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Adventitious Shoot and Plantlet Formation in Medium Staple Cotton Cultivar (*Gossypium hirsutum* L. cv Barac [67] B)

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ABSTRACT

In vitro adventitious shoot regeneration systems are considered most suitable for *Agrobacterium*- and biolistics-mediated genetic transformation to obtain transgenic plants. In the present investigation, adventitious shoot could be induced directly from medium staple cotton cotyledonary node devoid of cotyledons and apical meristem. Shoot development was induced on B5 medium supplemented with kinetin (Kin) or benzyladenine (BA) alone or in combination with α naphthalene acetic acid (NAA). Both types and concentrations of growth regulators significantly influenced shoot proliferation. Kin proved to be more effective than BA. The best response, however was obtained when 2.0 mg L^{-1} Kin was used. The efficiency of both BA and Kin for multiple shoot induction was negatively affected when combined with NAA. Elongation of multiple shoots was obtained on half strength agar-solidified B5 basal medium without phytohormones. More than 87% of the *in vitro* induced shoots produced roots when cultured on half strength B5 medium supplemented with 2.0% sucrose, 0.8% agar and 0.1 mg L^{-1} NAA. Rooted plants were hardened and 95% survived under greenhouse conditions.

Key Words: Shoot formation; Medium staple cotton; Kinetin; Hardening

INTRODUCTION

Cotton (*Gossypium hirsutum* L.) is one of the most commercially important fiber crops in the world. In addition to textile manufacturing, it produces seeds with a potential multiproduct base such as hulls, oil, linters and food for animals (Mishra *et al*., 2003; Aragao *et al*., 2005). In the Sudan cotton has been the most important cash crop and foreign-currency earner for the past 50 years. From seventies to late eighties, cotton alone contributed 45 - 65% of the total foreign-currency earnings (SCC, 1993).

In spite of the economic importance of cotton for the Sudan economy big fluctuations in production and yield occurred. This may be due to the cumulative effects of 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 in Sudan (Faki, 2006).

Although significant progress has been made in Sudan cotton breeding programs, traditional breeding techniques have several limitations, such as access to a limited gene pool, crossing barriers, inefficient selection and being time consuming. To overcome such problems of conventional breeding, advanced biotechnological method such as genetic transformation can be applied as alternative approach for development of disease and pest resistance for this crop.

Genetic transformation plays an important role in modern cotton breeding and has had a significant impact on production worldwide. Use of genetic transformation has produced insect resistant (Perlak *et al*., 1990; Jenkins *et al*., 1997) and herbicide resistant (Nida *et al*., 1996) cotton varieties. Transgenic lines are also valuable sources of germplasm for agronomic and fiber quality (Wilson *et al*., 1994) as well as contributing to reduced the usage of pesticides (Wilson *et al*., 1991). 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 *et al*., 1987; McCabe & Martinell, 1993; Gould & Magallanes-Cedeno, 1998).

Cotton regeneration was first observed in *Gossypium hirsutum* cv. Coker 310 (Davidonis & Hamilton, 1983) since then major work has been carried out for the development of protocol for an efficient regeneration system in cotton. Several scientists have successfully produced somatic embryoids and multiple shoots using various methods and media (Shoemaker *et al*., 1986; Chen *et al*., 1987; Trolinder & Goodin, 1987; Zhang & Wang, 1989; Voo *et al*., 1991; Kolganova *et al*., 1992). Although efficiency of cotton regeneration have been significantly improved but some difficulties still remains.

Sudan cotton breeding programs can highly benefit of adopting this modern technology, which allows the introduction of foreign genes into a germplasm, without modifying the genetic background of elite varieties. Therefore in order to, to pave the way for the application of transformation technology, the present study was undertaken to develop an efficient and reproducable regeneration protocol for elite Sudanese medium staple cotton cultivar (Barac [67] B).

