 67)

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ABSTRACT

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This study was carried out at department of Plant Cell and Tissue Culture, Commission for Biotechnology and Genetic Engineering, Khartoum, Sudan during August 2006 to June 2008 to develop a protocol for *in vitro* regeneration for elite Sudanese cotton cultivar. Induction of multiple shoots in medium stable cotton (*Gossypium hirsutum* L. cv. Barac - B - 67) has been achieved with cotyledonary nodes devoid of cotyledons and apical meristem. The induction of multiple shoots in explants varied with concentration and type of cytokinin as well as seedlings age. Explants from 35-day-old seedlings yielded the maximum number of shoots (2.8 shoots/explant) using Gamborg (B5) basal medium supplemented with 0.1 mg L^{-1} Benzyl adenine (BA) and kinetin (Kin) 2.5 mg L^{-1}. Elongation of multiple shoots was obtained on half-strength agar-solidified B5 basal medium without phytohormones. *In vitro* shoots were rooted on half-strength agar-solidified B5 basal medium without growth regulator or with 0.1, 0.5 or 1.0 mg L^{-1} naphthalene acetic acid (NAA). This procedure was rapid, reliable and with high-frequency. Thus, it can be advantageously used in application of modern gene transfer techniques for the improvement of medium staple cotton cultivar (Barac -B -67).

Key words: *Gossypium hirsutum*, multiple shoot, seedling age, coteledonary node

INTRODUCTION

Cotton **(***Gossypium hirsutum* L.) is one of the most important commercial crops of the world valued for its fiber, oil and other by-products. In Sudan, although over the past decade, the share of cotton in foreign currency export earnings has relatively declined where other products like oil and livestock have become strong competitors, cotton still maintains a major role in the economy. It forms an important source of livelihood for a large number (200,000) of its growers and their families, provides crop residues as feed for a large number of livestock from the pastoral sector, employs a considerable amount of hired seasonal labor in its picking and ginning operations, and maintains important forward and backward linkages that engender economic activities in the factor and post-harvest markets (Faki, 2006).

Cotton yield and production in Sudan is adversely affected by wide ranges of biotic and a biotic stresses such as disease, draught, salinity and its vulnerability to frequent insect and pest attacks. Pests and diseases infestations are notorious factors that reduce crop yields and inflate production costs. The costs of pest and weed control form a major cost component, reaching about one third and may be as high as 40% of pre-ginning production costs (Faki, 2006; Abdellatef and Khalafalla, 2008).

To attain sustainable cotton production such above mentioned constraints have been addressed by conventional breeding and enhanced management, though numerous excellent varieties of cotton have been produced, they seem insufficient to exploit the existing germplasm due to various incompatibility barriers. The use of genetic engineering techniques for introducing genes responsible for a biotic stress tolerance and production of agronomically desirable crops represent an attractive option for improving cotton production in Sudan. Cotton improvement using genetic transformation has now become a reality. Beginning in 1996, the commercialization of transgenic insect-resistant cotton (*Bt* cotton) in the United States represented a technological breakthrough and the first serious alternative to insecticides. Within 3 years, *Bt* cotton occupied 20% of world cotton acreage and was grown in several countries including the US, Australia, Mexico, China, Argentina, and South Africa (James, 2001). There is strong evidence that *Bt* cotton generates significant economic benefits to farmers, in addition to the beneficial effects on the environment, biodiversity and farmer's health (Edge *et al*., 2001).The potential benefits of this form of genetic improvement have not yet been realized in Sudan, mainly because the use of genetic engineering biotechnological tools such as biolistics and *Agrobacterium*-mediated transformation, require a prerequisite plant regeneration protocols that are genotype-independent, efficient, and which do not yield somaclonal variant. (Firoozabady, 1987; Gould and Magallanes-Cedeno, 1998; McCabe and Martinell, 1993). Therefore there is an urgent need for developing an efficient *in vitro* regeneration protocol involving Sudanese cotton cultivars with regard to multiple shoots induction from cotyledon explants, till date there is no report on *in vitro* regeneration for Sudanese cotton cultivar.

