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Mini Review Article

Cotton Ovule Culture: A Tool for Biological and Biotechnological Studies of Cotton

Maryam Behnam[1], Seyed Javad Davarpanah[2*], Ramin Karimian[2]

Abstract

Cotton is one of the most important crops in the world and increasing cotton yield is the main goal in the cotton industry. One important factor for crop improvement is increasing produced fibers by each developing seed. It is very interesting to manipulate fiber properties such as length, micronaire, color and strength which necessitate cotton ovule culture. Ovule culture is used as a tool to study physiology and biochemistry of secondary cell wall synthesis, effects of plant growth regulators, nutrition and environmental conditions on fiber and ovule development, inter-specific hybridization and embryo recue. This technique can be used for analysis of functional genes in fibers as transient expression systems. In this regard, fiber-specific promoters should be identified in developing fibers. Optimum growth regulator combinations can increase fiber yield and uniformity in vitro conditions. Despite mature ovules exogenous Indole-3-acetic acid and gibberellic acid are required for fiber development of unfertilized ovules in vitro condition even though these two hormones also induce fiber production in fertilized ovules. On the whole cotton ovule culture can be used as a model before permanent cotton transformation and field trials.

Keywords: Ovule Culture, Cotton, Transformation, Fiber

1. Department of Biotechnology, Faculty of Agricultural Sciences, Payam Nour University, Tehran, Iran
2. Applied Biotechnology Research Center, Baqiyatallah University of Medical Sciences, Tehran, Iran

* **Corresponding Author**
Seyed Javad Davarpanah
Applied Biotechnology Research Center, Baqiyatallah University of Medical Sciences, Tehran, Iran
E-mail: Davarpanah@bmsu.ac.ir

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Introduction

Cotton (*Gossypium hirsutum*) is an important fiber crop in the world [1]. Breakthroughs in yield increment by genetic manipulation has increased cotton production in USA to 7-10 kg/ha/year [2], Australia 23 kg/ha/year [3] and China 8-10 kg/ha/year [4]. Application of a regeneration system based on cotton vegetative tissues is an interesting approach for cotton improvement [1]. In addition, ovule culture is an appropriate tool for physio/Biochemical studies of fiber development.

Successful rescue of inter-specific hybrids of cotton in ovule culture may support embryo development [5-6]. Cotton ovule culture is a unique model for study of cellulose biosynthesis, embryo development and also a great target for assay of transient expression of gene constructs [6]. It is believed that ovule culture reveals relation of lint/linter with manipulation of plant growth regulators in vitro conditions and relations of growth, fertilization processes and embryo rescue [7].

Study of cotton fiber structure

Cotton fiber is an elongated epidermal cell with thickened secondary cell wall, enriched of cellulose. This cell before its death develops into a fiber of *G. hirsutum* with thickening the primary and secondary cell wall layers during a period of 50-60 DPA. Cotton fibers contain the most pure form of cellulose [8]. Almost cellulose constitutes 90% of cotton fiber whereas in many other fiber plants like linen, jute and hemp ¾ of plant shoot contains cellulose [8].

Cotton fibers are single-celled thus cell elongation can be evaluated independent of cell division. Therefore, trichome is a better term for cotton plant fibers as they are not a part of vascular tissue and originate from epidermis of ovule [9]. Cotton ovule culture was considered for two reasons: First, if appropriate hormones for fiber production to be identified in vitro conditions then it will be possible to enhance cotton fiber production using exogenous plant growth regulators in the farm Secondly, successful in vitro development of ovule will increase the chance of inter-specific hybrid rescue which is not possible by other means [5, 6].

G. hirsutum fibers grow normally 2-3 cm while in *G. barbadense* it may reach 6cm. cellulose constitutes more than 95% of its dry weight and despite many other plant cells has no lignin. It has a large central vacuole which is an important factor in fiber development [9]. Commercial varieties of cotton produce two types of fibers: lints which are used in textiles and linters with limited application in the industry but both are resulted from elongation of epidermal cells of mature seed and occupy the same position on the ovule surface [7].

Cotton fiber development has three overlapping steps: fiber initiation, thickening of the secondary cell wall and finally maturation. Fiber initiation is started in the pollination day. Nearly 25% of epidermal cells of ovule develop into lints which are commercially important. Fiber initiation and elongation occurs simultaneously, then ovules produce around 13000-21000 trichome [9], but cell elongation and cellulose deposition in secondary cell wall is strictly under control of gene regulation [7].