MATERIALS AND METHODS

Source of seed material, its delinting and sterilization. Seeds of medium staple cotton cultivar (Barac [67] B) used in this study were obtained from the Agricultural Research and Technology Corporation (ARTC), Wad Medani, Sudan. Seeds were delinted by using concentrated commercial H_2SO_4 (100 mL kg^{-1} of seeds). The seeds were continuously stirred in H_2SO_4 by spatula for one min then washed by continuously running tap water for other one min followed by thorough washing in sterile distilled water to remove traces of surface adherent. Under laminar flow cabinet seeds were disinfected by soaking in mercuric chloride $AgCl_2$ 0.2% (w/v) for 15 min with continuous shaking and finally washed for five times by sterilized distilled water.

Effect of basal media on *in vitro* seed germination. Three different basal media viz., full strength MS (Murashige & Skoog, 1962), full strength B5 (Gamborg, 1968) and MSB (MS salts & B5 vitamins) were evaluated for effects on *in vitro* germination of cotton seed. After surface sterilization, 100 seeds were directly inoculated on the media in culture bottles and incubated for 15 days at 25ºC ± 2 with a 16 h photoperiod. Data were recorded after two weeks.

Explant preparation and effect of phytohormones. Cotyledonary nodes were removed from seven days-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 throughout this experiment.

Different phytohormones were tested to assess the morphogenetic response of the explant. Explants were cultured in culture bottles containing B5 basal media supplemented with BA (0.1, 0.5, 1.0, 2.5 & 5.0 mg L^{-1}) or Kin (0.1, 0.5, 1.0, 2.5 & 5.0 mg L^{-1}) alone or in combination with NAA (0.1, 0.5, 1.0 & 2.5 mg L^{-1}). Cultures were incubated for six weeks at 25ºC ± 2 under 16 h photoperiod.

Elongation and rooting of *in vitro* induced shoots. Shoots were excised from the multiple shoot bunches obtained from cotyledonary node explant and transferred individually to culture bottles containing half - strength B5 basal media to assess their response for elongation. Elongated shoots (2 - 3 cm) derived from shoot bunches of cotyledonary node were excised and rooted on medium consisting of half strength B5 basal medium supplemented with 0.1, 0.5, 1.0 mg L^{-1} NAA either Indole Acetic Acid (IAA) or Indole Butric Acid (IBA). All the media used in this study were supplemented with 2% (w/v) sucrose, solidified with 0.8% (w/v) and the pH was adjusted to 5.5 before addition of the agar and autoclaving at 121°C and 15 lb psi for 15 min. Results were observed at regular intervals and data were collected from three independent experiments and presented as average ± standard error (SE).

RESULTS AND DISCUSSION

In order to establish an efficient *in vitro* regeneration protocol for medium staple cotton cultivar (Barac [67] B) seeds were delinted by using concentrated 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 by $HgCl_2$ has already been proved to be essential in cotton tissue culture (Rauf *et al*., 2004). Cotton seed germination was observed after 48 h and within 3 days produced well developed root system with expanded cotyledon on all media tested (Fig. 1a). Among the three different basal media evaluated for their effects on seed germination, B5 basal medium supported higher rate of germination (100%) followed by MSB (88%) and MS (78.7%) (Table I). These differences in *in vitro* germination rate between the basal media might be due to their basal salt formulation and the high germination percentage obtained on B5 is probably due to their low salt content compared to MS. Droste *et al*. (2005) attributed a lower *in vitro* germination rate of *Vriesea gigantea* and *Vriesea Philippocoburgii* seeds to the sensitivity of the species to high salt-concentrations present in MS medium. Higher germination rate is an important factor for establishing cotton tissue culture and be particularly useful when there is a need to submit a uniform set of seedlings to a treatment (Sakhanokho *et al*., 2001).

Direct shoot bud differentiation was observed after 2 weeks of culture initiation. Multiple shoots were initiated from the cotyledonary node explants after 4 weeks of culture (Fig. 1b). The frequency of shoot formation was influenced by types and concentrations of phytohormones used (Table II). Explants obtained from seven-days old seedling cultured on hormone-free B5 basal medium failed to show any response but remained green up to four weeks. However, on B5 basal medium supplemented with various concentrations of kin or BA alone or in combination with NAA enlarged in their size after one to two weeks of culture and adventitious shoots developed directly in another four weeks (Table II). These results agree 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.

