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Callus Induction, In vitro Shoot Development and Somaclonal Variations in Cotton (*Gossypium hirsutum* L.)

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Abstract

Callus induction and regeneration were studied in the two Turkish cotton varieties (Nazilli 84-S and Sahin 2000) possessing different sensitivity levels to salt. Also, the rate of polymorphisms was investigated using random amplified polymorphic DNA (RAPD) in the plant samples obtained from the shoot tip and node cultures. The cotyledon and hypocotyl explants gave better response in terms of callus induction when compared with the root explants. Hypocotyl explants were cultured in the Murashige-Skoog medium supplemented with 0.1 mg/l kinetin, 0.1 mg/l 2.4-D and 0.1 mg/l IAA and 77% and 84% callus induction was observed in Nazilli 84-S and Sahin 2000 cultivars, respectively. When MS medium was supplemented with various plant growth regulators, callus induction from the cotyledon and hypocotyl explants were observed. However, plant regeneration was not evident. When, 0.1 mg/l TDZ and 0.1 mg/l kinetin were added to the MS medium, 100% explant response was seen in both cultivars in the shoot tip cultures. Morever, in the cotyledonary nodes grown in the MS medium with the addition of 0.25 mg/l kinetin, the explant response was 86% and 68% for Nazilli 84-S and Sahin 2000, respectively. Genetic variation in the plants produced by direct regeneration of the shoot-tip and node cultures was determined via the RAPD technique. Genetic similarity was found to be highly similar for the both cultivars used in this study. The plants of Nazilli 84-S and Sahin 2000 had 93-99% and 94-99%, respectively.

Key words: Shoot tip culture, node culture, genetic similarity, RAPD

INTRODUCTION

One of the most crucial steps in plant breeding programs is to reveal and utilize genetic diversity. In this regard, using biotechnological methods complementing and supporting classical breeding programs has become an alternative way. One of the methods used for creating genetic diversity from available species is the use of tissue culture techniques. Somaclonal variation, which has notable advantages in plant breeding, is defined as genetic changes that emerge among the plants regenerated from somatically originated callus or cells using plant tissue techniques. Tissue culture techniques in combination with some molecular methods in genetic engineering studies have yielded fruitful results in plant genetics [1]. A tissue culture system properly designed and accurately setup is an important step for successful genetic transformations. Foremost, it is imperative to determine specific culture conditions optimized for every specific cultivar or species of interest in tissue culture [2, 3].

Tissue culture studies in cotton, which is an economically and commercially important crop have been in use since study by Davidonis and Hamilton (1983), when plant regeneration was achieved for the first time via somatic embryogenesis [4]. Due to the fact that the number of plants obtained from regeneration via in vitro techniques is relatively limited for cotton [5-10]. Because establishing an effective callus formation system from somatic tissues and an effective regeneration system needs to be developed [7, 11-13]. Since the success in cotton tissue cultures is largely dependent on the genotypes as stated by Trolinder and Chen (1989), determining tissue culture conditions appropriate for specific cultivars is crucial in genetic engineering studies [14]. There are reported studies on direct or indirect regeneration and callus induction with in vitro culture of different tissues in various cotton cultivars. Numerous studies have resulted in successful callus inductions from various explants including hypocotyl, cotyledon, root, leaf segments, immature embryo and nodal explants [3, 9, 13, 15]. Furthermore, shoot regeneration [16-18] and multiple-shoot induction [5, 10, 11, 19-23] have been achieved using apical meristem, shoot apex explants and cotyledon nodes. The explants were also used in the studies dealing with somatic embryogenesis [4, 6, 7, 23-26] and organogenesis [27], albeit resulting in a low success in regeneration. More studies are being conducted in our time with the goal of resolving tissue culture potentials of different plant varieties and increasing the rates of regeneration.

