INFLUENCE OF GROWTH REGULATORS ON CALLUS INDUCTION FROM HYPOCOTYLS OF MEDIUM STAPLE COTTON (*Gossypium hirsutum* L). CULTIVAR BARAC B -67

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ABSTRACT

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This study was carried out at laboratory of Plant tissue culture, Commission for Biotechnology and Genetic Engineering, Khartoum, Sudan during the period of April 2007 to February 2008 to develop a protocol for callus induction of elite Sudanese cotton. Callus cultures were initiated from hypocotyl explants of elite Sudanese medium staple cotton (*Gossypium hirsutum* L.) cultivar Barac B-67 on Gamborg's B5 basal media. Different types and concentrations of growth regulators were tested in order to obtain the best callus formation. Four auxin types, indole -3- acetic acid (IAA), α-naphthalene acetic acid (NAA), indole-3- butyric acid (IBA), 2,4-dichloro-phenoxyacetic acid (2,4-D) at five concentrations(0.1, 0.2, 0.5,1.0 and 1.5 mgL^{-1}) and two cytokinines, benzyl adenine (BA) and 6-furfuryl amino purine (Kin) at four levels (0.1, 0.5,1.0 and 1.5 mgL^{-1}) in combination with NAA at (1.0 and 1.5 mgL^{-1}) were used in this study. It was found that growth regulator type and concentration had a significant effect on the callus induction, the increment of callus index and callus physical appearance. The highest frequencies of callus growth index (8.13 and 8.0) were observed on hypoctyle explants cultured on B5 basal medium supplemented with 1.0 mgl^{-1} NAA in combination with 0.1 mgl^{-1} of Kin or BA, respectively. Medium containing Kin resulted in the formation of compact callus with large numbers of roots emerging from it. There was no callus formation on B5 basal medium. The callus induced on B5 medium containing 2, 4-D was brown in color and of low quality compared to that produced on B5 media containing NAA.

Key words: Callus induction, hypocotyls, growth regulator

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. It grown in 70 countries and over 180 million people around the globe are involved with the fiber industry which produces 20 - 30 billion US dollars of raw cotton (John, 1997).

In Sudan although over the past two decades, the share of cotton in foreign export earnings has relatively declined where other products like livestock and oil 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.

Cotton yield and production is affected by wide ranges of climatic and biotic factors. 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). Besides yields, pests and diseases may affect cotton quality and necessitate strenuous measures to combat quality influencing factors.

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 (Abdellatef and Khalafalla, 2007). To overcome such problems of conventional breeding, advanced biotechnological method which recently emerged as most important tool in agricultural research can be applied as alternative approach for genetic improvement of this crop.

The potential benefits of using advanced agricultural biotechnology in cotton genetic improvement have not yet been realized in Sudan. Establishment of an efficient callus induction protocol is an essential prerequisite in harnessing the advantage of cell and tissue culture for genetic improvement. For the successful application of the tissue culture technique in crop breeding, callus growth and plant regeneration potential of each crop must be determined (Khaleda and Al-Forkan, 2006). Many investigations on tissue culture and plant regeneration in a variety of plants including cotton have been well documented. Cotton callus induction was first reported by Price and Smith (1979) in *Gossypium koltzchianum*. Cotton hypocotyl explants were most responsive to callus induction and proliferation. (Zhang *et al*., 2001). Thus, first of all, for biotechnological research on cotton a reliable callus induction protocol using hypocotyl is essential.

The present investigation was undertaken with an objective to develop an efficient callus induction protocol which is a major prerequisite for *in vitro* plant regeneration system involving elite Sudanese medium staple cotton cultivar Barac B-67.

MATERIALS AND METHODS

Seed material

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

Seed surface sterilization and germination

Seeds were delinted by using commercial H_2SO_4 (100 ml/kg of seeds). The seeds were continuously stirred in H_2SO_4 by spatula for 1 minute then washed by running tap water under shaking for one minute and finally washed by distilled water for 3 mints. Under laminar flow cabinet seeds were immersed in mercuric chloride ($HgCl_2$) 0.3% for 15 mints then washed for five times by sterilized distilled water.

