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RESEARCH ARTICLE

Comparison of oil and fatty acid composition of seed, shoot and *in-vitro* regenerated organs of cotton (*Gossypium hirsutum* L. var. Sahel)

Babak Delnavaz Hashemloian* and Azra Ataei Azimi

Department of biology, Faculty of agriculture, Saveh branch, Islamic Azad University, Saveh, Iran

ABSTRACT: Cotton (*Gossypium hirsutum* L.) is a source of edible oil and fatty acids (FAs). In this study, embryos' explants were cultured in MS medium with different concentration of plant growth regulators (phytohormones) include BA, IAA, IBA, 2, 4- D and NAA, to finding the relationship of hormone and organogenesis with oil and FAs content. Oil was extracted with chloroform and methanol. FAs of oil were esterificated and methylated for GC-MS analysis. The seeds and shoots oil were 36.1 and 4.5%. Oil of regenerated shoots (33.3%) in T2 with 0.25 BA and 5 mgl^{-1} IAA was higher than to seeds, shoots and all regenerated organs. Unsaturated FAs increased in some treatments depending on the type of phytohormone and organogenesis. Amounts of all FAs of regenerated root of T4 in BA/IBA were lesser than to other samples. Our results showed that Sahel cultivar of cotton can synthesize 12 types of FAs, but some of those FAs are made under certain conditions depending on the type and concentration of phytohormones and organogenesis.

KEYWORDS: Phytohormone; Organogenesis; GC-MS; Saponification, Regeneration

ABBREVIATIONS: N-6 benzyladenine (BA), Indole Acetic Acid (IAA), 2, 4- Dichlrophenoxi acetic acid (2, 4-D) and Naphthalene Acetic Acid (NAA), regenerated plantlet (Pl), regenerated shoot (R-sh), Callus(Ca), regenerated root (R-ro), regenerated root of Cotyledon (R-ro-cot) and Somatic embryogenic callus(Som-ca).

INTRODUCTION

Cotton (*Gossypium hirsutum* L.) is one of the most economically important crops in the world. It is cultivated mainly for its filament and seeds oil [22]. Tissue culture regeneration protocol is very necessary for development of plants. But, cotton is recalcitrant to regenerate *in vitro* [6]. *G. hirsutum* cv Coker-312 showed good responses to gene transformation and in *vitro* regeneration through somatic embryogenesis and shoot tip organogenesis [4, 18, 23]. Somatic embryogenesis and plant regeneration has been reported from hypocotyl [3], but the response is highly genotype dependent [4, 20]. There are restrictions on the regeneration of cotton from protoplast, callus, or leaf tissue *in vitro* culture [15]. The oxidative stability of cottonseed oil can be lesser than other vegetable oils because of its high concentration of linoleic acid (18:2) [19]. The concentration of palmitic acid (16:0), a saturated fatty acid, is higher in cottonseed oil (~24%) than in many other vegetable oils [25]. Cottonseed oil also contains modest levels of cyclopropenoid fatty acids, which are considered anti-nutritional [22]. Limited variation in fatty acids composition of cottonseed oil was reported and the glandless trait did not significantly affect seed oil composition [10, 13]. The fatty acids composition of cotton seed oil is mainly linoleic (55.2–55.5%), palmitic (11.67–20.1%), and oleic acids (19.2-23.26%) [1]. The dominated fatty acids of cottonseed oil was linoleic acid (48.1 to 56.3 g/100 g) while in linseed oil mainly α-linolenic acid (53.2 g/100 g) and in fennel seed

*Corresponding author (✉): hashemloian1343@gmail.com
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oil mainly petroselinic acid (80.5 g/100 g) were dominated [14]. In this study, we investigated the effects of organogenesis and phytohormones on the oil content and fatty acids of cotton *in vitro* culture.

MATERIALS AND METHODS

Plant material

Seeds of cotton (*G. hirsutum* L. var. Sahel) were prepared by agriculture organization of Gonbad Kavoos (IRAN).

In vitro culture

In the present study, we selected the initiation concentrations according to previous studies to *in vitro* culture of cotton samples [9]. Cottonseeds fluff were cleaned by concentrated sulfuric acid. The cottonseeds were soaked in sulfuric acid for 1min and they were thoroughly washed with sterilized water 3 times for 15 min. They were then placed in ethanol (96%) for 1 min, and after complete evaporation of ethanol, the seeds coating was removed and the root region of embryos were cut.