Some variations occurred following tissue culture treatments are also utilized in plant breeding and such variations have been studied in recent years by some molecular techniques. RAPD (Random Amplified Polymorphic DNA), one of the molecular marker methods, has become an economic, widespread, reliable and fast method commonly and widely used in order to determine variations and polymorphism [28, 29].

Biotic and abiotic stresses are responsible for greatly reducing productivity in plants and salt stress is the leading factor [30]. In this study, two different cotton cultivars (sensitive and tolerant to salt) were used for callus induction and regeneration experiments to find out the most relatively optimized conditions. Also, RAPD analysis was employed to investigate the polymorphism of the plants developed from shoot tips and node cultures.

MATERIAL AND METHOD

Some cotton varieties (9 varieties) cultivated in Turkey were germinated in media containing different concentrations of NaCl and these varieties were then classified as sensitive and tolerant according to the criteria provided by Javid et al. [31]. Among them, Nazilli 84-S cultivar was determined as tolerant and Sahin 2000 was established as sensitive. These two cultivars were then used in the experiments.

Obtaining sterile seedlings and culturing explants

After dipping the seeds of the cultivars into concentrated H_2SO_4 (98%) solution, they were kept in 50% diluted-commercial sodium hypochlorite solution (containing 4.5% active chlorite) for 30 minutes and afterwards, they were washed in distilled water three times [19]. Sterilized seeds were cultured in half-strength Murashige-Skoog [32] (1/2 MS) medium with the addition of 20 g/l sucrose and 7 g/l agar. Callus induction and regeneration were assessed following the in vitro culture of hypocotyls (8-10 mm), cotyledon (6x6 mm) and root explants of the seven-day sterile seedlings, in the MS medium with the addition of different plant growth regulators. Also, shoot-tips (5-7 mm) and cotyledon nodes of 16-days old sterile seedlings of Nazilli 84-S and Sahin 2000 cultivars were cultured in the MS medium with the addition of different plant growth regulators, and their shoot developments were evaluated. Culture room conditions consisted of 16 hours of light and 8 hours of dark photo-periods with 100 µmol m^{-2} s^{-1} illumination and 25±2ºC temperature. The plants obtained from shoot-tip and cotyledon node cultures were transferred to the pots and genetic variation among the growing plants was evaluated via the RAPD method.

DNA isolation and RAPD analysis

Genomic DNA isolation was performed using a modified CTAB DNA isolation protocol from the samples of micropropagated plants [33]. DNA amount and purity were measured using a spectrophotometer. A number of 42 RAPD primers (OP-A11, OP- B [1-20], OP-C04 and OP-M [1-20] series) were used. RAPD-PCR reaction was optimized using per reaction in a final volume of 15 µl: 5 µl of genomic DNA (5 ng/µl), 1 µl of 10 µM primer, 2.4 µl of 25 mM $MgCl_2$, 1 µl of dNTP mix (10 mM), 0.25 µl of *Taq* DNA polymerase (5U/µl) with the *Taq* buffer without $MgCl_2$ (1X). RAPD-PCR program had pre-denaturation at 94ºC for 1 minute, and then 45 cycles of denaturation at 94ºC for 1 minute, annealing at 36ºC for 1 minute, elongation at 72ºC for 2 minutes and final elongation at 72ºC for 10 minutes. The PCR products were analyzed using agarose gel electrophoresis. The PCR bands were stained with EtBr and then visualized using DNR MiniBis gel imager [33].

Data Analysis

Callus induction, shoot tip and node culture data were subject to the ANOVA one-way analysis of variance and Duncan's multiple range test (P<0.05) was performed. Levels of differentiation were displayed by different letters. The differences among the cultivars were assessed by Mann-Whitney U test (P<0.05). For this purpose, the SAS Institute (1985) package software was used. The Statistica 7 software was used for drawing graphs and standard error (SE) values (±) were displayed.

