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The effects of hormonal components of nutrient medium, cultivar and explant type on cotton (*Gossypium hirsutum* L.) callus formation *in vitro*

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ABSTRACT

Objective: The study aimed to optimize tissue culture conditions of cotton (*Gossypium* sp.).

Methodology and results: The study was carried out at the Cotton Research Institute of Iran (CRII) in Gorgan with 4 cotton cultivars, i.e. Sahel, Sepid, Coker 312*349 and No.200. Cotyledonary leaves, hypocotyls and crown explants excised from 7 days-old seedlings grown under *in vitro* conditions were cultured on MS basal medium with B_5 medium vitamins containing various hormonal combinations of Naphthalene Acetic Acid (NAA) and 6-Benzylamino purine (BAP). Analysis of results showed that percent of callus formation was significant at 1% level for hormonal combinations in medium, cultivar, explant type and all interactions of studied factors. The highest callus formation was observed in cv. Sahel and Sepid at 41.9%. The hypocotyl explants with 51.3% had the highest frequency of callus formation, followed by crown and cotyledonary explants. The highest callus formation (75%) was observed in the hypocotyl explants cultured on MS medium containing 2 mg/l NAA without cytokinin. For crown and cotyledonary explants the highest callus formation was observed in MS media containing 1 mg/l NAA without cytokinin.

Conclusion and application of results: The results suggest that plant growth regulators are essential for callus formation. The higher levels of auxins can positively affect callus formation of different explants of cotton cultivars, but cytokinins have a negative effect. Therefore, low concentration of cytokinins with high concentration of auxins in nutrient medium increases callus formation frequency in cotton.

Keywords: Explant, Callus formation, Tissue culture, Cotton, Hormones.

Abbreviations: CRII, Cotton Research Institute of Iran; MS, Murashige and Skoog medium (1962); B_5, Gamborg *et al*., medium (1968); NAA, Naphtalene-3-acetic acid; BAP, 6-Benzylamino purine; CRD, Completely Randomized Design; ANOVA, Analysis of Variance; LSD, Least Significant Difference Test

INTRODUCTION

Cotton is one of the most important fiber crops in the world. Genetic improvement of cotton through conventional breeding is limited by several factors such as lack of useful variation and long time periods that are required to complete one breeding cycle. Although plant tissue culture is an attractive means for improving plants, its use requires an effective regeneration system from somatic tissues of cotton plants. Compared with many other crops, it is more difficult to obtain somatic embryogenesis and plant regeneration from cotton (Sakhanokho *et al*., 2004;; Ghasemi *et al*., 2007;; Ghaemi *et al*., 2011). Price and Smith (1979) were the first to report somatic embryogenesis in cotton,

Gossypium koltzchianum, although complete plants could not be regenerated. Davidonis and Hamilton (1983) first described plant regeneration from two years old callus of *Gossypium hirsutum* L. cv Coker 310 via somatic embryogenesis. Rajasekaran *et al.* (1996) obtained regenerative plantlets via somatic embryogenesis from cotton T25, GSA 78 and Acala, but the regeneration frequency was low in these varieties. Since then, significant progress has been reported in cotton tissue culture (Ghaemi *et al*., 2011). *In vitro* cultured cotton cells have been induced to undergo somatic embryogenesis in numerous laboratories using various strategies (Voo *et al*., 1991; Kumria *et al*., 2003; Ikram-ul-Haq, 2005; Wu *et al*., 2005; Hilarie *et al*., 2008; Ghaemi *et al*., 2011).

