In vitro plant regeneration of Iraqi cotton (*Gossypium hirsutum* L.) cultivars through embryonic axis

تجديد نبات القطن (*Gossypium hirsutum* L.) في الاصناف العراقية تحت ظروف المختبر من خلال محور الجنين

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Abstract

In plant tissue culture studies, obtaining new plantlets from different parts of plant is a very important feature with direct or indirect ways of regeneration. The plant show different regeneration capacities from species to species. In this present study, *in-vitro* direct shoots development from embryonic axis of cotton (*Gossypium hirsutum* L. cv. Lashata) were comparatively studied. Embryonic axis, embryonic hypocotyl and plumule obtained from five days old *in-vitro* grown seeds. All explants were cultured on MS media supplemented with 1- 4 mg/l Benzyl amino purine (BAP) + 0.1 mg/l Naphthalene acetic acid (NAA) and 3 mg/l activated charcoal. Cultured explants were kept at growth chamber with photoperiod for 16 hours light and 8 hours dark, at 25°C. Reproducible *in-vitro* plant regeneration was obtained from embryonic axis when cultured onto MS medium supplemented 1 mg/l BAP + 0.1 mg/l NAA and 3 g/l Activated charcoal. Shoot induction was to be noted 53.3%, 36.7% and 12.3 in embryonic axis, plumule and embryonic hypocotyl respectively. All these shoots were capable of rooting on MS medium supplemented with 0.1 mg/l NAA, and establishing in soil 3-4 weeks.

Key Words: Cotton, Direct organogenesis, Embryonic axis, *Gossypium hirsutum* L., *In-vitro*

المستخلص

تعتبر دراسات زراعة الانسجة النباتية مهمة للحصول على نباتات جديدة من اجزاء مختلفة من النبات هي ميزة هامة جدا بالاعتماد على وسائل مباشرة او غير مباشرة للتجديد وهي محطة اظهار قدرات التجديد المختلفة من نوع الى نوع اخر وبحسب النبات . اجريت دراسة الاكثار الدقيق في مختبرات قسم المحاصيل الحقلية ، كلية الزراعة ، جامعة انقرة . تم الاستفادة من اجزاء نبات القطن محور الجنين ، الرويشة و السويقة الفلقية السفلى وتم الحصول على اجزاء النبات من البذور بعد خمسة ايام من النمو تحت ظروف المختبر . تم وضع جميع اجزاء النبات في الوسط الغذائي MS واستكملت مع 1-4 ملغم/ لتر BAP بنزيل البيورين الامينية و 0.1 ملغم/لتر NAA نفثالين حامض الخليك مع 3 ملغم/ لتر من الفحم المنشط في الوسط الغذائي. تم رفع اجزاء النبات في غرفة النمو وكانت فترة الاضاءة 16 ساعة في النهار و 8 ساعات في الظلام وكانت درجة الحرارة 25 م . تم الحصول على تكاثر وتجديد النبات في المختبر من خلال جزء النبات محور الجنين التي وضعت في الوسط الغذائي واستكملت 1 ملغم/لتر BAP بنزيل البيورين الامينية و 0.1 ملغم/لتر NAA نفثالين حامض الخليك مع 3 ملغم/لتر الفحم المنشط. اظهرت النتائج ان اعلى نسبة لتحفيز البراعم كانت 53.3 %, 36.7 % و 12.3 % محور الجنين ، الرويشة و السويقة الفلقية السفلى على التوالي . وكانت جميع البراعم (الافرع) قابلة على التجذير في الوسط الغذائي SM والتي كانت قد استكملت مع 0.1 ملغم/ لتر AAN، وكان التاقلم في التربة . بغضون 3-4 اسابيع .

الكلمات المفتاحية: القطن ، توالد الاعضاء المباشر، محور الجنين ، المختبر ، *Gossypium hirsutum L*

Introduction

Cotton (*Gossypium hirsutum*L.) belongs to tribe Gossypieae and family Malvaceae, native to the tropical and subtropical regions from both the new and old world. The word "cotton" is derived from the Arabic word "*al qatan*" [1]. It is the world's most important natural fiber and has played a significant role in the economy, social structure, and history of many countries around the globe. It is also cultivated in many areas of Iraq. Cotton is an important crop for Iraq not only for industrial development but also play an important role in the economic development. Amongst the commercially cultivated species, over 90% of worldwide acreage is devoted to the tetraploid (2n = 4x = 52) or upland cotton [2]. Despite the availability of synthetic alternatives, it continues to be an important source of fiber for the textile industry.

