ANADOLU, J. of AARI
ISSN: 1300 - 0225
28 (2) 2018, 55 - 61
MAF

The Effect of Different Plant Growth Regulators on Callus Induction from Hypocotyl Explants and Plantlet Regeneration Through Somatic Embryo in Cotton (Gossypium hirsutum L.) Genotype Nazilli-143

Sadiye HAYTA-SMEDLEY[1] Nedim OZBEK[2] Arif ANSIZ[1]
Meltem BAYRAKTAR[3*] Aynur GUREL[1]

[1]Ege University, Faculty of Engineering, Bioengineering Department, Izmir / TURKEY
[2]Nazilli Cotton Research Institute, Aydin / TURKEY
[3]Ahi Evran University, Faculty of Engineering and Architecture, Genetic and Bioengineering Department, Kirsehir / TURKEY

** Corresponding Author (Sorumlu Yazar): e-mail: meltembayraktar5@gmail.com*

Received (Geliş tarihi): 08.06.2017 Accepted (Kabul tarihi): 05.06.2018

ABSTRACT: *A simple and reliable protocol has been developed for plantlet regeneration through somatic embryogenesis from suspension cultures of* ***Gossypium hirsutum*** *L. cv. Nazilli-143. Embryogenic callus was initiated from hypocotyl tissues of 7-day-old seedlings. High induction frequencies of the embryogenic callus were obtained on medium containing Murashige and Skoog (MS) salts, Gamborg's B5 medium (B5) vitamins, 30 g L^{-1} glucose, 0.75 g L^{-1} $MgCl_2$, 0.1 mg L^{-1} Kinetin and 0.1 mg L^{-1} 2,4-Dichlorophenoxyacetic acid (2,4-D) and the medium was solidified using 0.7% (w/v) agar (pH 5.8). Embryogenic calli were placed on plant growth regulator (PGR)-free liquid MS medium in order to establish suspension cultures for somatic embryo induction. Suspensions were sieved and the globular somatic embryos were collected and plated onto various types of semi-solid media. Embryo proliferation and maturation processes were best observed on medium containing 2/3 MS plus 1.3 g L^{-1} KNO_3 free from PGR. Plantlets were recovered from 36 % of the matured embryos. Plantlets with a root system and true leaves were removed from the sterile culture and were transferred into a plant growth chamber.*

Keywords: *Somatic embryogenesis, plantlet regeneration, suspension culture, cotton,* ***Gossypium hirsutum*** *L.*

Nazilli-143 Pamuk (Gossypium hirsutum L.) Genotipinde Farklı Bitki Büyüme Düzenleyicilerinin Hipokotil Eksplantlarından Kallus İndüksiyonu Üzerine Etkisi ve Somatik Embriyo Aracılığıyla Bitkicik Rejenerasyonu

ÖZ: ***Gossypium hirsutum*** *L. cv. Nazilli-143'ün hücre süspansiyon kültürlerinden somatik embriyo yolu ile bitkicik rejenerasyonu için basit ve güvenilir bir protokol geliştirilmiştir. Embriyogenik kallus 7 günlük fideciklerin hipokotil dokularından başlatılmıştır. En yüksek embriyogenik kallus oranı Murashige ve Skoog (MS) besin ortamının makro ve mikro elementleri, Gamborg (B5) besin ortamının vitaminleri ile 30 g L^{-1} glukoz, 0,75 g L^{-1} $MgCl_2$, 0,1 mg L^{-1} Kinetin ve 0,1 mg L^{-1} 2,4 Diklorofenoksi asetik asit (2,4-D) içeren ve % 0,7 (w/v) agar ile yarı katı hale getirilmiş besin ortamında (pH 5.8) elde edilmiştir. Somatik embriyo indüksiyonu için süspansiyon kültürlerini kurmak amacıyla, embriyogenik kalluslar bitki büyüme düzenleyicisi içermeyen sıvı MS besin ortamında kültüre alınmıştır. Süspansiyon kültürleri süzülmüş ve globular yapıdaki somatik embriyolar toplanarak çeşitli yarı katı besin ortamlarına aktarılmışlardır. Embriyo çoğaltımı ve olgunlaşması en iyi, 1,3 g L^{-1} KNO_3 eklenmiş, bitki büyüme düzenleyicisi içermeyen 2/3 MS besin ortamında gözlenmiştir. Olgunlaşmış embriyoların % 36'sından bitkiciğe dönüşüm gerçekleşmiştir. Köklü ve gerçek yapraklara sahip bitkicikler steril kültürden uzaklaştırılmış ve bitki büyüme kabinine transfer edilmişlerdir.*

