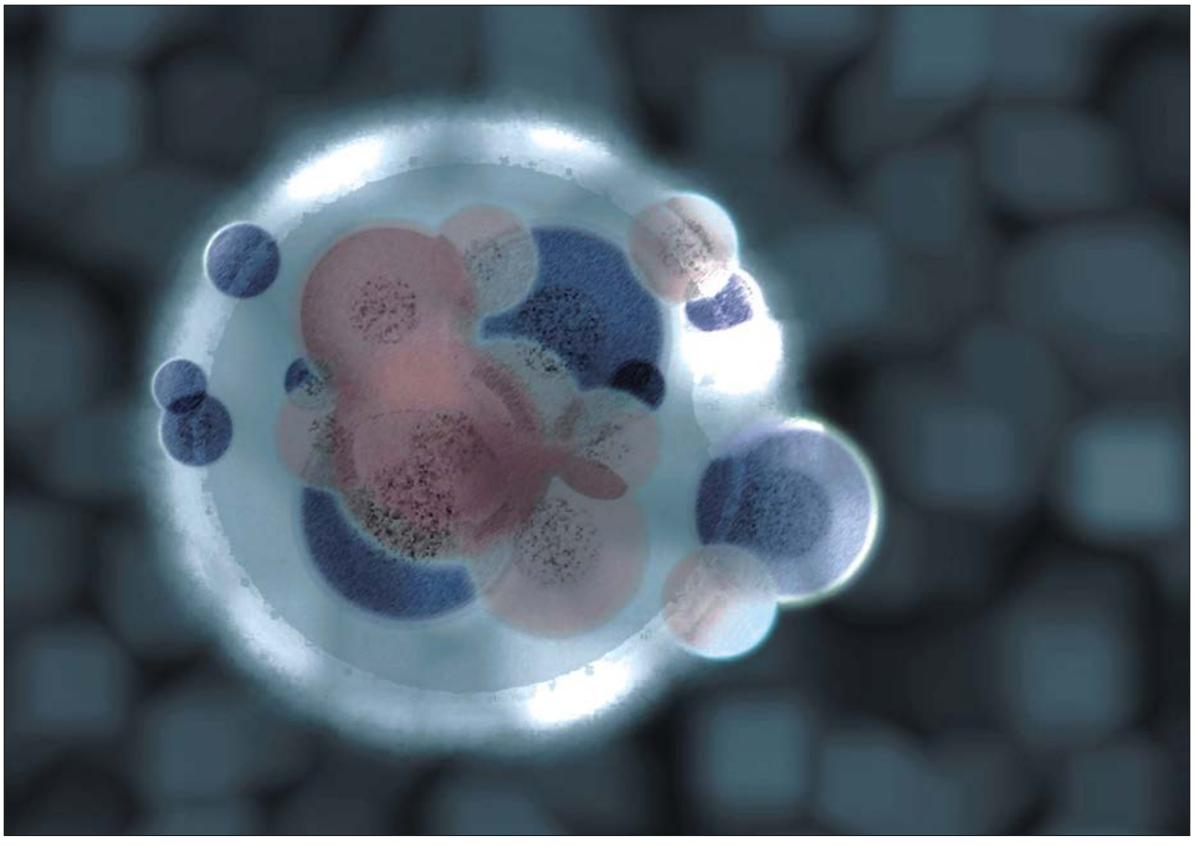

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Gossypol Accumulation and Morphogenesis in Cotton (*G. hirsutum* L.) Callus Culture

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Abstract: The objective of this study was to determine the influence of different plant hormones on gossypol accumulation in callus culture and morphogenesis of cotton (*G.hirsutum*.L). Conditions have been described for *in vitro* culturing of tissues from the seeds of two different types of cotton (glanded and glandless). The callus cultures were initiated using defined levels of Kinetin (Kn) and 6-Benzylaminopurine (BAP) and 2.4-dichlorophenoxyacetic acid (2:4-D) and 1-naphthaleneacetic Acid (NAA) and were then transferred on the same media and on media with no hormones also. The gossypol content of each callus cultures and different parts (root, stem and leaf) of aseptically grown (7 to 10 days old) seedlings and seeds was determined using HPLC for cotton plants. The different levels of auxin and cytokinin in the culture media led to differentiation and increased the accumulation of gossypol.