Cotton plant can grow in various types of soils in appropriate climate regions of the world but, the productivity of cotton is adversely affected by biotic and abiotic stresses. Therefore, cotton needs an efficient genetic manipulation approach for crop improvement. Thus, a reproducible and highly

efficient plant regeneration scheme is required for plant species. To date, somatic embryogenesis is the most routine method of plant regeneration in cotton [3, 4, 5]. A number of regeneration protocols via somatic embryogenesis have been developed for cotton using various explants and through manipulation of media composition and environment factors [5, 6, 7]. However, genotype-dependent response, a prolonged culture period, high frequency of abnormal embryo development, low conversion rate of somatic embryos into plants, frequency of somaclonal variants and a lack of shoot elongation, which has contributed to a decline in the regenerability of the culture, are the problems associated with cotton regeneration. The occurrence of loss of regenerability seemed to be influenced by the plant genotype, explants type, the number of subcultures, and culture medium [8]. In cotton, only a few cultivars regenerate very well in manipulate condition[9]. Cotton has proved to be difficult to manipulate in tissue culture [10]. Therefore, there is a need to find new and regenerable cotton lines for futher genetic manipulation [11]. Most cases of plant regeneration via somatic embryogenesis have utilized either wild or 'Coker', e.g. 310, 312, etc. varieties of cotton. Direct regeneration via organogenesis from callus has also been reported in some plants, but in cotton there is few reports [12, 13]; not many attempts have been made in this regard. In the present work, an attempt has been made to regenerate *G. hirsutum* cultivars through organogenesis and produce true to the type plant.

Materials and methods

Plant material

Seeds of cotton cv. Lashata and Coker 310 were used in this study obtained from Mabin El Nahrain Seed Company, Kirkuk, Iraq. The present study was carried out in Biotech Laboratory, Department of Field Crops, Faculty of Agriculture, Ankara University, Ankara (Turkey).

Seed sterilization

Seed were delinted with concentrated sulfuric acid H_2SO_4 for one min and then rinsed with running tap water and subsequently with double distilled water with few drops of disinfectant Teepol (Sigma, Germany). Thereafter, seed were dipped with 70% solution of sodium hypochlorite NaOCl for 20 min followed by three times washed with sterilized distilled water. To softening the seed coat and easily germination, seeds were soaked in sterile distilled water and kept for 24 hours at 25^{o}C in the dark.

Isolation of Explants

After soaking seeds were used for germination on Murashige and Skoog's [14] medium containing 3% sucrose and solidified with 2.8% phytagel. After 5 days of seed germination, embryonic axis,embryonic hypocotyls and plumule explants excited and inoculated onto MS (Murashige and Skoog's) medium containing 3% sucrose with different concentration of 1 to 4 mg/l BAP and 0.1 mg/l NAA with addition of 3 g/l activated charcoal for reducing browning caused by phenolics compound. Cultureswerekept in a growthchamber at 25^{o}C under dark for two days and subsequently under fluorescent light (35 µmol m^{-2} s^{-1}) following a 16/8 h light/darkcycle. Thereafter, for root induction, shoots obtained from direct shoot regeneration were allowed to root spontaneously or rooted on MS medium supplemented with 0.1, 0.2 and 1.0 mg/l NAA and 3 g/l activated charcoal for root induction.

Hardening

Rooted shoots obtained from direct organogenesis was first washed with sterile double-distilled water for removing agar or soldifying agent from the regenerated plant.Thereafter, regenerated plant transferred to plastic pots containing soil and peat moss. The plants were maintained in growth chamber for two weeks at temperature of 25–28°C and relative humidity 60–65% and watered once in three days. The established plantlets were finally transferred in clay pots containing mixture of soil and sand (1:1) and maintained in the greenhouse.