Embryos without root were used as explants. The explants were cultured in full strength MS (Murashige and Skoog) medium [16], supplemented with different concentrations of N-6 benzyladenine (BA), Indole Acetic Acid (IAA), Indole Butyric Acid (IBA), 2, 4- Dichlrophenoxi acetic acid (2,4-D) and Naphthalene Acetic Acid (NAA), (Table 1). The pH of the medium was adjusted at 5.8 and cultured samples were kept in a growth chamber with 8 h dark and 12 h light period (fluorescent, cool white light, 7 W/m^2), at 25^{o}C for 10 weeks. After 10 weeks; the *in vitro* culture samples were used for oil and FAs assay.

Oil extraction

Oil was extracted from cotton seeds, shoots, regenerated-plantlet (Pl), regenerated- shoot (R-sh), Callus (Ca), regenerated- -root (R-ro), regenerated- root of Cotyledon (R-ro-cot) and somatic embryogenic callus (Som-ca) from different treatments (T1-T8). Extraction of oil was performed according to the modified methods of Folch *et al.* (1957) [5] and Hubbard *et al.* (1977) [8] with chloroform and methanol mixtures. 5 grams of fresh weight (fw) of cotton samples were grinded and the oil was extracted with 24 ml of chloroform- methanol (2/1, v/v) by adding 6 ml water, at 4º C. The chloroform phase containing oil was collected. The chloroform was accumulated and concentrated at 60 ℃ by vacuum rotary evaporator and subjected to total oil measurement, oil esterification and methylation procedure for future analysis. The oil percentages was determined with weight measurement of chloroform residue.

Table 1. Regenerated plantlet (Pl), Regenerated shoot (R-sh), Callus(Ca), Regenerated -root (R-ro), Regenerated root of Cotyledon(R-ro-cot) and Somatic embryogenic callus(Som-ca) were produced by cotton explants in different hormone(mgl^{-1}) treatments(T1-T8) after 10 weeks incubation and total oil.

Treats	BA	IAA	2,4-D	IBA	NAA	organ	Total oil%
T1	0.25	2.5	-	-	-	Pl	5.4^{dc}
T2	0.25	5	-	-	-	R-sh	33.3^{a}
T3	0.25	-	5	-	-	Ca	15^{b}
T4	0.25	-	-	5	-	R-ro	7^{c}
T5	0.25	-	-	2.5	-	R-ro-cot	5^{dc}
T6	5	-	-	-	0.25	R-sh	7^{c}
T7	2.5	-	-	-	0.25	Som-ca	6^{c}
T8	0.25	-	-	-	5	Ca	3^{d}
Seed	-	-	-	-		-	36.1^{a}
Shoot	-	-	-	-		-	4.5^{d}

Esterification procedure

The oil saponification and FAs esterification performed according to the modified method of Dermaux et al. (1999) [2]. A solution of 1N KOH in methanol (80%) was added to the glass tube containing the 5 ml oil sample in chloroform. The tube was then capped tightly, shaked and heated for 1 h to oil saponification. After cooling the samples at room temperature, 30 ml of water: petroleum ether (1:1) was added and the sample was shaken with repeated inversions. The petroleum ether was separated and effused out. The aquatic saponification phase containing FAs were collected and mixed with 40 ml 4N chloridric acid (HCl) and 30 ml petroleum ether. The petroleum ether phase containing FAs concentrated at 60 ˚ C by vacuum rotary evaporator and collected in a vial.

Methylation procedure

Fatty acid methyl esters were prepared using sulfuric acid (H_2SO_4) in methanol according to the modified method of Hitchcock and Hammond *(1980) [7]. 5 ml of* sulfuric acid (0.1 M) in absolute methanol was added to the fatty acid vial and the tubes heated to 100 ˚C for 60 min. After cooling off was added 5 ml ethyl ether and 5 ml water. Fatty acid methyl esters were separated by ethyl ether. The ethyl ether containing fatty acid methyl esters were

transferred to a glass vial and stored in a freezer (-80ºC) For GC–MS analysis.

GC–MS analysis conditions

An agilent gas chromatograph from Hewlett-Packard (6890), equipped with an HP 5971 MS detector was used to analyze fatty acids. Separations were carried out on an Agilent (Hewlett-Packard) HP-5 fused silica capillary column (30 m × 0.25 mm I.D.; 0.25 µm film thickness). The GC-MS interface temperature was maintained at 300ºC. 1 µl of a cooled sample was injected manually in injector port (with split less mode and temperature at 200ºC). The helium carrier gas flow rate was1 ml min^{-1}. The column temperature program was as follows: 90ºC, held for 1 min; 12ºC min^{-1} to 150ºC, held for 1 min; 2ºC min^{-1} to 210ºC, held for 3 min; and 10ºC min^{-1} to 300ºC, held for 30 min. The selective ion mode was used in the analysis. The retention time and abundance of confirmation ions relative to that of quantification ions were used as criteria for identification. The start button and the injection of a sample were synchronized to have consistent retention time (RT) values. The mass-to-charge range was 50–500 amu. An oven temperature program was used with gradation at 50–300°C. The spectra were recorded on an interfaced computer. The same procedure was repeated for each all samples.