Anahtar Kelimeler: *Somatik embriyogenez, bitkicik rejenerasyonu, süspansiyon kültürü, pamuk,* ***Gossypium hirsutum*** *L.*

INTRODUCTION

Cotton is one of the most valuable fiber and seed oil crops in the world and is planted on a land area of about 32.4 million hectares worldwide (Wang *et al.,* 2006; Khan *et al.,* 2010; Juturu *et al.,* 2015). Since biotic and abiotic stresses highly influence the development of the fiber, its quality, and yield (Kumria *et al.,* 2003a; Khan *et al.,* 2010), conventional plant breeding methods have been generally used to improve these traits (Khan *et al.,* 2010).

Although conventional breeding programs have made steady improvements in the agronomic traits in cotton, genetic improvement is limited by several factors such as the lack of sufficient genetic variability in the existing germplasm pool and the requirement for long time periods (Wang *et al.,* 2006; Khan *et al.,* 2010). Both *in vitro* selection against different kinds of stress factors and the transgenic technology require the establishment of an effective plant regeneration system (Wang *et al.,* 2006). Some factors restrict the regeneration and transformation of cotton during the production of bio-engineered cotton as these processes are genotype dependent, and reproducible protocols have not yet been well established for the most elite cotton varieties (Kumria *et al.,* 2003b). Only a few cultivars of cotton have been successfully regenerated via somatic embryogenesis (Wang *et al.,* 2006).

Several methods have been used for the regeneration of cotton. But among them somatic embryogenesis is more preferred than organogenesis, since the regenerants have a probable unicellular origin and the somatic embryos have no vascular connections with the maternal tissue (Kumria *et al.,* 2003b; Khan *et al.,* 2010). The first study on somatic embryogenesis was reported in *Gossypium koltzschianum* (Price and Smith, 1979), but plant regeneration was not observed. Since then, regeneration via somatic embryogenesis was reported in *G. hirsutum* cv. 'Coker 310' (Davidonis and Hamilton, 1983), and *G. hirsutum* cvs 'Coker 201' and 'Coker 315' (Shoemaker *et al.,* 1986). Following these studies, some progress in regeneration was reported for different cotton genotypes in numerous laboratories (Trolinder and Goodin, 1988a, b; Finer, 1988; Firoozabady and DeBoer, 1993; Zhang *et al.,* 2000), but the frequency of embryogenesis described in these studies is lower than the Coker cultivars, which are agronomically very poor in their properties (Khan *et al.,* 2010). Thus, in comparison to many other crops, it is more difficult to obtain somatic embryos and regenerated plants from cotton (Wang *et al.,* 2006). The number of commercial cultivars and elite germplasm lines that have better-quality fiber and agronomic traits, which can undergo whole plant regeneration, still remains very low (Khan *et al.,* 2010).

The present study was designed to develop an efficient and simple protocol for somatic embryogenesis and plant regeneration in *G. hirsutum* L. cv. Nazilli-143, an elite cultivar with favorable agronomic traits.