Somatic embryogenesis and plant regeneration systems have been established from different tissues and regenerated plants have been obtained from explants such as hypocotyls, cotyledons and roots (Zhang, 2000; Ouma *et al*., 2004; Abdul Qayyum *et al*., 2006; Ghasemi *et al*., 2007; Ozyigit *et al*., 2007), anther (Zhang *et al*., 1996), embryo (Syed Sarfraz *et al*., 2004, 2005), protoplasts and ovules (Feng and Zhang, 1994), and from various cotton species and cultivars (Zhang, 1994b; Zhang *et al*., 2001; Tripathy and Reddy, 2002; Mishra *et al*., 2003; Sakhanokho *et al*., 2004; Jin *et al*., 2006; Han *et al*., 2009; Ghaemi *et al*., 2011). Regeneration procedures have been used to obtain genetically modified plants after *Agrobacterium*-mediated transformation of hypocotyls and cotyledons (Umbeck *et al*., 1987; Leelavathi *et al*., 2004; Mashayekhi *et al*., 2008) or by transformation of particle bombardment (Finer and McMullen, 1990; Rajasekaran *et al*., 2000).

Although efficiency of plantlets regeneration from embryogenic calli through somatic embryogenesis has been improved significantly in recent years, some difficulties remain.

Genotype dependent response restricts the application of cotton tissue culture and biotechnology in cotton breeding and production. Therefore, before plant tissue culture techniques are widely applied to cotton improvement programs, plant regeneration must be possible for a broad range of genotypes. Although several attempts have been made in the past on *in vitro* regeneration, little success has been made. In this research, the effects of different concentrations of NAA and BAP hormones in nutrient medium were studied on callus formation of different explants of some Iranian cotton (*G. hirsutum* L.) cultivars.

MATERIALS AND METHODS

Seeds of cotton cultivars Sahel, Sepid, Coker 312*349 and No.200 used as source material were obtained from Cotton Research Institute of Iran (CRII) in Gorgan, and were delinted by concentrated commercial sulfuric acid (100 ml/kg H_2SO_4/cotton seeds). The seeds were continuously stirred in H_2SO_4 for 10-15 seconds until the shiny surface of seeds appeared. Some water was then added and stirred for 5-6 seconds. The seeds were thoroughly washed with tap water to remove the acid completely, left in a water container for 10 minutes, after which those floating on the water surface were discarded. The delinted cotton seeds were sterilized with 70% ethanol for 1 min, and then surface sterilized for 20 min with 30% commercial sodium hypochlorite. The seeds were thoroughly washed with sterile distilled water (3-5 times for 15 min). After that, the delinted sterilized cotton seeds were kept on sterile blotting paper in Petri dishes. All sterilization process was performed in a laminar airflow cabinet. The cotton seeds were germinated on MS_0 medium (Table 2) and grown for 7 days in growth chamber at 25±2°C, under a 16/8 h (light/dark) photoperiod with light supplied by cool-white daylight fluorescent lights. Cotyledonary leaves with a small petiole, whose size did not exceed 5-10 mm, hypocotyl (10-15 mm) and crown with root (5-10 mm) segments were excided from 7 days-old cotton seedlings and cultured on different media combinations in growth chamber over a 3 weeks period at 20 - 22°C under a 16/8 h (light/dark) photoperiod. All experiments with the plant tissues were carried out *in vitro* on media based on MS (Murashige and Skoog, 1962) and vitamins based on B_5 medium (Gamborg *et al*., 1968), plus sucrose and plant growth regulators in different concentrations (Table 2). The influence of 14 varying concentrations of BAP and NAA hormones was studied with the purpose of finding the best cotton callus formation efficiency. The media were solidified with 7 g/l agar (Bacto-agar "DIFCO". USA). The MS_{10}- MS_{14}

media were liquid and agar was not used in these media. The experimental design was factorial experiment arranged in a completely randomized design (CRD) with 3 replications; 5 cultures being raised for each treatment. Callus formation frequency was calculated 21 days after culturing. The data were subjected to analysis of variance by SAS software and means compared by Least Significant Difference (LSD) Test at alpha=0.05.

RESULTS

Callus C formation varied significantly with treatment at 1% level for cultivar, explant type, hormonal combinations of nutrient medium and all their interactions (Table 1).