Band or bands were assessed in gel images of the reproducible results, as present "1" or absent "0". A matrix was obtained by taking into all of the bands account. This information was then used to calculate genetic similarities among the plants using Phylip (3.5c) software.

RESULTS AND DISCUSSION

Culture of root, hypocotyl and cotyledon explants

Root, hypocotyl and cotyledon explants were cultured in the MS nutrient medium with the addition of kinetin (K), 2.4-dichlorophenoxyacetic acid (2.4-D) and Indol-3-acetic acid (IAA), as described by Zhang et al. [4]. Also, the explants were cultivated in the MS medium with the addition of naphthaleneacetic acid (NAA), thidiazuron (TDZ) and $AgNO_3$ as suggested by Ouma et al. [21], where the success in direct shoot regeneration was reported. After 7 to 10 days, callus induction was seen in the cultures of root, hypocotyl and cotyledon of the seven-day sterile seedlings in the MS medium with addition of various plant growth regulators. Callus induction was detected from all of the explants (root, hypocotyl and cotyledon) of both cultivars in a medium with the addition of 0.1 mg/l K, 0.1 mg/l 2.4-D and 0.1 mg/l IAA (Table 1). On the other hand, while higher callus induction occurred from hypocotyl and cotyledon for Nazilli 84-S (77.00±6.06% and 78.26±6.58%, respectively), the highest callus induction for Sahin 2000 was observed chiefly in root explants (90.33 ± 3.17%) (Table 1) but callus development was insufficient. Moreover, no shoot regeneration was observed in the medium with the addition of NAA, TDZ and $AgNO_3$, which was expected to encourage shoot regeneration in both cultivars used in our study. Only callus induction was observed from some explants for the same media conditions (Table 1).

Numerous studies have been put forward to investigate the effects of various plant growth regulators on callus induction for cotton. For example, Haq and Zafar [25] achieved 93.33% callus induction from the hypocotyl with the same amount of plant growth regulators as used in our study (0.1 mg/l K and 0.1 mg/l 2.4-D without addition of IAA). Similarly, Kumria et al. [6] initiated an embryogenic callus formation from hypocotyl and cotyledon in a medium with addition of 0.1 mg/l 2.4-D + 0.5 mg/l K and 3% maltose and generated somatic embryos. Rajeswari et

Table 1. Callus induction from different explants of Nazilli 84-S and Sahin 2000 cultivars (%)

Cultivar name	Plant growth regulators (mg/l) and explants					
	K (0.1) + 2.4-D (0.1) + IAA (0.1)			NAA (0.01) + TDZ (0.175) + $AgNO_3$(5.1)		
	Root	Cotyledon	Hypocotyl	Root	Cotyledon	Hypocotyl
Nazilli 84-S	30.00 ± 2.88 [b]	78.26 ± 6.58 [a]	77.00 ± 6.06 [a]	-	53.80 ± 2.85	72.00 ± 4.07
Sahin 2000	90.33 ± 3.17 [b]	48.0 ± 6.87 [a]	84.00 ± 7.00 [b]	-	-	25.05 ± 3.40

Different letters indicate the statistical differences according to Duncan's Multiple Range test (P<0.05), ± SE.

al. [7] and Sultana and Hossain [13] found that an addition of 0.1 mg/l 2.4-D + 0.5 mg/l K to the MS medium was suitable for callus proliferation and suggested that low concentration of 2.4-D and higher concentration of kinetin for callus formation can be used. Furthermore, Kamal [9] proposed the use of high auxin and low cytokinin concentration and found that a medium with the addition of 2.4-D including (no cytokinin) was successful for callus formation. It was stated that increasing 2.4-D concentration (from 0.22 mg/l to 1.10 mg/l 2.4-D) in the presence of 0.20 mg/l benzylaminopurine (BAP) medium better encouraged proliferation but 2.4-D by itself did not produce callus [15]. Again similarly, it was reported that addition of benzyladenine (BA) along with 2.4-D caused 100% callus induction in cultures both under light and in dark [3]. Sakhanokho et al. [2] found that the medium with the addition of 2 mg/l NAA + 1 mg/l K was effective for embryogenic callus generation. Chowdhury et al. [8] investigated the effect of cytokinins on callus proliferation from the cotyledons of *Gossypium herbaceum* L. and reported that percentage of callus formation as well as shoot-developing callus and number of shoots obtained from the callus increased with higher cytokinin concentration. It was also stated that BA produced the best results in all of the hormones (BA, K and isopentenyl adenine) used in their study. It was shown that in vitro plant regeneration from non-organized callus tissue took great deal of time, regeneration frequency was quite low but still regeneration was possible [8].