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MATERIALS AND METHODS

Seed material

Seeds of medium staple cotton cultivar (Barac- B - 67) used in this study were obtained from the Agricultural Research and Technology Corporation (ARTC), Wad Medani, Sudan.

Surface sterilization and germination

Seeds were delinted by soaking in concentrated H_2SO_4 for 1 minute then washed by continuously running tap water for 1 minute followed by thorough washing in sterile distilled water to remove traces of surface adherent. Under laminar flow cabinet seeds were disinfected with mercuric chloride $HgCl_2$ 0.2% (w/v) for 15 mints with continuous shaking and then washed for five times by sterilized distilled water. After surface sterilization 5 seeds were transferred to sterile culture bottle containing 25 ml of B5 basal media (Gamborg *et al*., 1968) for germination at 25Cº± 1 and under 16 hrs light and 8 hrs dark photoperiod.

Media preparation

Sterilized distilled water was used for preparation of all medium used in this study. After addition of all macro- and micro-nutrients, vitamins, growth regulators and 3% sucrose as a carbohydrate source, the pH of the media was adjusted to 5.5 ±2 before autoclaving using 0.1N NaOH or 0.1N HCL. Volume was made and agar (0.8 %) was added. The medium was steamed to melt the gelling agent. Melted medium was then dispensed into, culture bottles and thereafter sterilized by autoclaving at 121°C and 15 lb psi for 15 min.

Explant preparation and experimental protocol

Cotyledonary nodes were removed from 7, 21 and 35 day-old *in vitro* raised seedlings. The cotyledons and apical meristem were excised and discarded. Thus, each explant had two dormant axillary buds. These decapitated cotyledonary nodes were used as explants in this experiment.

Explants were cultured in culture bottles containing B5 basal media supplemented with benzyladenine BA (0.1 – 5.0 mgL^{-1}) alone or in combinations with kinetin (0.1 – 5.0 mgL^{-1}). Cultures were incubated for six weeks under the same conditions as mentioned above. Twelve explants were cultured per treatment.

Shoots were excised from the multiple shoot bunches obtained from cotyledonary node explant and transferred individually to culture bottles containing different B5 basal media strength supplemented with 3% sucrose and 0.8% agar, to assess their response for elongation.

For root induction, *in vitro* elongated shoots (2-4 cm) were excised and cultured onto half strength B5 basal medium without growth regulator or with different concentrations (0.1-1.0 mgL^{-1}) NAA.

Results were observed at regular intervals and data were collected from three independent experiments and analyzed by using analysis of variance procedure (ANOVA) on excel computer programme. Means were separated by Duncan's multiple range test (DMRT) (Duncan, 1855).

RESULTS AND DISCUSSION

The use of modern techniques of cell, tissue and organ culture is central to many crop improvement programmes in both industrialized and developing countries. Indeed the limiting step to the successful development of transgenic plants of the major crops has not been transgene insertion itself, but rather the regeneration of viable plants from the transgenic explant material (Murphy, 2003).

In order to establish an efficient *in vitro* regeneration protocol for medium staple cotton cultivar (Barac [67] B) seeds were delinted by using H_2SO_4 and surface sterilized by $HgCl_2$ before *in vitro* germination. Disinfection of seeds through delinting with concentrated H_2SO_4 and then followed by $HgCl_2$ has already been proved to be essential in cotton tissue culture (Rauf *et al*., 2004; Abdellatef and Khalafalla, 2007; Abdellatef and Khalafalla 2008).