Table 1: Variance analysis of effect of cultivar, explant type, hormonal combinations of MS medium and their interactions on callus formation from various explant types of cotton cultivars.

Source of Variations	Degrees of Freedom	Mean Square
Cultivar (C)	3	**1989.09
Explant (E)	2	25648.56**
C * E	6	1834.08**
Hormonal Combinations of Medium (M)	13	3781.67**
C * M	39	1132.61**
E * M	26	711.33**
C * E * M	78	837.37**
Error	336	156.25
Total	503	297911.71

**: significant at 1% level

For all explants and cultivars, the greatest frequency of callus formation was observed on the MS_3 and MS_2 media (59.1 and 57.6%, respectively), while the least (27.8%) was obtained on the MS_4, MS_5, MS_6 and MS_7 media (Table 2).

Table 2: Components of different nutrient media for callus formation of cotton explants and the means comparisons of callus formation frequency (in %) in different media.

Media	Macrosalts and Microsalts	Vitamins	Hormonal Combination (mg/l)		Sucrose (g/l)	Agar (g/l)	Callus formation (LSD 0.05=5.795)	
			NAA	BAP		7		
MS_0	Based on MS	-	0	0	5			
MS_1	medium	Based on	1	4	30		31.9	ef
MS_2		B_5 medium	2	0			57.6	a
MS_3			1	0			59.1	a
MS_4			0	0			31.2	f
MS_5			0	4			27.8	f
MS_6			0	2			27.8	f
MS_7			2	2			27.8	f
MS_8			2	4			33.3	def
MS_9			1	2			40.9	c
MS_{10}			0.1	0.1		-	42.4	bc
MS_{11}			0.01	0.1		-	39.6	c
MS_{12}			0.1	0.01		-	47.9	b
MS_{13}			0	0.1		-	38.2	cd
MS_{14}			0.01	0.01		-	37.5	cde

- Means separated by Least Significant Difference (LSD) Test at alpha=0.05.
- Means with the common letters are not significantly different (at 5% level) and they are in the same group.

The first callus formation was observed 21 days after cultivation, irrespective of the studied cultivars. Hypocotyls isolated from 7 days-old seedlings had the greatest callus formation and were the best explants. Callus formation efficiency of hypocotyls was 51.3%; for cotyledons it was 26.6%, and for crown with roots it was 38.4% (Table 3). Therefore, in the next experiments, hypocotyl can be selected as the primary explant. Depending on cultivar, the average callus formation frequency rangedd from 33.5 to 41.9%, and cultivars Sahel and Sepid had higher levels of callus production. Although cultivars Sahel and Sepid had the highest callus formation in all experiments, analysis of cultivar * explant interaction showed that in the case of hypocotyl explants cv. No.200 had the highest callus formation (57.1%). However, in the case of cotyledon and crown explants the Sahel and Sepid were the best cultivars (Table 3).

Table 3: The means comparison of callus formation frequency (%) of cotton cultivars in relation to explant type. and their interactions.

Cotton cultivar	Explant type			Mean of cultivar (LSD 0.05=3.098)
	Cotyledon	Hypocotyl	Crown + Root	
Sahel	35.7	47.6	42.3	41.9 a
Sepid	33.9	51.2	40.5	41.9 a
Coker 312*349	20.2	49.4	30.9	33.5 c
No.200	16.7	57.1	39.9	37.9 b
Mean of explant type (LSD 0.05=2.683)	26.6 c	51.3 a	38.4 b	-

- Means separated by the Least Significant Difference (LSD) Test at alpha=0.05.
- Means with the common letters are not significantly different (at 5% level) and they are in the same group.