MATERIAL AND METHODS

Production of sterile seedlings

Cotton (*Gossypium hirsutum* L. cv. Nazilli-143) seeds were obtained from Nazilli Research Institute, Nazilli, Turkey. Mature seeds of cotton were delinted with sulfuric acid, then sterilized by sodium hypochlorite of 10% with a drop of Tween 20 for 10 min and rinsed three times with sterile water. The sterile seeds were kept in sterile water overnight to germinate at 25-30°C, then cultured on half strength Murashige and Skoog medium (MS; Murashige and Skoog, 1962) supplemented with 1.5% (w/v) sucrose and 0.7% (w/v) agar. Seven days after germination the seedlings had attained sufficient growth to be used for callus initiation.

Callus initiation and proliferation

Callus was initiated from hypocotyl tissues of 7-day-old-seedlings. Hypocotyls were cut into 5-6 mm segments, and they were cultured on media which consisted of MS salts, Gamborg's B5 medium (B5; Gamborg, 1968) vitamins (MSB), 0.75 g L^{-1} $MgCl_2$ (Trolinder and Goodin, 1988b) containing 3% (w/v) glucose, and 0.7% (w/v) agar. Then, combination of dichlorophenoxyacetic acid (2,4-D) (0.1, 0.5 mg L^{-1}), Kinetin (0.1, 0.5 mg L^{-1}) and indole-3-butyric acid (IBA) (0, 1.0 mg L^{-1}) were added to the culture media for investigation

of callus initiation and proliferation (Table 1). Callus cultures were subcultured monthly for callus proliferation.

Suspension cultures and somatic embryo induction

Approximately 15 g of embryogenic callus was placed into a 2 L erlenmeyer flask containing 400 mL of liquid MS medium, without the plant growth regulators (PGRs) for 4 weeks. The flask was shaken at 110 rpm. The pro-embryogenic tissues were formed from these embryogenic materials and pro-embryo formation was observed at a high frequency after 4 weeks. The cell suspension was sieved through 60-mesh stainless steel sieve and the pro-embryos were collected. 7.5 g of these pro-embryos were resuspended in a 1 L erlenmeyer flask containing 200 mL MS liquid medium. Following 4 weeks of culture, visible globular somatic embryos were formed from the pro-embryos.

Embryo maturation

Embryogenic cell suspensions were sieved through 60-mesh stainless steel sieve and the embryoids collected. Under a stereomicroscope 2-3 mm bipolar embryo were assessed and 200 per plate were transferred to various types of semi-solid media for the facilitation of embryo development. All embryogenic material was cultured on media which consisted of 2/3MS medium, 3% (w/v) glucose, excess KNO_3 in the amount of 1.3 g L^{-1} with or without plant growth regulators (Table 2).

Plant regeneration and acclimatization

Following complete regeneration of the embryoids into plantlets, they were transferred onto the semi-solid 2/3MSB medium without any plant growth regulators to be left to get hardened until the 2-4 leaves stage. Plantlets were removed from the culture vessels, and the roots were washed with water to remove agar medium residues. The plantlets were transferred to 5 cm in diameter pots containing a 1 : 3 peat : vermiculite mixture. They were covered with perforated transparent bags to prevent moisture loss and kept in a plant growth chamber at approximately 25 ºC, 70% humidity, 50 µmol $m^{-2}s^{-1}$ irradiance (cool white fluorescent light) and 16-h photoperiod. The potted plants were ventilated for 10–15 min by removing the bags from the plants once a day for a period of 2 weeks. The acclimation bags were removed at the end of the 2-week period. The plants were watered as needed with half strength MS medium.

Media and culture conditions

The pH of all the media was adjusted to 5.8 with 1 N HCl or 1 N NaOH prior to the addition of the gelling agent. They were autoclaved at 121 °C at 1.04 kg cm^{-2} for 15 min. All the cultures were incubated in a growth room at 26±1°C in cool white fluorescent light, with a florescence rate 50 µmol m^{-2} s^{-1} under a 16/8-h (light/dark) photoperiod.

Statistical analysis

Observations were recorded 4 weeks after culture response. The culture responses were expressed in terms of the percentage of explants forming callus. The experiments were arranged in a completely randomized design. Each treatment consisted of three replicates with 15 explants per replicate. The data were analyzed using standard ANOVA procedures. The differences between the means were determined by Fisher's least significant different (LSD) test using the SPSS statistical package (version 17.0).