In our study, due to better development of callus produced by the hypocotyl and cotyledon, subcultures were done for continuity of callus generated by these two explants (except root) as well as for formation of embryogenic callus cultures and some morphological properties of callus were established. The callus generated by cotyledon and hypocotyl in the MS medium with the addition of 0.1 mg/l K + 0.1 mg/l 2.4-D + 0.1 mg/l IAA were subcultured in the same medium at one month intervals. The subcultured callus of the Nazilli 84-S cotyledon was observed to be white, cream-light yellow and had compact structure. The greenish-white ones with fragile structure were also noticeable. The callus of hypocotyl had cream-light yellow and white colors with fragile and compact structures. Embryogenic callus was not observed in these subcultures. The callus, developed in a medium with the addition of 0.01 mg/l NAA + 0.175 mg/l TDZ + 5.1 mg/l $AgNO_3$ where direct shoot generation was expected, were subcultured only in the medium with 0.1 mg/l zeatin. Accordingly, greenish and white callus were obtained from the cotyledon and hypocotyl in addition to cream-light yellow callus with soft structures in general. When the callus, which were generated in a medium with the addition of 0.1 mg/l K + 0.1 mg/l 2.4-D + 0.1 mg/l IAA for the Sahin 2000 cultivar, were subcultured in the same medium, both cotyledon and hypocotyl explant-based callus generally had cream-light yellow, brown colors and soft tissue. The callus with white and greenish-white color were also seen. Cotyledon-based callus, subcultured in a medium with the addition of 0.1 mg/l IAA and 0.1 mg/l zeatin, turned to cream-light yellow and greenish-white with compact tissue; while the hypocotyl-based ones became greenish-white with fragile tissue. Zhang et al. [4] stated that zeatin concentration was critical on callus induction and callus growth from cotyledon, hypocotyl and root explants and found that lower zeatin concentration was advantageous for embryogenic callus induction. The best medium for embryogenic callus proliferation was given as 1 mg/l 2.4-D + 0.5 mg/l K + 0.5 mg/l zeatin but it was also argued that 2.4-D had negative impact on differentiation and germination of somatic embryos.

The medium with the addition of 0.01 mg/l NAA + 0.175 mg/l TDZ + 5.1 mg/l $AgNO_3$, which was found efficient by Ouma et al. [21] in their study for Delta pine cultivar, was also tested in our study and shoot regeneration was not observed in both cultivars. On the other hand, callus induction was observed from the hypocotyl and cotyledon for the Nazilli 84-S cultivar and only from hypocotyls for the Sahin 2000 cultivar. The callus were subcultured in a medium containing 0.1 mg/l IAA + 0.1 mg/l zeatin and cotyledon explant-based callus became white and cream-light yellow with compact structure. Hypocotyl proliferated the callus with compact as well as fragile structures in similar colors.

Shoot-tip cultures

Growth between 81.4% and 98.8% was observed in the shoot apices cultured in MS with the addition of different plant growth regulators. In both cultivars, 98% of the shoot apices cultured in the medium with TDZ + K addition successfully developed. Growth percentage of shoot tips was lower in the media with only kinetin or TDZ for both cultivars but it is higher for the Nazilli 84-S cultivar than the Sahin 2000 (Figure 1).