Key words: *In vitro*, cotton, gossypol, callus, secondary metabolite

INTRODUCTION

The phenolics are well known for disease resistance and may have chemical defense against various microorganisms and insect. Normally glandular cotton (*Gossypium hirsutum* L.) plant displays pigment glands in the leaves, stems and carpel walls in undiminishing numbers through out the life span of the plant. Pigment glands in cotton contain a variety of polyphenolic substances, one of this gossypol. The importance of gossypol as a source of resistance to insects in cotton (Bottger, Sheehan and Lukefahr 1964; Lukefahr and Houghtaling 1969). Mc Carty *et al.* (1996) also reported that the gossypol content of cotton *Gossypium* spp. has been of interest of plant breeders because those that have high levels often carry resistance to the tobacco budworm *Heliothis virescens* (F). Plant tissue cultures have been regarded as an attractive alternative to whole plants as a source of commercially important secondary products (Crocomo *et al*., 1981, Yeoman *et al.,* 1980). Little work has concentrated on the development of gossypol biosynthesis on *in vitro* conditions. A better understanding of the metabolic features of isolated cells *in vitro* may prove to be extremely valuable in future efforts to use plant tissue cultures as a commercial source of plant secondary products.The objective of this study was to determine the influence of different plant hormones on gossypol accumulation in callus culture and morphogenesis of cotton (*G. hirsutum* L).

MATERIALS AND METHODS

Plant material: The experiment was carried out at the Plant Biotechnology Laboratory of Institute of Genetics and Experimental Plant Biology Academy of Sciences Republic of Uzbekistan, in Tashkent, Uzbekistan during the period of 1995-1999. Five cotton (*Gossypium hirsutum* L.) varieties viz., AN Express-2, AN Bayut-2, L-479, L-475, Hybrid F1 (L-479 X L-475) were used as test material. Among them the variety L-479 was glandless. Seeds of variety L-479, L-475 and Hybrid F1 (L-479 X L-475) were obtained from the Tashkent State University, Uzbekistan. Seeds were surface sterilized for 2-3 minuets by immersing in 96% ethanol. Which were then rinsed three times in distilled water prior to germination. The seed materials were aseptically germinated on ¼ strength MS medium (Murashige and Skoog, 1962) in darkness at 28°C within two days.

Culture media: Segments of hypocotyles (5 mm) of seven days old seedlings were excised and placed on modified MS medium where glucose 30 g L^{-1}, B_5 Vitamin (Gamborg *et al*., 1968), supplemented with different concentrations and combinations of auxin and cytokinin, agar added 8 g L^{-1} and pH were 5.8-6.0. The media were dispensed into culture tubes and autoclaved at 120°C under 1.1 kg cm^{-2} pressure for 20 min. Cultures were maintained at 28°C with 16 h photoperiod providing light intensity of 2000 lux. After 30 days calli maintenance on

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media were visually scored for appearance, growth and general vigor and transferred on to the same media for increase and maintenance.

Extraction and analysis of gossypol: The seeds, leaf, root of seedlings (7-10 days old) and callus tissue of different cotton varieties were extracted with three volumes of chloroform. The extract was dried and the residue was taken up in 1 mL of HPLC grade methanol extract was extracted by HPLC (Hepshen *et al.,* 1988) with some modification the isocratic phase was replace by a linear gradient of mobile phase (methanol- acetonitrile-water). HPLC analysis was carried on Beckman System Gold model using C-18 column (Ultrasphere 0.46x25 cm), equipped with an IBM AT-486 computer operated high speed spectrophotometric detector. The solvent phase (80% methanol v/v in water was pumped at a flow rate of 1 mL/min. The gossypol was detected by its absorption at 280 nm. The Gossypol sample (specific activity of the sample 97.5%) as the standards obtained from the Institute of Bio-Organic Academy of Sciences Republic of Uzbekistan, Uzbekistan was used during the quantative analysis of the materials after identification.

Statistical analysis: The experiments were replicated three times and data on different parameters were subjected to statistical analysis and mean values were compared using LSD at 5% level of significant (Gomez and Gomez, 1984).