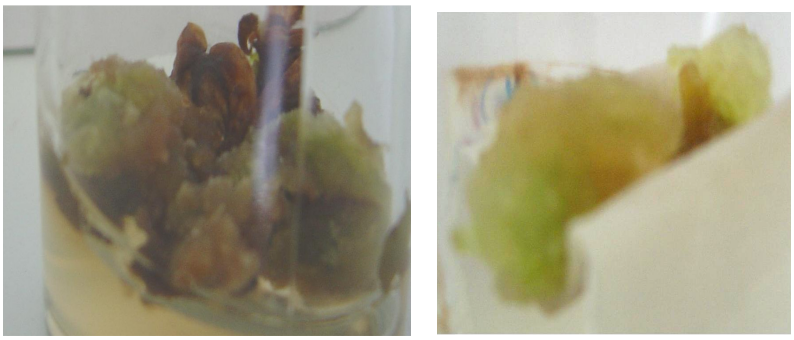
In general, hypocotyl explants possessed higher callus formation potential than cotyledon and crown with root segments on many of the nutrient media (table 4). Hypocotyls on the MS_2 medium containing the highest concentration of NAA (2 mg/l) without cytokinin expressed the greatest callus formation frequency (75%) of all treatments. However, MS_3 medium containing 1 mg/l NAA without cytokinin was the best nutrient medium for crown + roots and cotyledons (58.3 and 45.8%, respectively), which statistically was similar to MS_2 medium.

Table 4: The means comparison Cof callus formation frequency (%) in nutrient medium with varying hormonal components. , primary explant and their interactions.

Medium	Explant type			Mean of medium (LSD 0.05=5.795)	
	Cotyledon	Hypocotyl	Crown+ Root		
MS_1	22.9	41.7	31.2	31.9	ef
MS_2	41.7	75	56.2	57.6	a
MS_3	45.8	72.9	58.3	59.1	a
MS_4	20.8	43.7	29.2	31.2	f
MS_5	22.9	41.7	18.7	27.8	f
MS_6	16.7	43.7	22.9	27.8	f
MS_7	22.9	37.5	22.9	27.8	f
MS_8	14.6	54.2	31.2	33.3	def
MS_9	27	62.5	33.3	40.9	c
MS_{10}	33.3	45.8	74.9	42.4	bc
MS_{11}	16.7	47.9	54.2	39.6	c
MS_{12}	31.2	62.5	50	47.9	b
MS_{13}	22.9	39.5	52	38.2	cd
MS_{14}	33.3	50	29.2	37.5	cde
Mean of explant type (LSD 0.05=2.683)	26.6 c	51.3 a	38.4 b	-	

- Means separated by Least Significant Difference (LSD) Test at alpha=0.05.
- Means with the common letters are not significantly different (at 5% level) and they are in the same group.

This may be due to greater concentration of hormones i.e. NAA probably having positive effects on callus formation of cotton cultivars when used in greater concentration and by reducing BAP concentration led to somewhat satisfactory results in the case of many varieties of cotton. Calli formed from hypocotyls were rapidly formation, light or dark green or light yellow and loose. Cotyledonary formed calli were slow growing, compact, light or dark brown or dark green. Some of these turned brown and died (Figure 1). Also the hormonal components of nutrient medium affected the color and size of calli formed from different explants of cotton cultivars (Figure 2).



A **B**

Fig 1: **A**: Callus formed from cotyledonary explant of Sahel cotton cultivar on MS_9 medium.
B: Callus formed from hypocotyl explant of Sahel cotton cultivar on MS_9 medium.