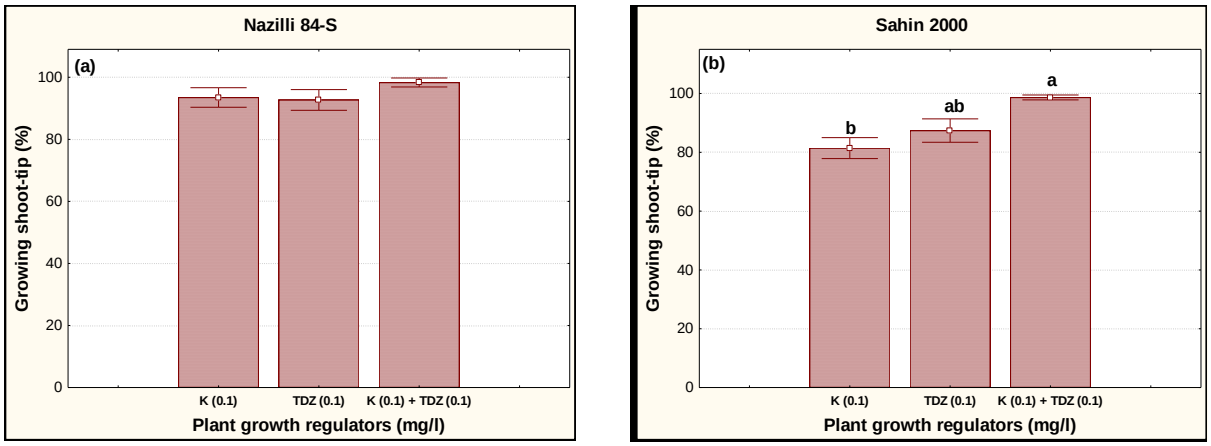

Figure 1. Growth of the shoot tips of Nazilli 84-S (a) and Sahin 2000 (b) cultured in the media with kinetin and/or TDZ (%). Different letters indicate the statistical differences according to Duncan's Multiple Range test (P<0.05), ± SE (A statistical difference was not observed in the Nazilli 84-S cultivar).

Taken all the conditions of hormonal treatments into account, the statistical analysis revealed that there is no significant difference in the development of the shoot tips of both cultivars (Figure 2).

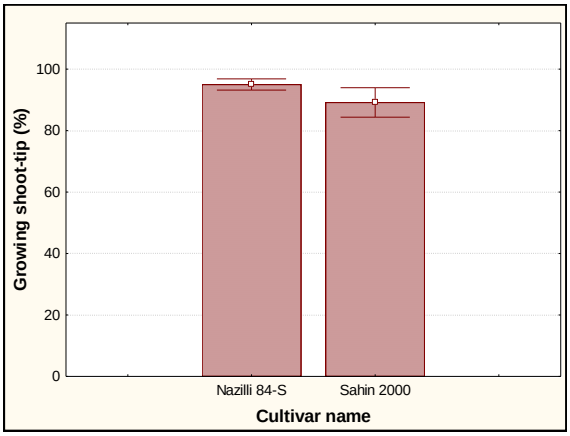

Figure 2. Percent development of the shoot tips of the cultivars cultured in the media with kinetin and/or TDZ with respect to Nazilli 84-S and Sahin 2000. Mann–Whitney U test was used (P<0.05), ± SE.