Results and discussion

In this present study, direct plant regeneration via organogenesis and better rooting were observed in cultivar Lashata. Regenerated plants did not show any changes in morphology, and they all were fertile. The regeneration media and hormone combinations were tested and different regeneration frequencies were observed in the both cultivars. Seeds were germinated onto hormone free medium and germination frequency were 90% for Coker-310 and 94% for Lashata (Data not shown). After five days, embryonic axis, embroyonic hypocotyl and plumule excised Fig. (1A) from germinated

seed and then cultured onto MS medium supplemented with 1 to 4 mg mg/l BAP and 0.1 mg/l NAA along with 3 mg/L activated charcoal. This experiment suggested that 1 mg/l BAP and 0.1 mg/l NAA were promoted shoot formation in embryonic axis compare with embryonic hypocotyle and plumule Fig. (1B) and responded better with Iraqi cultivars. These shoots were begun to emerge at first week of culture and elongated shoots formed after three week. In contrast, higher concentrations of BAP (3 mg/l and greater) suppressed shoot formation during the culture period. This observation agreed with previous study, as a Hemphill and colleagues [15] also concluded that higher concentrations of BAP suppressed shoot formation during the culture period. In a follow-up experiment, lower concentration (2 mg/l or Less) BAP induce of shoot formation from cultured embryonic hypocotyls and plumule in oth the cultivars which most probably developed from the pericycle region of explant Table (1).

After a three week culture period, explants such as embryonic hypocotyls and plumule were shown slightly callus at the cut ends of the explant when added the higher concentration of 4.0 mg/l BAP and 0.1 mg/l NAA. However, the most favorable BAP and NAA concentrations based on the development of elongated shoots (2 -3 cm in height) from embryonic axis after three weeks culture period were 1 mg/l or 2 mg/l BAP and 0.1 mg/l NAA in MS medium Table (1), Fig. (1C). Similar results were reported by Hemphill [15]; Gupta [16]; Ozyigit [17]. According to the earlier studies, BA is one of the most effective hormones for obtaining multiple shoots from different explant of cotton [15, 16]. A part from this, thidiazuron (TDZ) is also known as a promoter of multiple shoots in cotton explants, especially for cotyledonary nodes [17, 18]. It is obvious that tissue browning and blackening are also one of the major problems for in vitro culturing of cotton [19] like many other problematic species i.e. mango, coconut, etc [20].

There are also some possible alternative for preventing lethal browning caused by phenolics: using liquid media, frequent subculturing, addition of some antioxidants to medium, such as citric and ascorbic acids, PVP, and activated carbon [21, 22]. These methods usually reduce phenolic oxidation and favor regeneration from explants [21, 22]. In this present study, the addition of 3 g/l activated charcoal to media solved this problem. Rooting is a very important developmental period for tissue culture studies, especially for cotton-like problematic species. In this study, different concentrations of growth regulators apply for rooting processes and, as a result, the best rooting obtained with lower concentration of NAA with activated charcoal. After regeneration of shoots, plantlets of sub-cultured on MS medium supplemented with NAA and activated charcoal. Lashata and Coker-310 cultivars were showing root induction percentage and average number of root 58.4 and 47.2% with 3.8 and 2.2 cm respectively Table (2), Fig. (1D). Similar to our study, Nandeshwar and co-workers [23] reported that shoots were transferred to a rooting medium consisting of MS supplemented with 0.05–0.1 mg/l NAA and glucose 15 g/l better for rooting induction in cotton. Moreover, we could not observe any root at higher concentration of 1mg/l NAA and was showing lethal concentration for rooting induction in both the cultivars. Apart from this, Gupta and colleagues [24] reported that nutrient agar supplemented with 2.7 µM NAA as a stimulator for rooting, and they obtained 60% rooting with Khandwa-2 genotype. However, auxins and cytokinins are key players in cell differentiation and polarity development in plants and organogenesis is always triggered by these phytohormones [13]. There is a difference in the perception of strong and weak auxins by the explant, which could lead to the difference in the regeneration pathway.

After a hardening period (enclosed bags) of 2–3 weeks, the potted regenerants rooted, grew in height, and developed new leaves in regenerants Fig. (1E). All regenerated plant that were advanced to soil were phenotypically normal and all of the matured plants had initiated flowers and set viable R1 seeds under greenhouse conditions Fig (1F). Tissue culture of cotton plant are genotype dependent and different explants ourcess how different regeneration frequencies. Although worldwide, most of the planted cottons are transgenic, only a few cutivars could be genetically modified. Success with local cultivars have some advantageie adapted to their own area's climate, soil and altitude properties [17]. The conclusion is that all cultivars and possible explants ources are valuable and establishment of rapid *in vitro* regeneration systems from different cultivar is very important. A rapid regeneration protocol with local cultivars will become alternative solutions to some problems for genetically

modified plants in the future and can be make good contribution for cultivar improvement and development of transgenic plant.