Seed germination and explant

After surface sterilization 100 seeds were transferred to culture bottle and directly inoculated on the basic media at 25± 1ºC and photoperiod of 16 hrs light and 8 hrs dark. Four basal media namely, 1- full strength of MS (Murshige and Skoog's, 1962) salts and vitamins 2- half strength of MS salts and vitamins 3- full strength of B5 (Gamborg *et al*., 1958) salts and vitamins 4- MSB (Combination of MS salts and B5 vitamins) were tested for their effect on seed germination percentage. Hypocotyls segment of 0.5 cm length excised from 7-10 days- old seedling were used as explant in this study.

Effect of auxin on callus induction

Four auxin types were used to assess their effect on callus induction. Hypocotyls explants were cultured in culture bottles containing B5 basal media supplemented with (0.1, 0.2, 0.5, 1.0 or 1.5 $mgl^{-1)}$ of indole -3- acetic acid (IAA), α-naphthalene acetic acid (NAA), indole-3- butyric acid (IBA) and 2,4-dichloro-phenoxyacetic acid (2,4-D).

Effect of auxin in combination with cytokinine on callus induction

To assess the effect of auxin in combination with cytokinine, NAA at two levels (1.0 and 1.5 mgl^{-1}) was used in combination with either BAP or Kin at 0.1, 0.5 or 1.5 mgl^{-1}.

All the media used throughout this study were supplemented with 20% sucrose and 8% agar. The pH was adjusted to 5.5 before autoclaving at 121°C and 15 lb psi for 15 min.

Statistical analysis

All cultures were incubated for six weeks and forty explants were cultured per treatment. Cotton hypocotyl cultures were rated at weekly intervals. To set numerical values that represented both qualitative and quantitative growth, scale rating from 0 to 9 was developed (Figure 2). The scale was defined as: 0- no tissue growth, 1- callus arising from one explant end, 2- callus arising from both explant ends, 3- callus arising from both explant ends and double the original explant size 4-callus arising from both explant ends and triple the original explant size 5- callus arising from both explant ends and four times the original explant size 6- callus arising from both explant ends and five times the original explant size 7- callus arising from both explant ends and six times the original explant size.8- callus arising from both explant ends and seven times the original explant size. 9-callus arising from both explants ends and eight times the original explant size.

All the experiments were repeated three times and standard errors of the means were calculated.

RESULTS AND DISCUSSION

The limiting step to the successful use of modern techniques in genetic improvement of the major crops has not been transgene insertion itself, but rather the regeneration of viable plants from the transgenic explant material (Murphy, 2003). Thus, first of all, agric biotechnological research on crops requires reliable callus induction and then efficient *in vitro* regeneration system.

Cotton seeds from the field are highly contaminated as they are covered with large numbers of small hairs that can hold spores of fungi and bacteria (Figure1A). Moreover, for any tissue culture study, the surface of explants must be fully sterilized. In this study cotton seeds were delinted by using concentrated H_2SO_4® (Figure1B) then surface sterilized by $HgCl_2$ before *in vitro* germination. Delinting with H_2SO_4 is a highly effective way to remove the hairs and reduce the risk of contamination in the cultures. 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 (Abdellatef and Khalafalla, 2007; Rauf *et al*., 2004).

Cotton seed germination was observed after 48 hrs and within 3 days produced well developed root system with expanded cotyledon on all media tested (Figure 1C). 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 1). 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).

Different plant growth regulator (PGR) at different concentration and various combinations were tried for their effects of on cotton callus culture. The results indicated that cotton callus was induced on B5 basal medium supplemented with PGR. However, differences based on PGRs type and concentrations were observed.