The induction of multiple shoots in explants varied with concentration and type of cytokinin as well as seedlings age (Table 1). Cotyledonary node explant obtained from different seedling ages (7, 14 and 35 days) cultured on growth regulator free B5 basal medium failed to show any response but remained green up to 4 weeks. However, on B5 basal medium supplemented with various concentrations of BA alone or in combination with kinetin enlarged in their size after 1–2 weeks of culture and adventitious shoots developed directly in another 4 weeks (Table 1). These results are in accordance with the finding of Jorge *et al*., (1998) who found that

cytokinin is directly responsible for reprogramming the embryonic apical meristem axes of cotton towards the multiplication of buds. Highest average multiple shoots were developed on explant cultured on media supplemented with low cytokinin concentration (Table 1). Further increase in shoot number was not observed with increasing concentrations of cytokinin. Jorge *et al*., (1998) found that higher concentration of growth hormone yields fewer shoots. Furthermore, in this study, at higher level (5.0 mgL^{-1}) of BA cotyledonary node explant produced excessive callus and failed to induce any shoot (Table 1). The callus growths on explant usually interfere with the propagation process. (Thiem, 2003).

The association BA and kinetin positively affected the multiplication rate of the cotton compared with BA alone. In our result it was found that explants obtained from 35-day-old seedlings cultured on B5 basal medium supplemented with BA (0.5 mgL^{-1}) in combination with kinetin (2.5 mgL^{-1}) produced the highest number of shoots (2.8 shoots). Similar result were reported by Agarwal *et al*., 1997, who obtained highest number of shoots by culturing cotyledonary nodes devoid of apical meristem in basal medium supplemented with BA in combination with Kin (Table 1). Explants obtained from 35 day-old seedlings gave the highest number of shoots per explant, compared to that obtained from 7 or 21-day-old seedlings in more than 80% of the treatment. Explant (Seedling) age already has been shown to effect multiple shoot induction in cotton (Agrawal *et al*., 1997) and other plant species including faba bean (Khalafalla and Hattori, 2001) and soybean (Kim *et al.,* 1990)

Table 1. Effects of cytokinin and seedling age on multiple shoots induction on cotyledonary node explants of medium staple cotton cultivar (Barac B - 67)

PGR'S (mgL^{-1})		Seedling age (days)					
		7		21		35	
BA	Kin	Reg. culture (%)	No shoot/ explant (mean ± SE)	Reg. culture (%)	No shoot/ explant (mean ± SE)	Reg. culture (%)	No shoot/ explant (mean ± SE)
0.1	-	100	1.00±0 ^{h}	100	1.17±0.1^{gh}	100	2.0±0^{d}
0.5	-	100	1.00±0 ^{h}	100	1.17±0.1^{gh}	100	2.0±0.23^{d}
1.0	-	100	1.00±0 ^{h}	100	1.60±0.1^{f}	100	2.4±0.14^{b}
2.5	-	50.0	0.52±0 ^{j}	100	1.33±0.1^{g}	100	2.0±0.0^{d}
5.0	-	0.0	0±0.00 ^{l}	0	0.00±0 ^{l}	0	0.0±0.0^{l}
0.1	0.1	100	1±0.00 ^{h}	100	1±.0.00 ^{h}	100	2.3±0.14^{bc}
	0.5	100	1±0.00 ^{h}	100	2±0.23 ^{d}	100	2±.0.00^{d}
	1.0	100	1±0.00 ^{h}	100	1±0.14 ^{h}	100	2.2±0.11^{c}
	2.5	100	1±0.00 ^{h}	100	1±0.11 ^{h}	100	2.8±0.14^{a}
	5.0	100	1±0.00 ^{h}	100	1±0.14 ^{h}	100	2.7±0.14^{a}
0.5	0.1	91.6	0.92±.08 ^{h}	100	1±0.14 ^{h}	100	1.6±0.14 ^{f}
	0.5	100	1±.0.00 ^{h}	100	1±0.08 ^{h}	100	2.0±.00^{d}
	1.0	91.6	0.92±.08 ^{h}	100	1±0.08 ^{h}	100	2.0±.01^{d}
	2.5	83.3	0.83±0.11^{i}	100	1±0.08 ^{h}	100	2.4±0.14^{b}
	5.0	83.3	0.83±0.11^{i}	100	1±0.11 ^{h}	100	1.8±0.17^{e}
1.0	0.1	83.3	0.83±0.11^{i}	100	1±0.11 ^{h}	100	2.3±0.14^{bc}
	0.5	66.6	0.67±0.14^{i}	100	1±0.11 ^{h}	100	2.0±0.00^{d}
	1.0	75.0	0.75±0.13^{i}	100	1±0.00 ^{h}	100	2.3±0.14^{bc}
	2.5	83.3	0.83±0.11^{i}	100	1±0.00 ^{h}	100	2.3±0.23^{bc}
	5.0	58.3	0.58±0.14^{j}	100	1±0.00 ^{h}	100	2.0±0.00^{d}
2.5	0.1	66.6	0.66±0.14^{i}	100	1±0.00 ^{h}	100	1.8±0.17^{e}
	0.5	66.6	0.66±0.14^{i}	100	1±0.00 ^{h}	25	0.5±0.26^{j}
	1.0	41.6	0.41±0.14^{k}	100	1±0.00 ^{h}	16.6	0.3±0.23^{k}
	2.5	25.0	0.41±0.14^{k}	50	1±0.15 ^{h}	0	0^{l}
	5.0	0.0	0.0 ±0.00^{l}	41.6	1±0.14 ^{h}	0	0^{l}
5	0.1	callus	callus	callus	callus	callus	callus
	0.5	callus	callus	callus	callus	callus	callus