Table (1): Shoot induction, number and shoot length with different explants of *Gossypium hirsutum*in MS medium supplemented with variable concentrations of growth regulators and 3 g/l activated charcoal after 21 days of inoculation.

Explants	Growth regulator (mg/l)		Shoot induction (%)		Mean number of shoots/plant		Shoot length (cms)	
	BAP	NAA	Lashata	Coker-310	Lashata	Coker-310	Lashata	Coker-310
	1.0	0.1	53.3	-	3.7	-	3.1	-
Embryonic	2.0	0.1	33.3	-	1.8	-	2.9	-
Axis	3.0	0.1	26.7	30.0	1.2	1.9.	1.0	2.1
	4.0	0.1	-	-	-	-	-	-
	1.0	0.1	-	-	-	-	-	-
Embryonic	2.0	0.1	6.7	-	0.6	-	0.8	-
Hypocotyl	3.0	0.1	12.0	9.3	0.9	1.0	1.3	1.2
	4.0	0.1	-	-	-	-	-	-
	1.0	0.1	28.7	-	1.4	-	1.6	-
Plumule	2.0	0.1	-	12.3	-	1.1	-	1.7
	3.0	0.1	-	-	-	-	-	-
	4.0	0.1	-	-	-	-	-	-

Note: * Relativevalue of responce of explants: -, Nil

Table (2): Rootin ductionresponce in regenerated shoots obtained from embryonic axicex plant of cotton on MS medium supplemented with NAA andactivated charcoal.

MS Medium + NAA (mg/L)	Root induction (%)		Average number of root	
	Lashata	Coker-310	Lashata	Coker-310
0.1 + 3 g/L AC*	58.40	47.20	3.8	2.2
0.2 + 3 g/L AC*	11.92	-	1.6	-
1.0 + 3 g/L AC*	-	-	-	-

AC*= Activated Charcoal

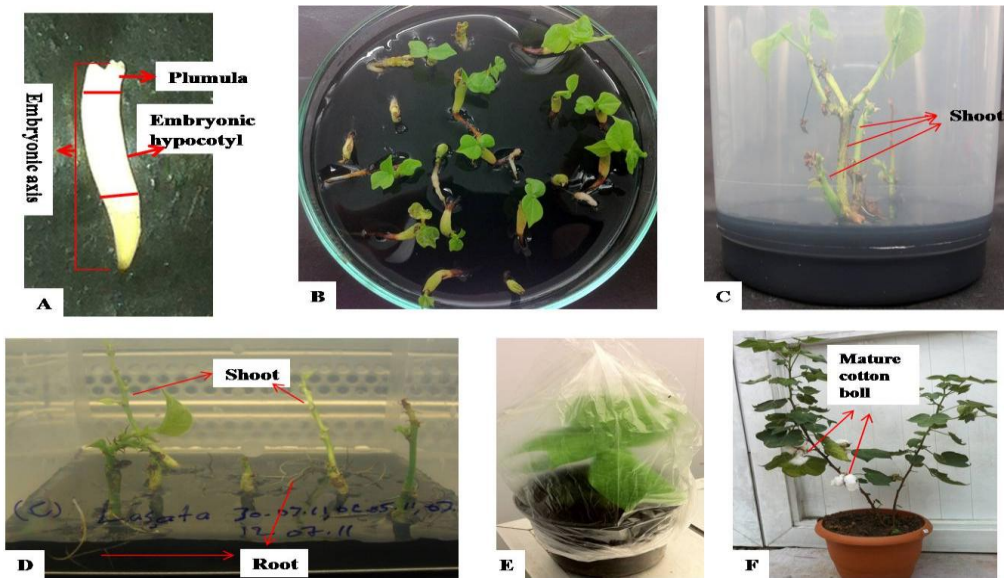

Fig (1): (A) Five-day-old embryonic axisex plants of cotton (*Gossypium hirsutum*L.) var. Lashata showing different explant. (B) Direct shoot development from embryonicaxis on MS medium supplemented with 1 mg L^{-1}BAP and 0.1 mg L^{-1} NAA. (C) Shoot multiplication and elongation from embryonicaxis. (D) Root induction on MS medium supplemented with 3 mg L^{-1}activated charcoal and 0.1 mg L^{-1} . (E) Pre-hardening for adaptation of culture raised cotton plant. (F) Matured plant bearing flower and cotton boll in plastic house.

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