RESULTS AND DISCUSSION

The gossypol level of the seed related closely with the overall express of glandulosity of individual plants of a particular genotype (Lee *et al*., 1968). Thus an assay of the gossypol at the seed and different parts of seedlings of different varieties could give a close estimate of the total gossypol content of the sample. The amounts of gossypol content of these samples were estimated by the HPLC (High Performance Liquid Chromatograph). Results showed that the content of gossypol was higher in seeds than any other parts of the seedlings (Table 1). Seeds of the variety AN Express-2 contain higher amount 64 mg g^{-1}, 62 mg g^{-1}, glandless cotton variety L-479 content 0.2 mg g^{-1}, 49.5 mg g^{-1} variety L-475 and Hybrid F_1 (L-479 X L-475) contain 26.6 mg g^{-1}, respectively. Among different parts of the seedlings contents of gossypol was higher in roots then stem and leaves, respectively. From this results showed that cotton variety L-479 contents a few amount of gossypol. Among different cotton varieties L-479 content gossypol 320 times less in seed, 55 times in root, 45 times in stem and 42 times in leaf than other glanded cotton varieties, respectively.

Table 1: Content of gossypol on seeds and different parts of seedlings of cotton

Variety	Seed (mg g^{-1})	Root (μg mg^{-1})	Stem (μg mg^{-1})	Leaf (μg mg^{-1})
AN Express-2	64±1.5	49±1.5	19±0.2	3.0±0.0
AN Bayut-2	62±1.5	50±1.5	15±0.2	3.9±0.0
L-479	0.2±0.0	0.9±0.0	0.5±0.1	0.1±1.5
L-475	49.5±1.5	42±1.5	22.5±0.5	4.2±0.0
F_1(L-479x L-475)	26.6±1.5	24±1.5	20±0.5	1.8±0.0

Table 2: Response of hypocotyles explant of different cotton varieties towards callus formation in MS medium with different concentrations of 2.4-D, Kn, BAP and NAA

2.4-D	Kn	BAP	NAA	Callus characteristics
0.1	0.1	-	-	White friable callus in 5 days
1.0	1.0			White friable callus in 5 days
	0.1	0.1		White friable callus in 5 days
	1.0	1.0		White greenish friable callus in 5 days
	1.0		5.0	Compact white yellow callus in 5 days
		1.0	5.0	Compact white yellow callus in 7 days

For callus induction hypocotyls of 3 to 5 cm in length were excised and cultured on MS medium supplemented with different concentrations of 2.4-D, Kn, BAP and NAA. Callus initiation was observed after 3 to 5 days of inoculation. Result showed that among different concentrations 0.1 mg L^{-1} 2.4-D with Kn (0.1 mg L^{-1}) was found to be the best for callus induction for all varieties, in this combination 100% of calli formed from hypocotyls, except the variety L-479. The higher concentration of NAA (5.0 mg L^{-1}) with Kn (1.0 mg L^{-1}) or BAP (1.0 mg L^{-1}) did not showed best response for callus induction. In texture of the calli ranged from very hard and compact to watery and friable (Table 2). Among different concentrations low concentrations of 2.4-D with Kinetin or BAP resulted vigorous and medium friable callus. Higher concentrations of Kn (1.0 mg L^{-1}), BAP (1.0 mg L^{-1}) and NAA (5.0 mg L^{-1}) appeared to decrease friability and often formed hard, compact callus. These results are agreement with those reported by Trolinder and Goodin (1987, 1988), Davidonis and Hamilton (1983).

Media supplemented with different concentrations of 2.4-D, NAA, Kn and BAP resulted the different growth and fresh weight rate of callus and gossypol production (Table 3). Results of the contents of gossypol on different medium showed that medium supplemented with 2.4-D with Kn or BAP exhibited intensive callus production but inhibitory effect of biosynthesis of gossypol. Thus, the hypocotyles of the variety ANBayut-2 on the medium with 2.4 D (0.1 mg L^{-1}) and Kn (0.1 mg L^{-1}) produced 1340 mg callus within 30 days and 5020 mg after another 30 days and the amounts of gossypol was 0.044 (μ kg g^{-1}). The amount of callus was lower (315 and 850 mg, respectively) when callus were transferred on the medium containing Kn (1.0 mg L^{-1}) and NAA (5.0 mg L^{-1}) and the amount of gossypol was higher but the amount of gossypol was gradually decreased (Table 4). Similar

Table 3: Effect of the different auxin and cytokinin on callus and gossypol accumulation. Data were recorded after 30 and 60 days of incubation