Saeed et al. [16] observed that the best shoot development for meristem shoot tip cultures of 19 cultivars was in the MS medium that only contained kinetin (0.1 mg/l). Rashid et al. [35] observed a 13%-100% growth in shoot apex (2, 4, 6 mm) culture of 10-days old seedlings of 22 cotton varieties in non-hormone MS medium and emphasized change of growth according to the varieties. As a result of a study performed on the effect of BA, multiple-shoots (the highest was 3.4 shoots per shooted explant) were obtained from apical embryonic axes (caulinar apex, apices) in a medium with the addition of BA [5]. Also, more successful results were seen with the addition of TDZ to medium in the culture of meristem (5 mm) and caulinar apices (5.8 shoots per explant) [19]. Ozyigit and Gozukirmizi [35], who studied with the cultivar Nazilli 84-S, observed the best response of 98% in the MS medium with the addition of 0.1 mg/l K + 1 mg/l polyvinylpyrrolidone (PVP) for the culture of shoot apices (2-3 mm) of 7-days old seedlings. The response was 93.5% in our study in a medium containing only kinetin (0.1 mg/l) as the regulator.

Node cultures

The cotyledon nodes of the 16-days old seedlings were cultured in media with only kinetin and combinations of K + BAP and NAA + IBA. Shoot developed from only one node in most of these explants and a small number of explants displayed shoot developments from both nodes. The best result was obtained using the medium with 0.25 mg/l K addition in both cultivars (Figure 3).

The effect of plant growth regulators in explant response was found to be statistically significant. A statistical difference was not observed between the node cultures of the cultivars in response to different plant growth regulators, when all the data are considered (Figure 4).

Agrawal et al. [11] cultured cotyledon nodes of five, twenty and thirty-five days old sterile seedlings (by separating cotyledon leafs from apical meristem) in media with only K, BAP and a combination of K + BAP. The best explant response (100%) was seen in 35-days old seedlings using combination of 2.5 mg/l K and 2.5 mg/l BAP. In our study, explant response in medium with the addition of 1 mg/l K and 1 mg/l BAP was 34% for Nazilli 84-S and 42.5% for Sahin 2000. Ozyigit [36], who observed higher shoot development than our study for Nazilli 84-S, obtained shoot developments of 74.2%, 60.7% and 41.6% respectively in medium with 0.1 mg/l K from cultures of the first, the second leaf nodes and from cotyledon nodes of 35-days old seedlings.

Rauf et al. [20] investigated the effect of kinetin on multiple-shoots from cotyledon nodes in *Gossypium hirsutum* L. cultivar NIAB-999 and received 100% explant response from the culture of shoot apices with both cotyledons in the MS medium with the addition of 0.25 mg/l K. Explant response in the nodes without cotyledon was 91.2%. Khalafalla and Abdellatef [22] obtained the best result from the cotyledon nodes of 35-days of seedlings with the maximum shoot number (2.8) in Gamborg (B5) medium with 0.1 mg/l BA + 2.5 mg/l K. They reported that the type and concentration of cytokinin was important as much as age of seedlings in multiple-shoot induction from the cotyledon nodes.

In our study, the shoots developed from the nodes were taken to a fresh medium with combination of the same plant growth regulators and subsequently, after their transfer to a medium with 0.1 mg/l K + 0.05 mg/l NAA, developing plants were taken into pots to study somoclonal variation.