Statistical Analysis

The results were subjected to the analysis of variance and least significant difference test (LSD) to ascertain the variability in oil content.

RESULTS

In vitro culture

After 10 weeks incubation, Pl, R-sh, Ca, R-ro, R-ro-cot and Som-ca were produced by cotton explants in different hormone (mgl^{-1}) treatments (T1-T8) and some samples with different organogenesis and phytohormne concentrations were used for oil assay (Table 1). Some organs derived from embryonic explants, concurrent regenerated plantlet (Pl) in T1; Regenerated shoot (R-sh) in T2 (BA/IAA) and T6 (BA/NAA); Callus(Ca) in T3(BA/2, 4-D) and T8 (BA/NAA), Regenerated-root (R-ro) in T4 and Regenerated-root- cotyledon (R-ro-cot) in T5 (BA/IBA) and somatic embryogenic callus(Som-ca) in T7 (BA/NAA).

Total oil (Table 1): Oil of seeds and shoots were 36.1 and 4.5% (g/g). Oil of R-sh in T2 with 0.25 mgl^{-1} BA and 5 mgl^{-1} IAA with 33.3% was the highest among all samples but lesser than the seeds. Oil content of all regenerated organs and shoots reduced significantly to seed with organogenesis and plant growth regulators ($P \leq 0.05$).

GC-MS analysis results **(**Table2, 3)**:** 16 compounds were detected in cotton oilseed but some of those were FAs. Those FAs included tetradecanoic (myristic), Benzenedicarboxylic (Phthalic), Hexadecanoic (Palmitic), 10,13- Octadecadienoic acid, Octadecadienoic (linoleic), Octadecenoic (Oleic), Octadecanoic (Stearic). Some compounds were FAs or their derivatives. Except for nonadecane alkane, 9 compounds of T1 oil Plantlet were FAs. 13- Octadecenoic (petroselinic) and 8,11-Octadecadienoic of oil of this sample weren't find in oilseed. All 5 compounds of plant oil shoot were FAs and T2 oil regenerated shoot (R-sh) had 7 FAs. 8 compounds were detected from T3 oil regenerated root (R-ro) but 6 of them were FAs. T4 oil Callus (Ca) with 12 compound had 6 FAs. Unsaturated and saturate FAs and some other compounds were observed in oilseed, shoot and regenerated organs (T1-T8) by GC-MS analysis. The number and entity of these compounds were different. R-ro-cot of T5 in 0.25 mgl^{-1} BA and 2.5 mgl^{-1} IBA had the highest number of compounds. In this treatment; number of saturated FAs increased but unsaturated FAs reduced in the seed. Number of unsaturated FAs were more than saturated FAs in all treatments, except for T5. Cotton oilseed that had the maximum number of FAs. In all treatments, the number of unsaturated FAs were lower than seeds. These results showed that the type and concentration of hormone and the type of organogenesis affect number of plant compounds.

FAs of cotton oilseed included phthalic acid (0.81%), myristic acid (11.40%), palmitic acid (21.69%), linoleic acid (26.68%), oleic acid (7.64%), stearic acid (11.04%), linolenic acid (9.33%) and other FAs (10.88%). In the shoot, amount of oleic and linoleic acid increased significantly in contrast to seed but stearic acid decreased. Myristic and linolenic of seed absented in the shoot. The amount of phthalic acid increased in the shoots to seed and all regeneration organs. Myristic acid was observed only in oilseed and oil R-ro-cot of T5. The amount of palmitic acid of shoot was similar to that of seed. Kind of FAs of oil extract of Pl in T1 was more similar to the shoot, except for petroselinic. Petroselinic acid with high

Table 2. GC-MS analysis results: Number and entity of unsaturated (un) and saturate (sa) FAs (FA) and other compounds of oil of seed, shoot and *in vitro* samples (T1-T8) of cotton

Compound	seed	shoot	T1	T2	T3	T4	T5	T6	T7	T8
N. un FA	5	3	4	3	2	3	3	4	3	4
N. sa FA	3	2	2	2	1	2	4	2	2	2
Alkane	1	0	1	1	0	2	1	1	1	0
Alkene	1	0	0	0	0	0	1	0	0	1
Other	1	0	0	0	3	5	4	2	2	2
Organ	Seed	Shoot	Sh-ro-ca	Sh-ca	Ca	Ro- ca	Cot-ro	Sh	Som-ca	Ca
Total compound	16	5	10	7	8	12	22	13	9	13