Genetic engineering application for cotton breeding

Nowadays, genetic engineering is not only used for yield improvement and pest-resistance but it also is used for

improvement of cotton fiber properties which includes length, micronaire, color and strength [8].

Cotton is an important crop and genetics play an important role in its modern cultivation and has a prominent effect on its production [10].To apply genetic engineering, a trust-worthy and genotype-independent regeneration system is required. Although somatic embryogenesis has been developed however, some problems have been remained unsolved. For example only a few verities such as Coker can produce somatic embryos and regenerate [10]. Ovule culture is an efficient step toward cotton breeding [1]. This system is a proper model to study transient expression of gene constructs because gene expression in cotton fiber can be studied at different steps of fiber growth and properties [8].

Cotton cultivars for ovule culture

Ovules of three genotypes Reshmi, Reshmani, Th3/83 have been used as explants for callus induction [1]. Commercial importance of *G. hirsutum* and *G. barbadense* due to their longer fibers have made researchers to use their cultivars in studies for cotton fiber modification [6, 7, 11, 12,]. Also growth of submerged fibers and ovules in several cultivars including Texas Market, Coker312, DP50 and S501 was studied [13].

Proper time for ovule isolation

Cotton flower petals are white in color in the pollination day and the first day after pollination they turn pink [14]. The culture of 1, 2, 3, 4, 7 Day Post-Anthesis (DPA) cotton ovules for 72 h in a hormone-free medium determined that cell division is rare in 1DPA and does not happen in 7 DPA ovules whereas the highest frequency of cell division occurs in 2-4 DPA ovules. Therefore, fiber cell division is a function of ovule age and it happens in absence of exogenous hormones [15]. Regarding this, 48h DPA is the best time for ovule harvest and isolation which is enough for pollen to reach ovules and assure of maximum chance of fertilization for each ovary. In addition, 2DPA ovules submerge in the liquid medium but 1DPA ones sink. This buoyancy results in maximum ovule growth and fiber production [14].

In presence of 0.5 µm Gibberellic acid (GA_3) and 5-20 µm indole-3-acetic acid (IAA), 0DPA has been reported the best age for ovule culture and fiber elongation [13-14]. On the whole, 24-48h DPA has been reported the best age for ovule isolation because multicellular fibers are rarely visible in this age [12, 14, 15].

Sterilization of ovaries for ovule culture

Isolated ovaries are immersed in 70% ethanol for 5-10 minutes and 3-5 times rinsed with sterile distilled water [13]. Beasley has immersed ovaries for 20 min in a solution of NaOCl 6% with 0, 0.5 and 2 % Tween, then rinsing with sterile distilled water and finally sterilization on the flame [15].

Ovule culture media

The first cotton ovule culture was reported on a low salt medium containing casein hydrolysate, vitamins, IAA and GA applied for 6DPA ovules resulted in unusual growth of ovules but normal fibers growth [16]. Many published protocols advise low salt formulations such as White, 1957 or Nich, 1951 which ovules are submerged in them but these media do not support optimum condition for ovule culture [5-6] therefore, low salt media are not proper ones for ovule culture [16]. Addition of coconut milk can also increase young ovule lifetime and agar will cause ovule browning [13]. Murashig-Skoog (MS) medium with two modifications can be used successfully for ovule culture. Firstly, substitution of NH_4NO_3 with KNO_3 as a sole nitrogen source and secondly, using a mixture of glucose and sucrose instead of sucrose [6, 7]. It also has been reported that respiration inhibitors rotenone and thiourea has no effect on fiber elongation and ovule fresh weight [17].

Addition of 0.09-0.19 mM exogenous calcium to Beasley and Ting (BT) medium has increased fiber length while normal calcium concentration of 3 mM in BT medium does not seems has any effect on fiber elongation. Therefore, usually high concentration of calcium inhibits fiber development [18].

Also, addition of osmotic materials such as sorbitol to BT medium express inhibitory effects of cotton ovule development and pH differences of the medium at the end of culture period [6].

Plant growth regulators in cotton ovule culture

Immature cotton ovules were cultured as explants for callus induction on MS medium containing different concentrations of IAA, 2,4 –D , IBA and kinetin (3-4 mg/L) which 3 mg/L 2,4-D showed the best results [1]. Exogenous plant growth regulators are required for in vitro culture of immature cotton ovule and fiber growth. Auxin and gibberellic acid increase fiber production of fertilized ovules [13, 19] and GA_3 increases fiber chains production. A combination of ABA and kinetin in a basal medium without PGRs causes ovule browning, further experiments revealed their antagonistic effects in ovule culture [7, 19, 20].