Means with same letters are not significantly different at 5% using Duncan's multiple range test

Cotton regenerated shoots did not elongate on the same induction media. Therefore, shoots produced from 35 day-old seedlings on B5 basal medium supplemented with BA (0.1 mgL^{-1}) and Kin (2.5 mgL^{-1}) were transferred individually to culture bottles containing hormone free B5 basal medium at different salt strength and supplemented with 3% sucrose and 0.8% agar for 15–20 days for elongation. Elongation of one shoot per culture tube with an average length of 2.9 cm (Figure 1) was obtained in half strength B5 basal medium without phytohormone (Table 2). BA has been reported to regenerate cotton plants with short and compact shoots (Banerjee *et al*., 2000). Moreover, as in this study, cytokinins have often been reported to stimulate shoot proliferation while inhibiting shoot elongation (Brassard *et al*., 1996). The use of hormone-free medium for shoot elongation has already been reported for cotton (Abdellatef and Khalafalla, 2007), soybean (Kaneda *et al*., 1997) and faba bean (Khalafalla and Hattori, 2001).

Table 2. Effects of B5 basal medium salt strength on elongation of shoots regenerated from cotyledonary nodes explant of cotton cultivar (Barac -B -67)

Medium strength	Responded shoot (%)	Length of elongated shoots (cm) (mean ±SE)
Full B5	100	1.76±0.23[c]
½ B5	100	2.9±0.17[a]
¼ B5	100	2.27±0.19[b]

Means with same letters are not significantly different at 5% using Duncan's multiple range test

Root initiation on elongated shoots occurred after 6 weeks on half-strength B5 basal medium or with addition of 0.1, 0.5 or 1.0 mgL^{-1} of NAA. Rooting of cotton shoots was higher (87.5%) on medium containing 0.1 mgL^{-1} NAA compared to basal medium alone or that containing higher level (0.5 or 1.0 mgL^{-1}) of NAA (Table 3). The use of NAA and low salt basal medium for rooting of *in vitro* induced shoots has already been reported for cotton (Agrawal et al., 1997; Abdellatef and Khalafalla, 2007).

Table 3. Effects of naphthalene acetic acid (NAA) on *in vitro* rooting of regenerated shoots after 6 weeks of culture on half strength B5 medium

Phytohormones (mgL^{-1}) NAA	Rooting %	No of roots/shoot (Mean ± SE)
0.0	45.0	0.8±0.3 [b]
0.1	87.5	1.4±0.3 [a]
0.5	75.0	0.8±0.2 [b]
1.0	50.0	1.1±0.5 [a]

Means with same letters are not significantly different at 5% using Duncan's multiple range test