The hypocotyl explant induced callusing in the presence of auxin on the other hand failed to produced callus in media free auxins, this declared that, the presence of auxins was capable to inducing callus. However, the callusing percentage, degree of callusing and callus appearance are auxin types and concentration dependant (Table 2). Essentially effects of auxins on cotton callus induction already have been reported (Trolinder and Goodin, 1987; Firoozabady *et al*, 1987; Sakhanokho *et al*., 1998. Among the auxins types tested, NAA induced the highest callusing rate and best callus appearance, compared to other types (Table 2). Among the different concentrations of NAA, 1.0 mgl^{-1} promoted rapid growth and produced the highest callusing rate (7.7) (Table 2). Several types of callus were distinguishable based on the physical appearance. Callus induced on B5 medium containing NAA was initially healthy invariably greener and more granular compared to other auxins (Figure 1D).However, the callus induced on B5 medium containing 2, 4-D was brown in color and of low quality (Figure 1E).

To study the combined effect of auxins and cytokinins on callus induction, two levels (1.0 and 1.5 mg/l) of NAA were used in combination with BAP and Kin at 0.1, 0.5, 1.0 and 1.5 mg/l. The result indicated that combination of NAA with BAP and Kin promoted the induction and growth of cotton callus. B5 medium supplemented with 1.0 mg/L NAA in combination with kin at 0.1 mg/l is the best medium for the induction of cotton embryogenic callus and develop maximum callus rate (8.13) (Table 3). However, medium containing Kin resulted in the formation of compact callus with large numbers of roots emerging from it. Combinations of NAA with BA induced grainy, nodular texture of high quality callus (Figure 1E).

Development of an efficient tissue culture and plant regeneration protocol for elite cotton varieties is the first step towards the application of transgenic technology to improve cotton breeding and is, thus, the foundation of cotton biotechnology research programme (Zhang *et al*., 2001). We have established callus induction protocol for Sudanese medium staple cotton cultivar Barac B -67. This protocol will pave the way for the development of *in vitro* regeneration system for this elite cultivar and consequently will promote the application of plant tissue culture technology in the area of selection resistance, production of artificial seeds, and genetic transformation.

Table 1. The effects of different basal media on *in vitro* germination of cotton seed after 3 weeks

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

Table 2. Effect of different auxins on callus induction from hypocotyls explant obtained from 7 days old seedlings of cotton (*Gossypium hirsutum* L.) cultivar Barac B-67 after 6 weeks of culture.

Plant Growth Regulators				Callusing response	Callus growth index	Callus color
2,4-D	NAA	IAA	IBA	(%)	(Mean ± SE)	
0.00	0.00	0.00	0.00	0.00	0.00±0.0	_
0.1	0.00	0.00	0.00	100	6.07±0.1	yellow to brown
0.2	0.00	0.00	0.00	100	7.00±0.2	yellow to brown
0.5	0.00	0.00	0.00	100	5.20±0.1	yellow to brown
1.0	0.00	0.00	0.00	100	4.00±0.0	yellow to brown
1.5	0.00	0.00	0.00	100	3.00±0.2	yellow to brown
0.00	0.1	0.00	0.00	100	4.40±0.4	Green yellowish
0.00	0.2	0.00	0.00	100	4.73±0.4	Green yellowish
0.00	0.5	0.00	0.00	100	6.37±0.3	Green yellowish
0.00	1.0	0.00	0.00	100	7.70±0.1	Green yellowish
0.00	1.5	0.00	0.00	100	6.77±0.1	Green yellowish
0.00	0.00	0.1	0.00	83.3	2.90±0.2	Green
0.00	0.00	0.2	0.00	90	3.17±0.2	Green
0.00	0.00	0.5	0.00	93.3	3.37±0.3	Green
0.00	0.00	1.0	0.00	96.6	4.80±0.3	Green
0.00	0.00	1.5	0.00	96.6	5.00±0.2	Green
0.00	0.00	0.00	0.1	0.00	0.00±0.0	-
0.00	0.00	0.00	0.2	63.3	0.80±0.2	Green
0.00	0.00	0.00	0.5	70	1.60±0.1	Green
0.00	0.00	0.00	1.0	80	1.80±0.2	Green
0.00	0.00	0.00	1.5	86.6	2.93±0.2	Green