A comparison of cotton fiber growth revealed that NAA affects more on genes involved in cellulose synthesis of secondary cell wall rather than IAA [12]. IAA induces more fiber production in unfertilized ovules compared with GA_3 and both together showed synergistic effect. Exogenous kinetin increases the size of unfertilized ovules but has no effect on fiber growth alone, while ABA inhibits fiber development in unfertilized ovules [6-7]. In such ovules fiber cell division occurs in absence of exogenous plant growth regulators and addition of IAA and GA_3 decrease frequency of multi-cellular fibers. IAA alone can decrease this frequency but GA_3 has no effect alone. After 72 h ovule culture in presence of IAA frequency of multicellular fibers is about 5% whereas during the same period, in presence of GA_3 this frequency is $1/5^{th}$ of when both exist in the medium, indicating they modulate each other effects [15]. It has been reported that temperature lower than 34°C inhibited fiber elongation but not in presence of exogenous auxin or ammonium [11].

Study of plant growth regulators on fiber elongation determined that fiber elongation does not occur in absence of IAA or GA_3 while Kinetin with the two PGRs is used for ovule growth without epidermal fiber production. ABA, similar to high concentrations of kinetin decreased ovule capacity for fiber production in presence of IAA and GA_3 [14].

In conclusion, it seems that IAA provides optimum condition for fiber production effectively while GA_3 provides this condition for fertilized ovules and in all performed experiments IAA has produced more fiber than GA_3 [7, 12, 15, 19].

Optimum temperature for in vitro ovule culture

Growth circles of cotton fibers seldom can be detected in temperature below 28°C whereas in temperature over 34°C growth circles are continuously established on secondary cell wall of fibers in ovule culture. Therefore, it seems that 28°C is the threshold temperature for in vitro ovule culture [11]. It has been reported that development of immature ovules and fiber elongation increases with a temperature shift of 20°C to 30°C. Ovules remained white in temperatures higher than 30°C during a 3 week culture period, but temperature increment to 35°C resulted in ovule browning after 10-15 days. It is also worth mentioning that ovules from greenhouse plants in the middle of summer produce more fiber than ovules from plants in the middle of winter [14]. Most ovules were not able to produce fiber in temperatures below 34°C, but in presence of NH_4^+ they could produce more fibers in temperatures below 28°C-32°C [16].

Ovule transformation

Ovule culture can be used for the analysis of functional genes in transient expression system which requires identification of fiber-specific promoters [9]. Ovules were transformed with fiber-specific construct directing *gus* expression in fibers using biolistic method. Expression of reporter gene *gus* occurred several hours after particle bombardment and it was determined that fiber cells continue their growth after transformation [21]. Also, sonication assisted Agrobacterium- mediated transformation (SAAT) method has been reported a highly effective method for ovule transformation [22]. Ovule transformation of "Varamin" cultivar using *Agrobacterium* vacuum infiltration technique showed successful expression of *gfp* under control of CaMV35S promoter and also fiber-specific expression of a spider silk protein gene *MaSp1* under control of GaRD22-like1core promoter [23].

Visualization of elongated fibers in ovule culture

To determine effect of culture conditions on fiber growth, ovules are immersed in a solution of 0.018% Toluidine blue, washed in distilled water for 60 seconds, then are washed with formalin: acetic acid: ethanol (FAA) and the absorption of the retained color is measured using a colorimeter or spectrophotometer and is compared with the control [6, 7, 18 , 24].

Conclusion

Cotton ovule culture is a proper model for study of transient expression of functional genes in fibers, effects of hormones, temperature and many other effective factors on fiber growth. More elongated and stronger fibers will have more application in the industry; therefore more attention is paid to study of efficient factors on length and strength of fibers. The best time for ovule isolation is 48h DPA and the best temperature for ovule culture is 28°C-34°C. Also, study of effect of different plant growth regulators on ovule culture revealed that IAA is the most efficient PGR for fiber elongation and GA_3 has synergistic effect on fiber development. Regarding all fore-mentioned ovule culture is a fast and reliable method to study gene function in fiber development before permanent cotton transformation and its application in agriculture and textile industry.

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