RESULTS

Callus initiation

The surface sterilized seeds showed 100% germination on ½ MS medium (Figure 1). Hypocotyl explants were excised and cultured on various embryogenic callus induction media (Table 1). The hypocotyl segments began to produce callus 7 days after cultured (Figure 1). Callus induction frequencies were significantly influenced by the concentration and the composition of the plant growth regulators (Table 1). The combined effect of 0.1 mg L^{-1} 2,4-D with 0.1 mg L^{-1} Kinetin provided the best response for the embryogenic callus formation on MSB media with 3% (w/v) glucose and 0.7% (w/v) agar (100%) ($P < 0.05$). The creamy white and friable calli were produced in all plant growth regulator combinations (Figure 1) and they displayed potential to develop into somatic embryos.

Table 1 Effect of different growth regulators (mg L^{-1}) on callus induction from hypocotyl explants in cotton[§].
Çizelge 1. Pamukta farklı büyüme düzenleyicilerinin (mg L^{-1}) hipokotil eksplantlarından kallus indüksiyonu üzerine etkisi[§].

2,4-D	Kinetin	IBA	Number of explants Eksplant sayısı	Explants forming callus (%) ± SD Kallus oluşturan eksplantlar (%)	
0.1	0.1	1	45	70 ± 8.2	ab
0.1	0.1	-	45	100 ± 0.0	a
0.1	0.5	1	45	70 ± 7.1	ab
0.1	0.5	-	45	70 ± 7.1	ab
0.5	0.1	1	45	60 ± 7.1	bc
0.5	0.1	-	45	50 ± 16.3	cd
0.5	0.5	1	45	70 ± 4.1	ab
0.5	0.5	-	45	20 ± 4.1	d

[§] Values are the means of three experiments constituting a total of 45 explants per treatment (*n*=45). Mean values within a *column* followed by the same *letter* are not significantly different ($P< 0.05$) according to the Least Significant Difference (LSD) test.
[§] Değerler, deneme başına toplam 45 eksplanttan oluşan üç tekerrürün ortalamasıdır (n = 45). Aynı sütunda aynı harfin izlediği ortalama değerler, en küçük anlamlı farklar (Least Significant Differences =LSD) testine göre önemli ölçüde farklı değildir ($P <0,05$).

Somatic embryo induction and maturation

Somatic embryo induction from hypocotyl derived callus of *G. hirsutum* L. cv. Nazilli-143 was obtained in MS liquid medium. In this medium, cell suspensions with small cell aggregates and visible globular embryos were formed 4 weeks after culture incubation (Figure 1). Somatic embryos were developed from the pro-embryos which were usually characterized by multiple cell aggregate stages (Ganesan *et al.,* 2007).

Globular staged embryos were collected and plated onto various types of semi-solid media (Table 2). They were subcultured in the same medium for maturation and development. 14 days after subculture incubation, heart and cotyledonary shaped embryos were formed simultaneously from globular staged embryos (Figure 1). The best medium for somatic embryo proliferation and maturation was determined as the 2/3MS medium containing 1.3 g L^{-1} KNO_3 without plant growth regulators.

Table 2. Plantlet regeneration (%) from somatic embryos cultured in different media.
Çizelge 2. Farklı besin ortamlarında kültüre alınan somatik embriyolardan bitkicik rejenerasyonu (%).