Table 3. Kind and rate (%) of fatty acid of seed, shoot and treatment samples (T1-T8) oil of cotton

Fatty acid	seed	shoot	T1	T2	T3	T4	T5	T6	T7	T8
Phthalic	0.81	11.66	2.81	4.25	4.27	6.10	0.94	2.71	4.51	3.22
Palmitic	21.69	20.41	22.46	22.25	17.63	7.39	28.8	26.63	17.18	28.36
Linoleic	26.68	37.6	2.49	44.52	32.6	18.09	45.45	46.78	38.79	45.71
Oleic	7.64	22.9	22.52	25.78	14.41	9.69	12.33	3.7	24.53	13.63
Stearic	11.04	3.01	13.3	3.02	-	1.3	2.11	3.02	5.17	6.67
Margaric	-	-	-	-	-	-	0.17	-	-	-
Palmitoleic	-	-	-	-	-	-	0.98	-	-	-
Myristic	11.4	-	-	-	-	-	1.88	-	-	-
Linolenic	9.33	-	-	-	-	-	-	-	-	0.83
Petroselinic	-	-	29.32	-	-	-	-	-	-	-
Ricinoleic	-	-	-	-	-	-	0.44	-	-	-
Pentadecenoic	-	-	-	-	-	-	-	-	-	2.25
Other FA	10.88	-	10.73	-	-	-	-	10.9	-	-
Un FA%	55.34c	72.16a	57.87b	70.45a	51.28c	23.88d	60.14b	64.09ab	67.63a	55.64c
Sa FA%	44.50a	23.42d	35.76b	25.27d	17.63e	8.69f	32.79bc	29.65c	22.35d	35.05b
Organs	Seed	Shoot	Pl	R-sh	Ca	R- ro	R-ro-cot	R-sh	Som-ca	Ca

amount (29.32%) in Pl wasn't found in seed, shoot and other regeneration organs. Linoleic acid in this sample was low in seed, shoot and other regenerated organs. Stearic acid absented in Ca of T3 with 0.25 mgl^{-1} BA supplemented with 5 mgl^{-1} 2,4-D but in Ca of T7 (2.5 mgl^{-1} BA with 0.25 mgl^{-1}NAA) and T8 (0.25 mgl^{-1} BA with 5 mgl^{-1}NAA) was 5.17 and 6.67%, respectively (Table 9). Phthalic, palmitic, linoleic, oleic, stearic were detected in all samples except for stearic in Ca of T3.

Some FAs include myristic in seed and R-ro-cot of T5, linolenic in seed and Ca of T8, margaric, palmitoleic and ricinoleic in R-ro-cot of T5; and pentadecenoic acid in Ca of T8 were absented in other organs. In R- ro of T4 unsaturated and saturated FAs were low in contrast to other samples. Unsaturated FAs in shoot, R-sh of T2 and Som-ca of T7 were higher significantly in compare with other organs.

DISCUSSION

McCabe and Martinell, (1993) [15] and Yan-Xia et al., (2006) [24] reported that *in vitro* cotton regeneration to callus, protoplast, or leaf tissue is strongly restricted but in this research, regenerated plant, shoot, root and callus were obtained from cotton embryonic explants in MS medium with BA / IAA, BA / 2, 4-D, BA / IBA and BA / NAA (Table 1), according Khan et al. (2006) [9]. The cotton oilseed contains 17–25% oil [1]. Oil content of all regenerated organs and shoot were reduced by organogenesis and plant growth regulators. Total oil of seed and shoot of cotton var. Sahel were 36.1 and 4.5%. Total oil of R-sh in T2 with 0.25 mgl^{-1} BA and 5 mgl^{-1} IAA with 33.3% was highest among all samples but lesser than of the seed. The fatty acid composition of cottonseed oil included phthalic acid (0.81%), meristic acid