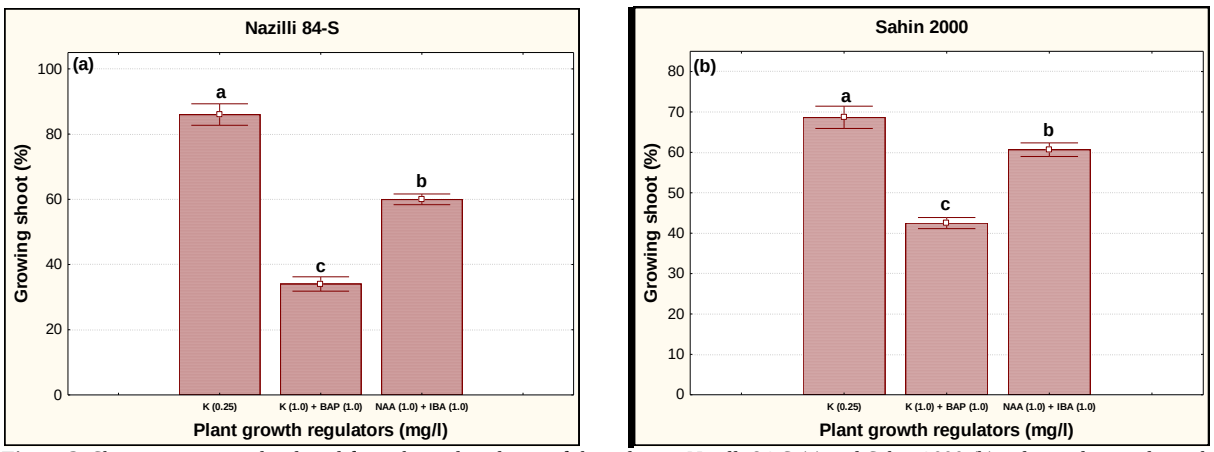

Figure 3. Shoot percentages developed from the node cultures of the cultivars Nazilli 84-S (a) and Sahin 2000 (b) cultivated in media with the addition of different plant growth regulators. Different letters indicate the statistical differences according to Duncan's Multiple Range test (P<0.05), ± SE.

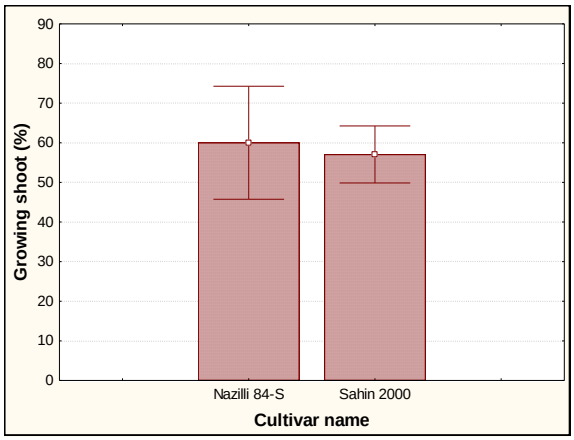

Figure 4. Shoots (%) developed from the node cultures in the cultivars Nazilli 84-S and Sahin 2000. Mann–Whitney U test was used (P<0.05), ± SE.

Somaclonal variation

Genomic DNAs were successfully isolated from the micro-propagated plants belonging to the cultivars Nazilli 84-S and Sahin 2000. A total of 42 primers were used in RAPD-PCR reactions. Five primers did not yield any PCR products and 3 primers did not produce scorable bands. The PCR bands obtained from remaining primers were analyzed to determine genetic variation of the plants. Thirty four micro-propagated plants were obtained from the shoot-tip and node cultures of the cultivar Nazilli 84-S. RAPD analysis of these plants resulted in a total of 320 amplification products. Thirty (10.6%) and 286 (89.3%) bands were found to be polymorphic and monomorphic, respectively (Figure 5). While genetic similarity among the plants varied between 93% and 99%. Genetic similarity among the four plants developed from the same cotyledon node was calculated to be 98%.

For Sahin 2000, 236 amplification products were obtained from the 12 plants developed from only shoot-tip cultures. While 23 of these products were determined to be polymorphic (9.74%), 213 bands were monomorphic (90.25%) (Figure 6). Genetic similarity values varied between 94% and 99%.

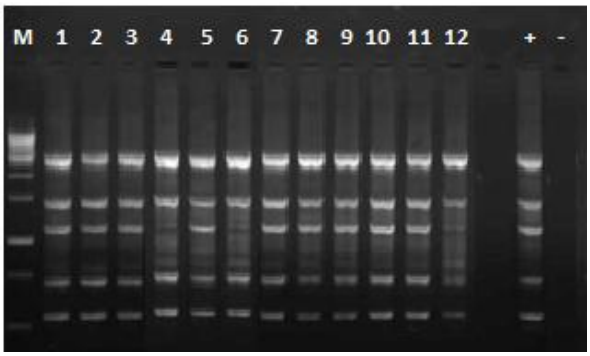

Figure 6. RAPD-PCR result of primer OPB-04 for the micropropagated plants of Sahin 2000. Marker (M):1 kb ladder ("+" positive, "-" negative control).