Embryo Maturation Media Embriyo Olgunlaştırma Ortamı	Number of somatic embryos Somatik embriyo sayısı	Plantlet regeneration rate (%) Bitkicik rejenerasyon oranı (%)
2/3 MS + 1.3 g L^{-1} KNO_3	200	36
2/3 MS + 1.3 g L^{-1} KNO_3 + 0.1 mg L^{-1} Kinetin + 0.1 mg L^{-1} NAA + 0.1 mg L^{-1} GA_3	200	7

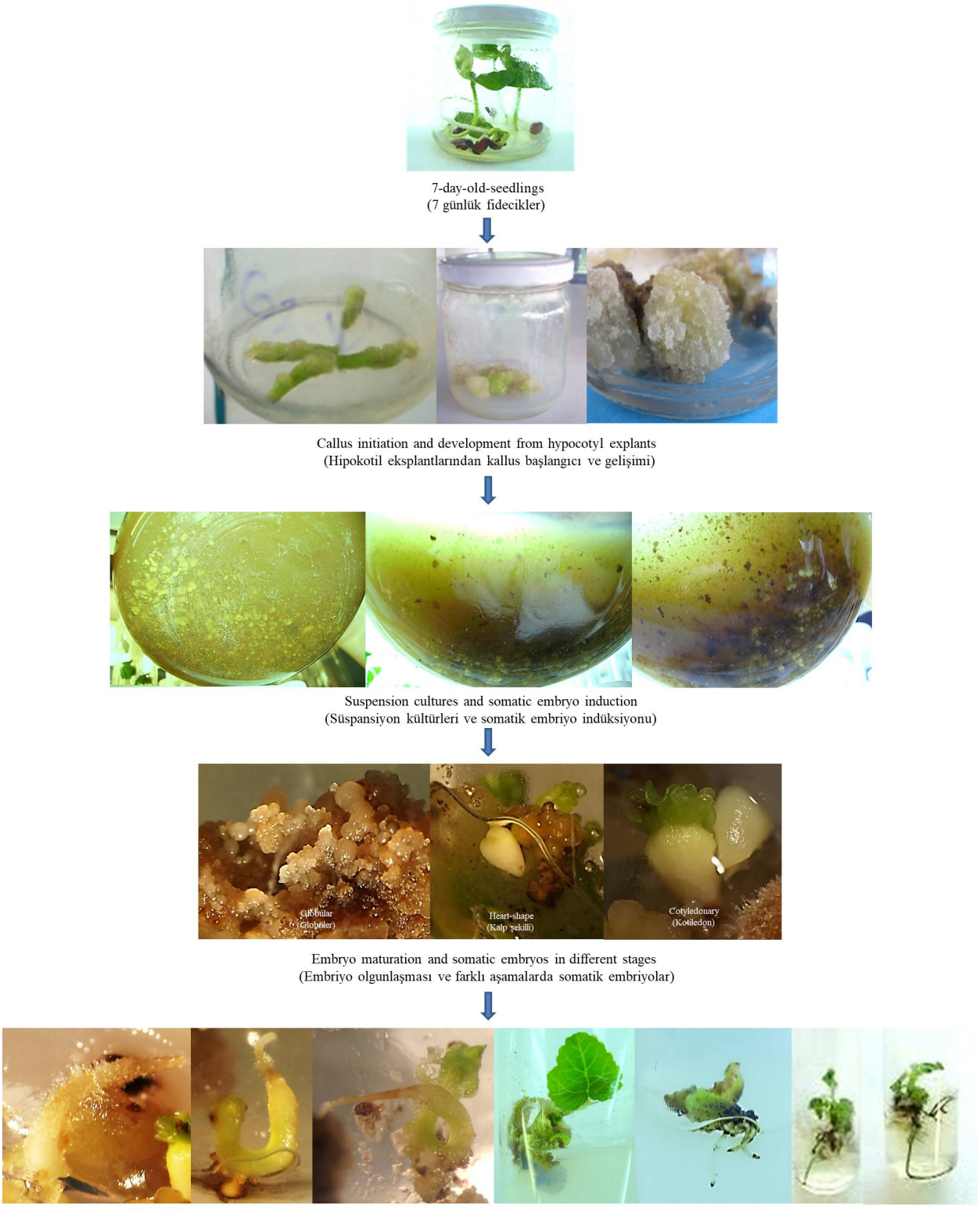

Figure 1. Somatic embryogenesis and plantlet regeneration in cotton (*Gossypium hirsutum* L.) genotype Nazilli-143.
Şekil 1. Nazilli-143 pamuk (*Gossypium hirsutum* L.) genotipinde somatik embriyogenez ve bitkicik rejenerasyonu.

