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HIGH EFFICIENCY SHOOT AND ROOT FORMATION FROM COTYLEDONARY NODES OF COTTON (*GOSSYPIUM HIRSUTUM* L.)

İBRAHIM İLKER ÖZYİĞİT [1*] AND NERMİN GÖZÜKIRMIZI [2]

[1]*Marmara University, Science and Arts Faculty, Department of Biology, 34722, Goztepe/Istanbul/Turkey*
[2]*Istanbul University, Faculty of Science, Department of Molecular Biology & Genetics, 34118, Vezneciler/Istanbul/Turkey*

Abstract

The purpose of this study was to develop an efficient micropropagation system for cotton (*Gossypium hirsutum* L.), a worldwide commercially important fiber crops. In this study, successful shoot and root induction were achieved from cotyledonary nodes of two different cotton genotypes, Nazilli 84S and Çukurova 1518 which are widely planting in Turkey. Plant tissue culture systems were established on Murashige and Skoog (MS) media supplemented with various plant growth regulators using cotyledonary nodes with hypocotyl pieces as explant. Explants were placed on different MS media supplemented with different combinations of kinetin (KIN) and α-Naphtaleneacetic acid (NAA). Seven-day-old explants were used and germination, regeneration and rooting processes were consisted in 5 weeks. The best regeneration responses were from cotyledonary nodes of Nazilli 84S - 80 % and Çukurova 1518 - 75 %. Successful direct regeneration and rooting were obtained and significant differences were not seen between two genotypes. In addition, regenerated young plants were phenotypically normal and they set seeds. This rapid *in vitro* regeneration and rooting procedure can be also available for use of particle gun and *Agrobacterium*-mediated transformation.

Introduction

Modern plant biotechnology involves establishment of tissue culture and gene transfer systems obtaining desired specific characteristics to import for crop improvement implies the ability to regenerate a great number of plants (Naz *et al.*, 2007). In cotton (*Gossypium hirsutum* L.), regeneration methods have been developed since the middle of the 80's. Price & Smith (1979) were first reported somatic embryogenesis in cotton, *Gossypium koltzchianum*, although complete plants could not be regenerated. Davidonis & Hamilton (1983) first described plant regeneration from two-year-old calli of *G. hirsutum* L. cv. Coker 310 via somatic embryogenesis. Since then, significant progress has been reported in cotton tissue culture (Ikram-ul-Haq & Zafar, 2004, Méndez-Natera *et al.*, 2007).

There are two main types of regeneration methods for cotton biotechnology: organogenesis (Gould *et al.,* 1991; Hemphill *et al.,* 1998; Zapata *et al.,* 1999) and somatic embryogenesis (Trolinder & Goodin 1987; Finer, 1988; Firoozabady & DeBoer, 1993). Regenerated plants have been obtained from explants such as hypocotyl, cotyledon, root, anther and from various cotton species (Ozyigit *et al.,* 2007). Somatic embryogenesis and

* Corresponding Author: ilkozyigit@marmara.edu.tr; Phone: (+90216) 3487759/1182; Fax: (+90216) 3478783

plant regeneration systems have been established from cotton tissue, protoplasts and ovules (Ikram-ul-Haq & Zafar, 2004). However, both processes have many disadvantages, which effects to obtain fertile plants from explants. Some of those are; genotype dependent regeneration, poor rooting, a prolonged culture period, browning that caused death of explants, high frequency of abnormal embryo development, low conversion rate of somatic embryos into plantlets, lack of shoot elongation (Kumria *et al*., 2003, Ouma *et al.,* 2004; Ozyigit *et al.,* 2007). In addition, efficient *in vitro* techniques for regeneration of large numbers of plantlets from cotton are limited when compared to other major commercial crops (Ikram-ul-Haq & Zafar, 2004; Ozyigit *et al.,* 2007).

Furthermore, regeneration frequency depends on genotype and the most responsive lines are Coker varieties, which are no longer under cultivation. Genotype dependent response restricts the application of cotton biotechnology to cotton breeding and therefore, before plant tissue culture techniques are widely applied to cotton improvement programs, plant regeneration must be possible for a broad range of genotypes (Zhang *et al.,* 2001, Ozyigit *et al.,* 2007).

Although cotton was problematic in tissue culture studies, Firoozabady *et al.* (1987) and Umbeck *et al.* (1987) reported the first *Agrobacterium*-mediated transformation to cotton in 1987. Since then, genetically modified cottons which carry insect- and herbicide-resistant genes were obtained successfully at the beginning of the 1990's and transgenic cotton cultivars became commercially available in 1995 (Collins, 1996; Song *et al*., 2000, Hussain *et al*., 2007). As it is known Coker and Acala genotypes are amenable for genetic transformation because of their high regeneration potential (Katageri *et al.,* 2007). In addition, this genotype dependent transformation capacity also makes cotton problematic (Ozyigit *et al.,* 2007). The transgene from the Coker 310FR can then be transferred to elite genotypes by conventional breeding. However, this could also lead to introgression of undesirable characters from Coker 310FR. Thus transformation of elite genotypes is desirable (Katageri *et al.,* 2007). Successful efforts to directly transform elite genotypes by alternate methods have been reported. Satyavathi *et al.* (2002) reported genetic transformation of two Indian genotypes of cotton using shoot apices. Lately, Katageri *et al.* (2007) attempted to transform an elite Indian genotype of *G. hirsutum* by regenerating *Agrobacterium*-treated shoot apical meristems as described by Gould *et al.* (1991) with minor modifications.