The dose of cytokinin is known to be critical in multiple shoots induction. Therefore, in this study we compared the response of cotyledonary node explants to various concentrations of BA and kin. Kin at different concentrations induced more shoots per explant compared to BA at the same concentrations. Indicating that, kin was more efficient than BA for multiple shoot production from cotton cotyledonary node explant. The shoot regeneration frequency increased with increases in concentration of kin until it reaches 2.5 mg L^{-1}, which was found to be the optimal concentration for maximum frequency of shoot bud formation (two shoots per explant). However, at higher concentration the number of shoot per explant was reduced (Table II). This is mainly, because at higher cytokinin level cotyledonary node explant produced excessive callus and failed to improve the efficiency of shoot multiplication. Thiem (2003) reported that callus growth on explant usually interfere with the propagation process.

Table I. The effects of different basal media on *in vitro* germination of cotton seed

Basal media	%Germination (Mean ± SE)
MS	78.7 ± 3.5
B5	100.0 ± 0.0
MSB	88.0 ± 2.3

Table II. Effects of phytohormones types and concentrations on shoot induction from cotyledonary nodes of medium staple cotton cultivar BARAC-(B) -67 in B5 medium

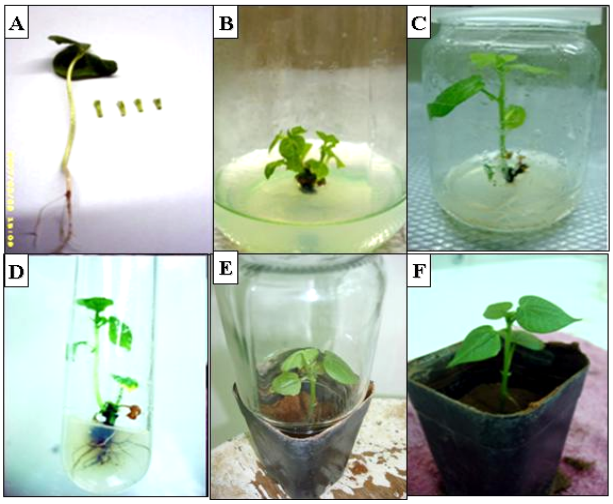
Phytohormones (mg/l)			Regenerating culture (%)	Number of shoots per explant (Mean ± SE)
BA	Kin	NAA		
0.0	0.0	0.0	0.0	0.00 ± 0.00
0.1	-	-	100	1.00 ± 0.00
0.5	-	-	100	1.00 ± 0.00
1.0	-	-	100	1.00 ± 0.00
2.5	-	-	50	0.52 ± 0.15
5.0	-	-	41	0.40 ± 0.14
0.1	-	0.1	75	0.80 ± 0.13
0.1	-	0.5	66	0.70 ± 0.14
0.1	-	1.0	66	0.70 ± 0.14
0.1	-	2.5	41	0.40 ± 0.14
0.5	-	0.1	38	0.30 ± 0.15
0.5	-	0.5	35	0.20 ± 0.12
0.5	-	1.0	33	0.30 ± 0.14
0.5	-	2.5	50	0.50 ± 0.14
-	0.1	-	100	1.67 ± 0.14
-	0.5	-	100	1.75 ± 0.13
-	1.0	-	100	1.75 ± 0.13
-	2.5	-	100	2.00 ± 0.00
-	5.0	-	100	1.75 ± 0.13
-	0.1	0.1	100	1.30 ± 0.14
-	0.1	0.5	100	1.10 ± 0.08
-	0.1	1.0	100	1.00 ± 0.00
-	0.1	2.5	100	1.00 ± 0.00
-	0.5	0.1	100	1.00 ± 0.00
-	0.5	0.5	100	1.00 ± 0.00
-	0.5	1.0	100	1.00 ± 0.00
-	0.5	2.5	100	1.00 ± 0.00