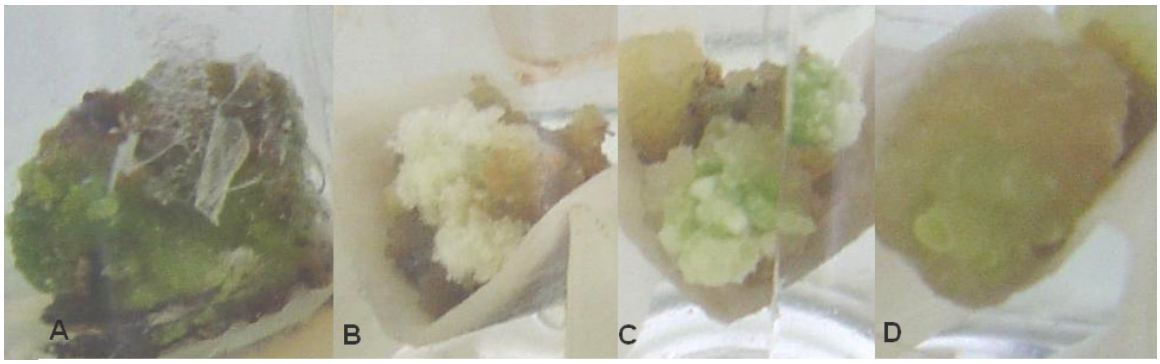


Fig 2: Effects of nutrient medium components on color and size of calli formed from hypocotyl explants of Sahel cultivar (A: MS_8 medium; B: MS_{12} medium; C: MS_{13} medium; D: MS_9 medium).

DISCUSSION

Plant tissue culture is an important step in any successful plant transformation scheme. Stable transformation requires that a single cell gives rise to a plant. The ideal transformation scheme is that via somatic embryogenesis, because each transformed cell of callus has the potential to produce a plant. Somatic embryogenesis and subsequent plant regeneration has been reported in most of the major crop species (Abdol Qayyum *et al*., 2006; Ghasemi *et al*., 2007; Mashayekhi *et al*., 2008; Ghaemi *et al*., 2011). In this study different callus induction media with various concentrations of NAA and BAP hormones were tested using different explants (hypocotyls, cotyledons, and crowns with root) and different cotton cultivars.

Trolinder and Xhixian ((1989) and Zhang *et al*. ((2001) classified cotton varieties on the basis of somatic embryogenesis and plant regeneration into four categories. First were the varieties with high ability for somatic embryogenesis and plant regeneration, such as Coker 201, Coker 312 which have become the model varieties in cotton tissue culture and genetic transformation. Second were those with moderate ability for somatic embryogenesis and plant regeneration that could produce some embryos and plantlets after many subcultures. Many varieties were

included in this class e.g. Coker 310, Siokra 1-4, and Coker 315, among others. The third category had poor ability, although somatic embryogenesis could occur but without regeneration. Fourth category included some genotypes on which embryo formation had not been observed. The varieties used during the present study belonged to the second and third categories.

In this study, hypocotyl explants produced more suitable, greater and friable callus than other explants used in the experiments. These results were similar to those of Zhang (2000); Ouma *et al*. (2004); Rajasekaran *et al*. (2004); Abdul Qayyum *et al*., (2006) and Abdellatef and Khalafallah (2008).

Induction media with 1 and 2 mg/l NAA without cytokinin achieved 57.6 and 59.1% callus formation, respectively. The effect of MS_2 and MS_3 media showed that high concentration of NAA without BAP leads to suitable results from the callogenic point of view. According to observations, the suitable medium for callus induction was NAA, probably having positive effects on callus formation of cotton cultivars when used in greater concentration, and by reducing BAP concentration led to somewhat satisfactory results with many cotton cultivars. Results showed that shoot induction media with 4 and 2 mg/l BAP without NAA achieved 27.8% callus formation which was lower than other callus induction media. MS_5 and MS_6 media with high BAP concentration (2 and 4 mg/l) without NAA had slow proliferation and less callus formation. MS_7 and MS_8 media containing 2 mg/l NAA and 2 or 4 mg/l BAP also did not show good results from the callogenic point of view. Also, MS_1 medium containing 1 or 4 mg/l NAA and BAP had low callus formation. These results were similar to Abdul Qayyum *et al*. (2006).

Between the evaluated media, there was not any hormone in MS_4 medium, and other media had high concentrations of cytokinin BAP. , By decreasing and increasing the concentration of NAA in MS medium we can get different results. Therefore, we can conclude that hormones are essential for callus formation, however, high concentration of cytokinins reduce callus formation, but low concentration of cytokinins with high concentration of auxins play positive role in this respect and increase callus formation frequency.