Genetic similarity having been found high among the micropropagated plants was an expected situation in shoot-tip and node culture studies and they are preferred culture techniques for preserving genetic stability. Many strategies such as cytogenic analysis, iso-enzyme markers and different DNA markers are used for evaluating genetic differences of the plant clones obtained from using in vitro media. In a study conducted by Tafvizi et al. [37], somaclonal variation in two different cotton varieties obtained with meristem culture and their hybrids were analyzed with the RAPD technique. Different somaclonal variation rates were seen in different varieties and as number of sub-cultures increased, genetic variation also increased. Also, they emphasized the critical importance of explant source in somaclonal variation. Somaclonal variation in plant tissue cultures is one of the practical resources for the purpose of creating genetic diversity in plant breeding programs. Diversity generally results from changes in the genomes of vegetatively differing cells via epigenetic influence or as a result of tissue culture [37]. Evans and Sharp [38] sorted 4 critical reasons that can cause somaclonal variation: genotype, explant origin, cultivation time and culture condition. Furthermore, different tissue source (changes in donor plant origin or explant-based variation), lack of nucleic acid precursors, anomalies in cell division and stresses are the other possible reasons that can potentially cause somaclonal variation [39].

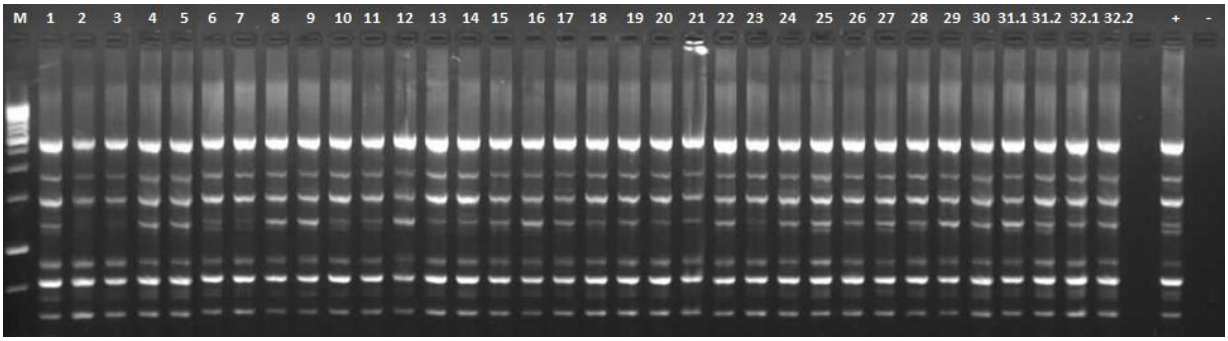

Figure 5. RAPD-PCR result of primer OPB-01 for the micropropagated plants of Nazilli 84-S (1-30: samples developed from shoot-tip culture, 31.1-31.2 and 32.1-32.2: samples developed from same node culture). Marker (M):1 kb ladder ("+" positive, "-" negative control).

As a result of callus culture, shoot-tip and node culture protocols for cotton varieties of Nazilli 84-S and Sahin 2000 were applied using different explant types and with the addition of different growth regulators into medium and sub-culture studies were conducted. Even though healthy callus were observed, indirect regeneration could not be obtained. Genetic variation in the plants produced by direct regeneration of the shoot-tip and node cultures was determined via the RAPD technique. Genetic similarity was found to be highly similar for the both cultivars used in this study. The plants of Nazilli 84-S and Sahin 2000 had 93-99% and 94-99%, respectively.

Acknowledgments

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