Table 3. Effect of NAA in combination with BA and Kin on callus induction from hypocotyls explant obtained from 7 days old seedlings of cotton (*Gossypium hirsutum* L.) cultivar Barac B-67 after 6 weeks of culture

Plant Growth Regulators (mg/l)			Callusing response (%)	Callus growth index (Mean ± SE)	Callus color
BA	Kin	NAA			
1.0	0.1	0.0	100	8.00±0.28	Yellow, red
1.0	0.5	0.0	100	6.73±0.27	Yellow, red
1.0	1.0	0.0	100	4.00±0.26	yellow
1.0	1.5	0.0	0.0	0.00±0.00	-
1.5	0.1	0.0	100	3.47±0.19	yellow
1.5	0.5	0.0	70	2.43±0.24	yellow
1.5	1.0	0.0	0.0	0.00±0.00	-
1.5	1.5	0.0	0.0	0.00±0.00	-
1.0	0.0	0.1	100	8.13±0.30	yellow
1.0	0.0	0.5	100	5.07±0.25	yellow
1.0	0.0	1.0	80	3.03±0.27	yellow
1.0	0.0	1.5	60	2.67±0.23	yellow
1.5	0.0	0.1	53.3	3.83±0.37	yellow
1.5	0.0	0.5	53.3	3.43±0.20	yellow
1.5	0.0	1.0	46.6	1.90±0.21	yellow
1.5	0.0	1.5	33.3	0.67±0.10	yellow