Throughout germination, the matured cotyledonary staged embryos turned green color and then most of these embryos produced root and shoot simultaneously (Figure 1). The highest plantlet regeneration (36%) from matured somatic embryos was observed in 2/3MS medium supplemented with 1.3 g L^{-1} KNO_3 without plant growth regulators (Table 2). The same medium composition together with the addition of Kinetin, Naphthaleneacetic acid (NAA) and Gibberellic acid (GA_3) provided a slow response for the conversion of the embryo on the media and abnormal embryo formation was observed.

The somatic embryos were transformed into plantlets in the medium after 4-5 weeks. Twenty-nine plantlets, which had 2-4 leaves, were removed from the sterile culture and transformed into a plant growth chamber. After proper acclimatization, the regenerated plantlets displayed 100% survival rate.

DISCUSSION

Even though regeneration via somatic embryogenesis has been improved for several cotton genotypes, some problems still remain such as the genotype-dependent response and the prolonged culture period. However, many other factors could also affect the tissue culture response such as the types of media, hormone regimes, nitrogen sources, carbon sources, and amino acids (Wang *et al.,* 2006). Further studies of such factors would facilitate the development of genotype-dependent culture methods to enhance the growth performance of the tissue culture response of recalcitrant cotton genotypes.

The plant growth regulators and their concentrations have obvious effects on callus initiation and development in cotton (Wang *et al.,* 2006). In the present study, low concentrations of 2,4-D and Kinetin could induce embryogenic callus in *cv.* Nazilli-143. These results are consistent with previously conducted study (Wang *et al.,* 2006), in which the combination of auxin with cytokinin (2,4-D with Kinetin) gave a significantly more successful response for the embryogenic callus induction. In generally, the induction of embryogenic callus has been effectively achieved by the combined treatment of auxin and cytokinin.

Suspension culture is one of the most important means for somatic embryo production (Wang *et al.,* 2006). In this study, 4 weeks after culture incubation, the somatic embryos were produced in MS liquid medium. Our results show that, once induced, somatic embryogenesis does not require any additional exogenous plant growth regulators. The shoot and the root appeared simultaneously and did not require any additional treatments as reported earlier (Kumria *et al.,* 2003a) from initiation callus to the production of rooted clone took five months.

Several reports indicated that the reduction of macronutrients played an important role in somatic embryogenesis and embryo maturation in cotton (Trolinder and Goodin, 1988b; Wang *et al.,* 2006). Trolinder and Goodin (1988b) have reported that, the addition of 1.3 g L^{-1} KNO_3 to MS medium had some beneficial effect on embryo development. In our study, heart and cotyledonary shaped somatic embryos were formed simultaneously from globular staged somatic embryos in 2/3MS medium supplemented with 1.3 g L^{-1} KNO_3.

Wang *et al.* (2006) indicated that solid cultures combined with liquid culture protocols could be a feasible approach for achieving somatic embryo formation and plant regeneration of recalcitrant genotypes. We obtained 36% plantlet regeneration frequency in Nazilli-143. As reported by Aydin *et al.* (2004), plantlet regeneration frequency of Nazilli-143 somatic embryos were previously determined as 29.78% from hypocotyl explants on plant growth regulator-free MS solid medium without any liquid culture stage.

This research has shown that large numbers of somatic embryos of cotton can be produced in a short period of time (2-3 months). The protocol of somatic embryogenesis and plantlet regeneration in *cv.* Nazilli-143 developed in this study could serve as a potential tool for studying gene manipulation, plant transformation, somaclonal variation, as well as for *in vitro* selection against different kind of stress factors. This method could also be applied to other *Gossypium* genotypes.

ACKNOWLEDGEMENTS

This research is financially supported by the General Directorate of Agricultural Research (GDAR) through project no TAGEM/TA/08/05/02/001.