Name of varieties	Hormonal conc. (mg L^{-1})		Amounts of callus (mg) days after culture		Amounts of gossypol (µg mg^{-1}) days after culture	
	2.4-D	Kn				
ANExpress-2	0.1	0.1	760fg	4650b	0.043a	0.042b
ANBayut-2			1340	5020a	0.044a	0.01c
L-479			720fg	3625e	0.007e	0.001f
L-475			780fg	4548c	0.042a	0.01d
F_1(L-479xL-475)			720fg	4073d	0.038b	0.01d
	2.4-D	BAP	30	60	30	60
ANExpress-2	0.1	0.1	737cd	855c	0.044a	0.040 b
ANBayut-2			783cd	880c	0.045a	0.030c
L-479			625de	745cd	0.006f	0.001g
L-475			715d	1220a	0.043a	0.020d
F_1(L-479xL-475)			681de	950b	0.041b	0.010e
	2.4-D	Kn	30	60	30	60
ANExpress-2	1.0	1.0	510c	875a	0.043b	0.02d
ANBayut-2			649bc	900a	0.044b	0.05a
L-479			230e	687b	0.007d	0.003d
L-475			498c	880a	0.045b	0.05a
F_1(L-479xL-475)			386d	688b	0.040c	0.04c
	NAA	Kn	30	60	30	60
ANExpress-2	5.0	1.0	220cd	845c	0.057a	0.06b
ANBayut-2			315cd	850c	0.089a	0.05c
L-479			125de	540cd	0.007f	0.003g
L-475			212d	825a	0.073a	0.05d
F_1(L-479xL-475)			176de	688b	0.052b	0.04e

In each column, mean not bearing similar superscripts differ significantly ($p<0.05$)

Table 4: The amount of callus and gossypol accumulation on the hormone and hormone free medium. Data were recorded after 90 days of incubation

Name of varieties	Hormonal conc. (mg L^{-1})		Amounts of callus (mg) days after culture	Amounts of gossypol (µg mg^{-1}) days after culture
	Kn 1.0	NAA 5.0	90	90
ANExpress-2			1400a	0.035a
ANBayut-2			1400a	0.034a
L-479			1100de	0.016c
L-475			1300ab	0.034a
F_1(L-479xL-475)			1200cd	0.032b
		Nil	90	90
ANExpress-2			2500b	0.029b
ANBayut-2			2900a	0.030b
L-479			2000cd	0.050a
L-475			2200c	0.020c
F_1(L-479xL-475)			2100cd	0.018cd

In each column, mean not bearing similar superscripts differ significantly ($p<0.05$)

observation also reported by Stickland and Sunderland (1972) that 2.4-D added to the medium reduced the amounts of anthocyanin produced by callus of *H. gracilis*. Blakely and Steward (1961) found that anthocyamin production in callus of *H. gracilis* was markedly influence by the medium constituents, particularly the auxin concentration. After the first sub culture the callus were transferred on the same medium and also maintained on media with no hormones (Table 4). Significant amounts of gossypol were produced on the hormone free medium. For the L-479 variety the levels of gossypol increased upon transfer of the cultures on the hormone free medium but decreased among other varieties. The ability of the cultures to thrive in the absence of exogenous auxin and cytokinin suggests that these cells produce gossypol on the endogenous level. Some morphological differentiation was also observed in the callus cultures used in this investigation. Medium containing Kn and NAA formed more differentiated callus tissue and in some cases, root structure was observed. Meimeth *et al.* (1982) found the higher levels of a lectin from *Psophocarpus tetragonolobus* in those cultures that were rapidly differentiating. Cell cultures provide excellent system for the study of secondary metabolism. Simple alterations of growth media and environmental factors (light), often have a dramatic effect on the type and pattern of products produced. From present results, it may be concluded that, the gossypol biosynthesis could furthermore, be influenced by using the media. There was a significant effect of the media on the gossypol levels but the expression of gossypol on the exogenous hormones free medium indicate that differentiation and tissue organization are the factors which influenced the production. Similar observations have been made by other workers (Tabata *et al.*, 1972; Yeoman *et al.*, 1982; Collinge and Yeoman, 1986) with other explants. Hence, this

regulation of the gossypol synthesis and turnover was due to a direct hormonal action or to a secondary effect remains to be determined.

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