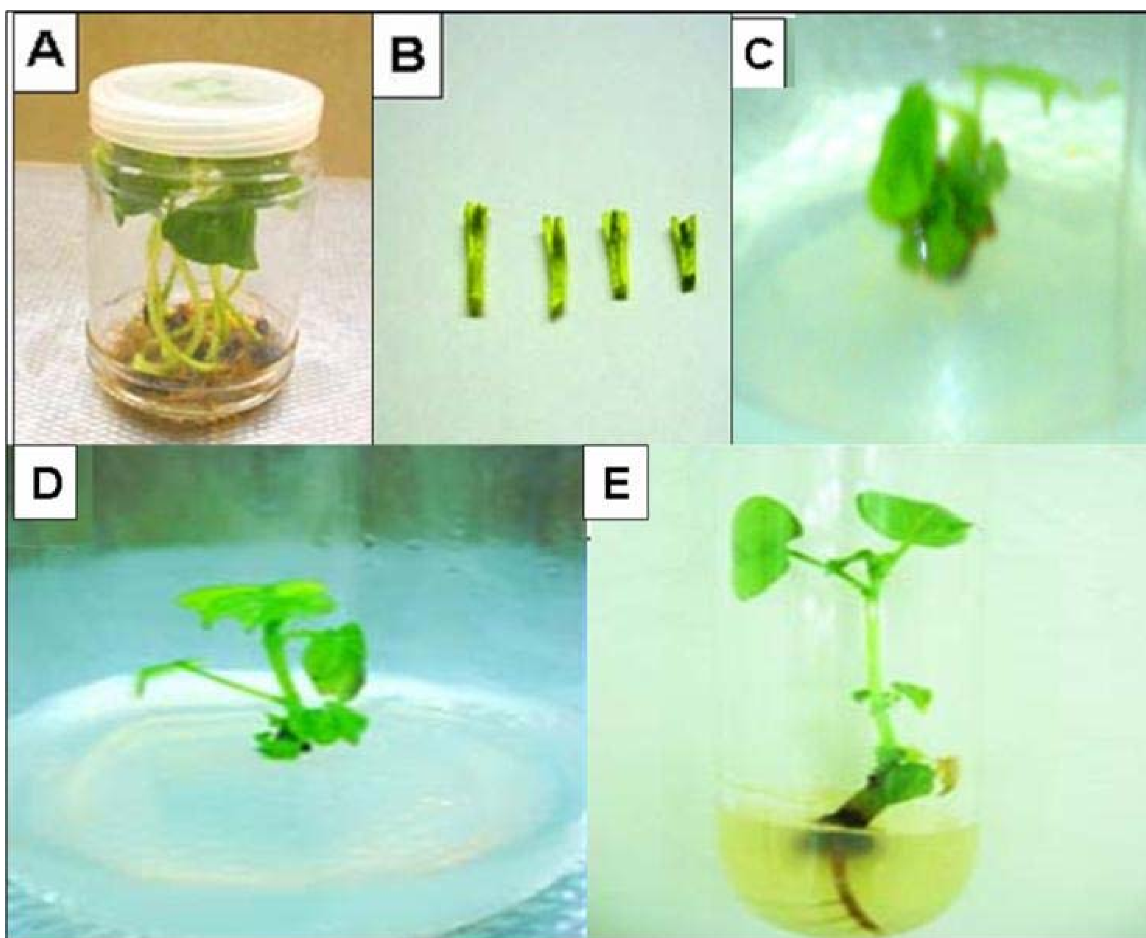

Figure 1. *In vitro* induction of multiple shoots and plant regeneration of medium staple cotton cultivar (Barac-B-67) A. *In vitro* germinated seedling. B. Cotyledonary explant obtained from 21 days - old – seedling. C. Multiple shoots bunches induced from cotyledonary node explants. D. Shoot elongated on half strength B5 basal medium. E. *In vitro* rooted shoot on half -strength B5 medium supplemented with NAA (0.1 mg L^{-1}).

In conclusion, development of an efficient tissue culture and plant regeneration protocol for elite Sudanese cotton cultivars is the first step towards the application of transgenic technology to improve cotton breeding and is, thus, the foundation of cotton biotechnology. Furthermore, the present finding of enhancement of multiple shoot induction by the addition of various additives will promote the application of plant tissue culture technology in the area of selection resistance and production of cotton artificial seeds.

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