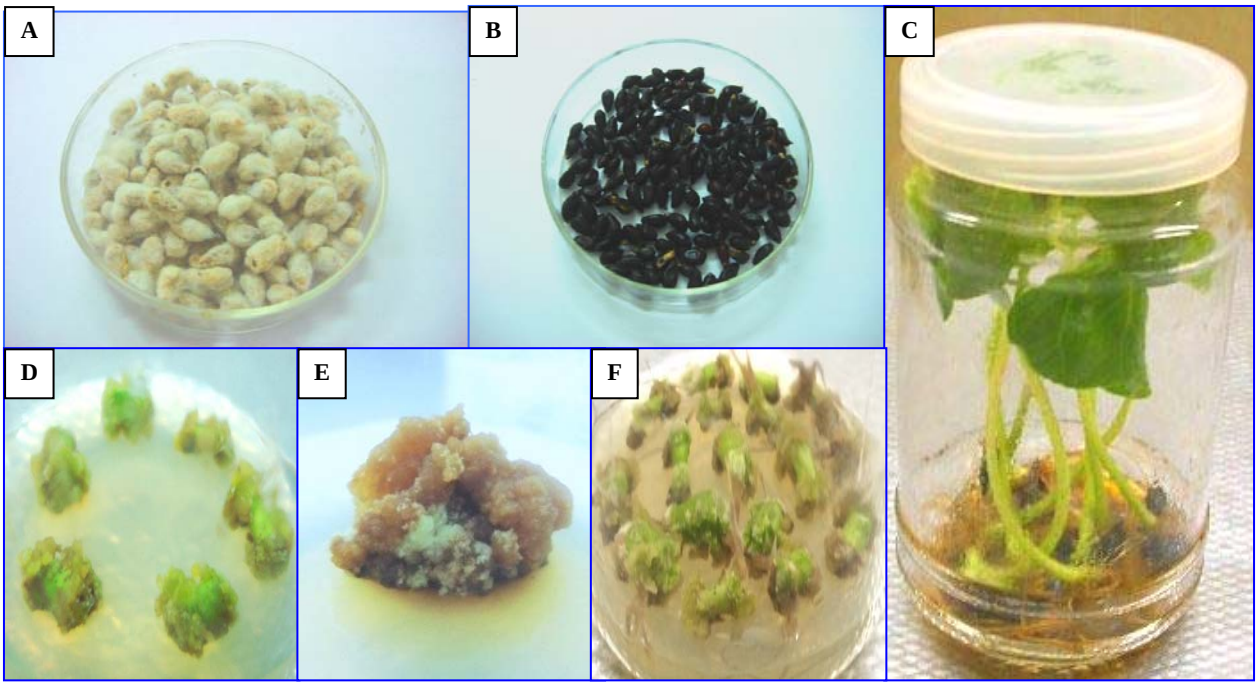

Figure 1. Cotton (*Gossypium hirsutum* L.) cultivar Barac B -67 callus formation A. Cotton seed with lint. B. Delinted seeds C. 7 days- old *in vitro* germinated seedling D. Yellowish-green callus produced on B5 basal media supplemented with NAA. E. Brown callus produced on B5 basal media supplemented with 2.4-D.F. Rooted callus produced on B5 medium supplemented with NAA in combination with Kinetin

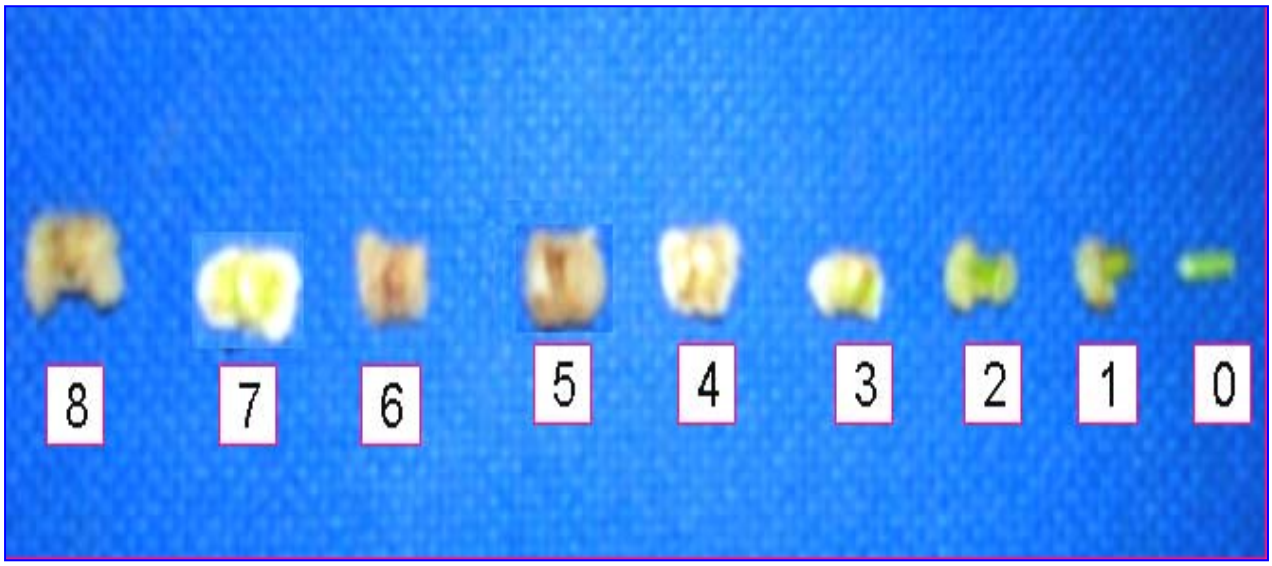

Figure 2. Growth scale rating of cotton callus induced to set numerical values that represented both qualitative and quantitative growth. 0 – no tissue growth, 1- callus arising from one explant end, 2- callus arising from both explants ends and doubles the original explant size, 3- callus arising from both explant ends and triple the original explant size, 4- callus arising from both explant ends and four times the original explant size, 5- callus arising from both explant ends and five times the original explant size, 6- callus arising from both explant ends and six times the original explant size, 7- callus arising from both explant ends and seven times the original explant size, 8 callus arising from both explant ends and eight times the original explant size

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