(11.40%), palmitic acid (21.69%), linoleic acid (26.68%), oleic acid (7.64%), stearic acid (11.04%), linolenic acid (9.33%) and other FAs (10.88%). The fatty acids composition of cotton seed oil reported mainly linoleic (55.2–55.5%), palmitic (11.67–20.1%), and oleic acids (19.2–23.26%) [1]. The dominating FA are linoleic acid in cottonseed oil with 48.1 to 56.3% [14], while in cotton var. Sahel was 26.68% and is very lesser than other reports [14, 1]. In fennel seed oil mainly 18:1 FAs (80.5 g/100 g) with petroselinic acid are predominated [13]. Petroselinic acid with high amount (29.32%) in Pl of T1 wasn't found in oil of seed, shoot and other regeneration organs. As with the Lawhon *et al.* (1977) study [12], these studies showed fairly limited variation in fatty acid composition. Some FAs include meristic in seed and R-ro-cot of T5, linolenic in seed and Ca of T8, margaric, palmitoleic and ricinoleic in R-ro-cot of T5; and pentadecenoic acid in Ca of T8 absented in other organs. In R- ro of T4 unsaturated and saturated FAs were low to other sampels. Unsaturated FAs in shoot, R-sh of T2 and Som-ca of T7 were significantly higher than other organs. Shimizu *et al.* (2001) found 4.9% of total oil in *Salicornia herbacea* shoots [21], whereas Kulis *et al.* (2010) [11], obtained between 3.2% and 7.1% of total oil from shoots of *S. europaea* and *S. virginica*, depending on the extraction method. Similar to their seeds, FAs composition of shoots of *Salicornia* and *Sarcocornia* species are dominated by polyunsaturated acids with 16 and 18 carbons, but a significant amount of saturated Fas are also present [11]. The FAs synthesized in the shoot and root were mainly C (16), C (18), and C (18:1) but in the seed about 18% to 32% of the FAs were C (12) FAs. Root FAs were intermediate between the seed and shoot [17].

CONCLUSION

Using embryonic explants of cotton can obtain regeneration plants, shoots, roots, somatic embryo and callus *in vitro* culture. Plant hormones and organogenesis effected on total oil**.** Cotton var Sahel can synthesize 12 types of FAs, but some of these FAs are made under certain conditions depending on the type and concentration of hormones and the type of regenerated organ. Hormones showed some positive effects on enhancing the production of certain FAs in cotton. Saturated and unsaturated FAs content depended on the type of organogenesis and type of hormone. Decreasing the number of FAs, increasing unsaturated FAs, reducing saturated FAs, increasing the unsaturated/ saturation and increasing other different substances indicate changes in the direction of organogenesis. Thus plant growth hormones are going to act as an interesting field of research for studying their effect on various important metabolites of *in vitro* culture of plants.

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مقایسه روغن و اسیدهای چرب دانه، شاخساره و اندام های باززایی شده پنبه رقم ساحل (*Gossypium hirsutum* L. var. Sahel) در کشت درون شیشه

بابک دلنواز هاشم لوئیان *، عذرا عطائی عظیمی

گروه زیست شناسی، دانشکده کشاورزی، دانشگاه آزاد اسلامی واحد ساوه، ساوه، ایران

*نویسنده مسئول: delnavaz@iau-saveh.ac.ir

چکیده

پنبه (*Gossypium hirsutum* L.) یکی از منابع روغن و اسیدهای چرب (FAs) خوراکی است. در این تحقیق، جداکشت های رویانی در محیط MS با مقادیر متفاوتی از تنظیم کننده های رشد (فیتوهورمون ها) شامل بنزیل آدنین(BA)، اندول استیک اسید (IAA)، ۲و۴- دی کلروفنوکسی استیک اسید (2, 4-D) و نفتالین استیک اسید (NAA)، برای یافتن ارتباط هورمون و اندامزایی با محتوای روغن و اسیدهای چرب کشت شدند. روغن با کلروفرم و متانول استخراج شد. اسیدهای چرب روغن برای آنالیز با گاز کروماتوگرافی جرمی، استری و متیله شدند. دانه ها و شاخساره ها به ترتیب ۳۶/۱ و ۴/۵٪ روغن داشتند. روغن شاخساره های باززایی شده (۳۳/۳٪) در تیمار ۲(T2) با ۰/۲۵ میلی گرم بر لیتر BA و ۵ میلی گرم بر لیتر IAA، بیشتر از دانه ها، شاخساره ها و همه اندام های بازایی شده بود. اسیدهای چرب غیر اشباع در برخی از تیمارها بسته به نوع فیتوهورمون و نوع اندامزایی افزایش داشتند. مقادیر همه اسیدهای چرب ریشه های باززایی شده در T4 با BA/IBA کمتر از دیگر نمونه ها بود. نتایج ما نشان داد که پنبه رقم ساحل می تواند ۱۲ نوع اسید چرب را بسازد ولی برخی از آن ها، در شرایط خاص بسته به نوع و غلظت فیتوهورمون ها و اندامزایی ساخته می شوند.

کلمات کلیدی: فیتوهورمون؛ اندامزایی؛ گاز کروماتوگرافی جرمی؛ صابونی شدن؛ باززایی