Table III. The Effect of auxin on rooting of *In vitro* derived shoots after three weeks of culture on half-strength B5 medium

Phytohormones (mg L^{-1})		Rooting %	No of roots/shoot (Mean ± SE)
NAA	0.0	37.0	0.8±0.3
	0.1	87.0	1.4±0.3
	0.5	75.0	0.8±0.2
	1.0	50.0	1.1±0.5
IAA	0.1	37.0	0.9±0.5
	0.5	50.0	0.5±0.2
	1.0	62.5	1.1±0.4
IBA	0.1	50.0.	0.4±0.2
	0.5	50.0	0.8±0.3
	1.0	62.5	0.8±0.3

In order to evaluate the synergistic effect of BA and Kin with NAA for direct shoot regeneration, it was found that, the combination of NAA with BA or Kin negatively affected the multiplication rate of the cotton compared with cytokinine. The inhibitory effect of auxin on multiple shoot induction has been demonstrated in numbers of plants. In faba bean (Khalafalla & Hattori, 2000) and mung bean (Gulati & Jaiwal, 1992) it was reported that the addition of

Fig. 1. *In vitro* induction of multiple shoots and plant regeneration of medium staple cotton cultivar (Barac [67] B), (A) Seven days – old *in vitro* germinated seedling and cotyledonary explants, (B) Multiple shoots bunches induced from cotyledonary node explants, (C) Shoot elongated on half strength B5 basal medium, (D) *In vitro* rooted shoot on half – strength B5 basal medium supplemented with NAA (0.1 mg/L), (E) Potting up and hardening of the plantlets, (F) Cotton plant established in soil



NAA to medium containing cytokinin did not improve shoot multiplication rate. In the present study, Kin alone was found to be suitable for both multiple shoot bud induction and proliferation. However, the multiple shoots obtained on various concentrations of kinetin failed to elongate on the same medium (Fig. 1c).

Based on previous study carried in our laboratory (submitted for publication), it was found that half strength B5 basal medium without phytohormone was the most suitable media for shoot elongation, therefore shoot produced from seven days-old seedlings on B5 basal medium supplemented with at 2.5 mg L^{-1} Kin were transferred individually to culture bottles containing half-strength B5 basal medium without phytohormones and supplemented with 2% sucrose and 0.8% agar for 15 - 20 days for elongation. Cytokinin has been reported to regenerate cotton plants with short and compact shoots (Banerjee, 2000). Moreover, as in this study, cytokinins have often been reported to stimulate shoot proliferation, while inhibiting shoot elongation (Brassard, 1996). The use of hormone-free medium for shoot elongation has already been reported for soybean (Kaneda *et al*., 1997) and faba bean (Khalafalla & Hattori, 1999).

Elongated shoots were excised and rooted on half-strength B5 medium without or with different levels of either NAA, IAA, or IBA was found to be more effective for root induction compared to basal media without or with IAA or IBA. Rooting of cotton shoots was higher (87.5%) on half-strength B5 medium containing 0.1 mg L^{-1} NAA (Table III) (Fig. 1d). The promotion effect of reducing the salt concentration of basal medium and using of NAA on

rooting of *in vitro* induced shoots has already been reported for cotton (Agrawal *et al*., 1997).

For acclimatization, plantlets were removed from rooting medium after three weeks of incubation and transferred to plastic pots containing autoclaved soil and covered with glass bottle to maintain humidity and were kept under culture room conditions for one week (Fig. 1e). After three weeks, glass bottles were removed and transferred to green house and placed under shade until growth was observed. 95% of the plants survived and all were morphologically normal (Fig. 1f).

In conclusion development of an efficient tissue culture and plant regeneration protocol for elite Sudanese medium staple cotton varieties is the first step towards the application of transgenic technology in improving cotton breeding. Moreover, this is likely to promote the application of plant tissue culture technology in the area of selection resistance and production of artificial seeds.

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