REFERENCES

Aydin, Y., Z. Ipekci, T. Talas-Ogras, A. Zehir, A, K. Bayrovic, and N. Gozukirmizi. 2004 High frequency somatic embryogenesis in cotton. Biol. Plantarum 48 (4): 491-495.

Davidonis, G. H., and R. H. Hamilton. 1983. Plant regeneration from callus tissue of *Gossypium hirsutum* L. Plant Science Letters 32: 89-93.

Finer, J. J. 1988. Plant regeneration from somatic embryogenesis in many cultivars of cotton (*Gossypium hirsutum* L.). Plant Cell Rep. 7**:** 481-494.

Firoozabady, E., and D. L. Deboer. 1993. Plant regeneration via somatic embryogenesis in many cultivars of cotton (*Gossypium hirsutum* L.). *In Vitro* Cell. Dev. Plant 299: 166-173.

Gamborg, O. L., R. A. Miller, and K. Ojima. 1968. Nutrition requirements of suspension cultures of soybean root cells. Exp. Cell Res. 50: 151-159.

Ganesan, M., R. Chandrasekar, B. D. Ranjitha Kumari, and N. Jayabalan. 2007. Somatic embryogenesis and plant regeneration of *Abelmoschus esculentus* through suspension culture. Biol. Plantarum 51 (3): 414-420.

Juturu, V. N., G. K. Mekala, and P. B. Kirti 2015. Current status of tissue culture and genetic transformation research in cotton (*Gossypium* spp.). Plant Cell Tissue and Organ Cult. 120: 813-839.

Khan, T., V. S. Reddy, and S. Leelavathi. 2010. High-frequency regeneration via somatic embryogenesis of an elite recalcitrant cotton genotype *(Gossypium hirsutum* L.) and efficient *Agrobacterium*-mediated transformation. Plant Cell Tissue Organ Cult. 101: 323-330.

Kumria, R., V. G. Sunnichan, D. K. Das, S. K. Gupta, V. S. Reddy, R. K. Bhatnagar, and S. Leelavathi. 2003a. High-frequency somatic embryo production and maturation into normal plants in cotton (*Gossypium hirsutum*) through metabolic stress. Plant Cell Rep. 21: 635-639.

Kumria, R., S. Leelavathi, R. K. Bhatnagar, and V. S. Reddy. 2003b. Regeneration and genetic transformation of cotton**:** Present status and future perspectives**.** Plant Tissue Culture 13: 211-225.

Murashige, T., and F. Skoog. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Plant Physiol. 15: 473-497.

Price, H. J., and R. H. Smith. 1979. Somatic embryogenesis in suspension cultures of *Gossypium klotzschianum* Anderss. Planta 145: 305-307.

Shoemaker, R.C., L. J. Couche, and D. W. Galbraith. 1986. Characterization of somatic embryogenesis and plant regeneration in cotton (*Gossypium hirsutum* L.). Plant Cell Rep. 3: 178-181.

Trolinder, N. L. and J. R. Goodin. 1988a. Somatic embryogenesis in cotton (*Gossypium*) I. Effects of source of explant and hormone regime. Plant Cell Tissue Organ Cult. 12: 178-181.

Trolinder, N. L., and J. R. Goodin. 1988b. Somatic embryogenesis in cotton (*Gossypium*) II. Requirements for embryo development and plant regeneration. Plant Cell Tissue Organ Cult. 12: 43-53.

Wang, Y. X., X. F. Wang, Z. Y. Ma, G. Y. Zhang, and G. Y. Han. 2006. Somatic embryogenesis and plant regeneration from two recalcitrant genotypes of *Gossypium hirsutum* L. Agricultural Sciences in China 5 (5): 323-329.

Zhang, B. H., F. Liu, and C. B. Yak. 2000. Plant regeneration via somatic embryogenesis in cotton. Plant Cell Tissue Organ Cult. 60: 89-94.