Although phytohormones especially auxins could enhance callus growth, after two months, they were not necessary for growth of calli, and the embryogenicity of calli could be obtained on simple MS medium without any plant growth regulator.

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REFERENCES

Abdellatef E,. and Khalafallah MM, 2008. Influence of growth regulators on callus induction from hypocotyls of medium staple cotton (*Gossypium hirsutum* L.). Cultivar barac B-67. J. Soil Nature 2(1): 17-22.

Abdul Qayyum RS, Sarfraz Hussain M, Saqib Shahzad S, Yassir Abbas Bokhari M, Hashim Raza A, Allah Rakha A, Majeed A, Shahid A, Saleem Z, Husnain T, and Riazuddin S, 2006. Regeneration in cotyledon of cotton. J Zhejiang Univ Sci B. 7(4): 291–298.

Davidonis GH,. and Hamilton RH, 1983. Plant regeneration from callus tissue of *Gossypium hirsutum* L. Plant Sci. Lett. 32: 89-93.

Feng R,. and Zhang BH, 1994. Ovule culture and rescue of cotton hybrid embryos. Shaanxi J. Agric. Sci. 2: 46-48.

Fioozabady E, Deboer DL, and Merlo DJ, 1987. Transformation of cotton (*Gossypium hirsutum* L.) by *Agrobacterium tumefaciens* and regeneration of transgenic plants. Plant Mol. Biol. 10: 105-116.

Ghaemi Mahin, Majd Ahmad, Fallahian Fathalah, and Ghasemi Bezdi Kamal, 2011. Comparison of callus induction and somatic embryogenesis of some Iranian cottons (*Gossypium* Spp.) with Coker 312 and histology of somatic embryogenesis. Afr. J. Biotechnology. 10(15): 2915-2922.

Ghasemi Bezdi K, Karlov Gennady I, and Ahmadikhah A, 2007. Effects of genotype, explant type and nutrient medium components on canola (*Brassica napus* L.) shoot *in vitro* organogenesis. Afr. J. Biotechnol. 6(7): 861-867.

Han GY, Wang F, Zhang GY, and Ma ZY, 2009. Somatic embryogenesis and plant regeneration of recalcitrant cottons (*G. hirsutum* L.). Afr. J. Biotechnol. 8(3): 432-437.

Hilarie KT, Mongomake K, Daouda K, Justin KY, Georges NG, Pierre TW, Alain D, and Michel MJ, 2008. Trans-resveratrol as phenolic indicator of somatic embryogenesis induction in cotton (*Gossypium hirsutum* L.) cell suspensions. Afr. J. Biochem. Res. 2 (1): 015-023.

Ikram-ul-Haq, 2005. Callus proliferation and somatic embryogenesis in cotton (*Gossypium hirsutum* L.). Afr. J. Biotechnol. 4(2): 206-209.

Jin S, Zhang X, Nie Y, Guo X, Liang S, and Zhu H, 2006. Identification of a novel elite genotype for in vitro culture and genetic transformation of cotton. Biol Plant. 50(4): 519-524.

Kumria R, Sunnichan VG, Das DK, Gupta SK, Reddy VS, Bhatnagar RK, and Leelavathi S, 2003. High-frequency somatic embryo production and maturation into normal plants in cotton (*Gossypium hirsutum* L.) through metabolic stress. Plant Cell Rep. 21(7): 635-639.

Leelavathi S, Sunnichan SG, Kumria R, Vijaykanth GP, Bhatnagar RK, and Reddy VS, 2004. A simple and rapid Agrobacterium-mediated transformation protocol for cotton (*Gossypium hirsutum* L.): Embryogenic calli as a source to generate large numbers of transgenic plants. Plant Cell Rep. 22: 465-470.

Mashayekhi M, Shakib AM, Ahmad-Raji M, and Ghasemi Bezdi K, 2008. Gene transformation potential of commercial canola (*Brassica napus* L.) cultivars using cotyledon and hypocotyl explants. Afr. J. of Biotechnol. 7(24): 4459-4463.

Mishra R, Wang H, Yadav NR, and Wilkins TA, 2003. Development of a highly regenerable elite Acala cotton (*Gossypium hirsutum* cv. Maxxa) - a step towards genotype-independent regeneration. Plant Cell, Tissue and Organ Culture 73: 21-35.

Murashige T,. and Skoog F, .1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant 80: 662-668.

Ouma JP, Young MM, and Reichert NA, 2004. Optimization of in vitro regeneration of multiple shoots from hypocotyl sections of cotton (*Gossypium hirsutum* L.). Afr. J. Biotechnol. 3(3): 169-173.

Ozyigit II, Kahraman MV, and Ercan O, 2007. Relation between explant age, total phenols and regeneration response of tissue cultured cotton (*Gossypium hirsutum* L.). Afr. J. Biotechnol. 6(1): 3-8.

Price HJ,. and Smith RH, 1979. Somatic embryogenesis in suspension cultures of *Gossypium klotzschiaanum* Anderss. Planta 145: 305-307.

Rajasekaran K, Sakhanokho HF, Zipf A, Saha S, Sharma GC, and Peng WC, 2004. Somatic embryo initiation and germination in diploid cotton (*Gossypium arboreum* L.). *In Vitro* Cell Dev. Biol. Plant 40: 177-181.

Sakhanokho HF, Ozias-Akins P, May OL, and Chee PW, 2004. Induction of somatic embryogenesis and plant regeneration in select Georgia and Pee Dee cotton lines. Crop Sci. 44:2199-2205.

Syed Sarfraz H, Husnain T, and Riazuddin S, 2004. Somatic Embryo germination and Plant Development from Immature Zygotic Embryos in Cotton. Pak. J. Biol. Sci. 7(11): 1946-1949.

Syed Sarfraz H, Husnain T, and Riazuddin S, 2005. In-ovule Embryo Culture: A Novel Method of Cotton Transformation. Pak. J. Biol. Sci. 8(2): 297-301.

Tripathy S,. and Reddy GM, 2002. *In vitro* callus induction and plantlet regeneration from Indian cotton cultivars. Plant Cell Biotechnol. Mol. Biol. 3(3&4): 137-142.

Trolinder NL,. and. and Xhiaxian C, 1989. Genotype specificity of the somatic embryogenesis response in cotton. Plant Cell Rep., 8: 133-136.

Umbeck P, Johnson G, and Barton K, 1987. Genetically transformed cotton (*Gossypium hirsutum* L.) plants. Biotechnology 5: 235-236.

Voo KS, Rugh CL, and Kamalay JC, 1991. Indirect somatic embryogenesis and plant recovery from cotton (*Gossypium hirsutum* L.). *In Vitro* Cell. Dev. Biol. 27: 117-124.

Wu J, Zhang X, Nie Y, Jin S, and Liang S, 2005. Factors affecting somatic embryogenesis and plant regeneration from a range of recalcitrant genotypes of Chinese cottons (*Gossypium hirsutum* L.). *In Vitro* Cell. Dev. Biol. 40(4): 371-375.

Zhang BH, 1994b. List of cotton tissue culture (Continuous). Plant Physiol. 30: 386-391.

Zhang BH, 2000. Regulation of plant growth regulators on cotton somatic embryogenesis and plant regeneration. Biochemistry 39: 1567-1567.

Zhang BH, Feng R, Li XL, and Li FL, 1996. Anther culture and plant regeneration of cotton (*Gossypium klotzschianum* Anderss). Chinese Sci. Bull. 41: 145-148.

Zhang BH, Feng R, Liu F, and Wang Q, 2001. High frequency somatic embryogenesis and plant regeneration of an elite Chinese cotton variety. Bot. Bull. Acad. Sin. 42: 9-16.