In this study, we reported direct shoot regeneration and efficient rooting by using cotyledonary nodes of two commercially important cultivars of cotton in Turkey. The usage of cotyledonary nodes in direct regeneration is important, because in direct regeneration from nodes, incidence of genetic mutations and somaclonal variations were low in plants regenerated from other sides of explants. This may be the absence of tissue differentiation phases like callus induction or somatic embryo formations (Gould, 1998). This rapid *in vitro* regeneration and rooting procedure can be applied to plant transformation either by particle bombardment or to *Agrobacterium*-mediated transformation of cotton.

Materials and Methods

In this study, two different cotton varieties (Nazilli 84S and Çukurova 1518), which are being widely planted in Turkey were cultured for *in vitro* direct regeneration. Nazilli 84S's seeds were obtained from Nazilli Cotton Research Institute, Aydın-Turkey while

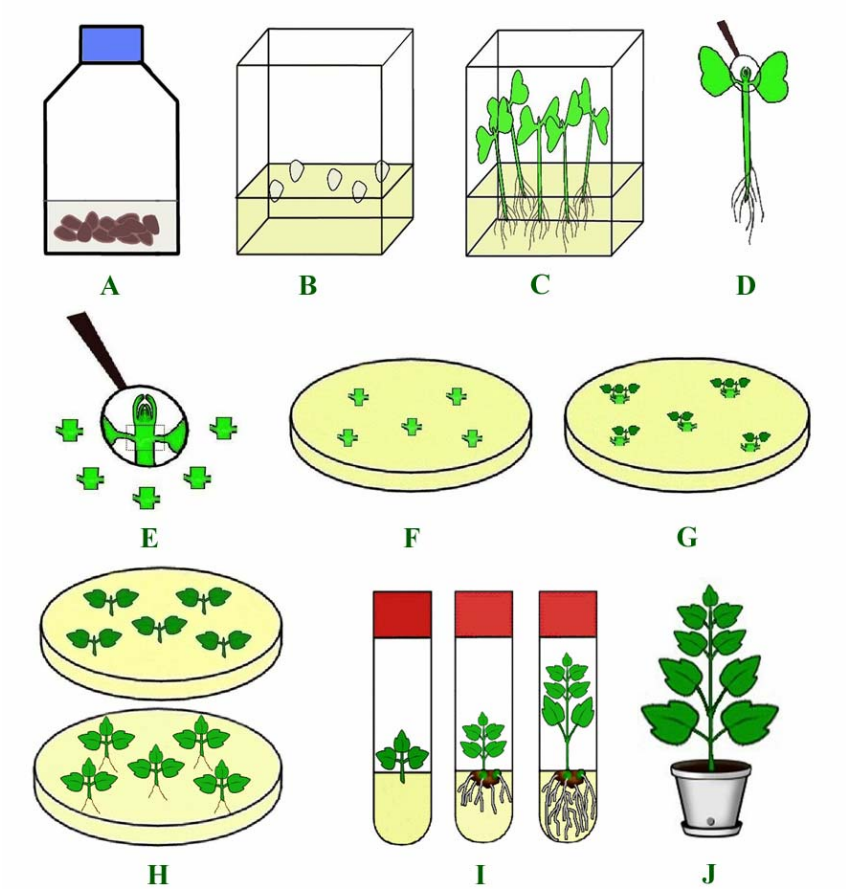

Figure 1: *In vitro* direct regeneration protocol for cotton (*Gossypium hirsutum* L.), A- Surface sterilization, B- Planting seeds on MSØ media, C- 1-week-old young plantlets, D- Cotyledonary nodes, E- Isolation of cotyledonary nodes, F- Culturing on MS + 0.1 mg/L KIN + 1 g/L PVP, G- In vitro direct regeneration, H-Subculturing on MS + 0.1 mg/L KIN + 2 mg/L NAA + 1 g/l PVP, I- Rooting in WPM and 1 mg/L IBA, J- Adaptation to the soil.

Çukurova 1518's seeds were obtained from Çukurova Agricultural Research Institute, Adana-Turkey.

Cottonseeds were kept under flowing tap water for approximately one hour and they were surface sterilized by immersion in 70 % ethanol for three minutes, followed by stirring in 20 % commercial bleach (ACE Mark) for 20 minutes (Figure 1-A). The surface sterilized seeds were rinsed three times with sterile distilled water for five minutes and they were dried onto filter papers. Seed coats were removed with sterile sculpture and pliers prior to germination. The seeds were germinated on hormone free MS (Murashige & Skoog) medium, which contained 1 mL MS vitamin solution, 30 g sucrose and 2.2 g phytagel (Figure 1-B). The pH of the media was adjusted to 5.7 with 1 M NaOH before autoclaving. 20 mL MS media were poured into Magenta vessels and

five seeds were germinated in each Magenta vessel (Figure 1-C). Seeds were kept at growth chamber with photoperiod of 16 hours light (7500 lux) and eight hours dark, at 25 ^{o}C and 70 % humidity.

After 7 days of germination, cotyledonary nodes, which contained hypocotyl pieces, were dissected out from seedlings and then cultured on MS media supplemented with 0.1 mg/L KIN (kinetin) + 1 g/L PVP (polyvinylpyrrolidone) and MS + 0.1 mg/L KIN + 2 mg/L NAA (α-Naphthaleneacetic acid) + 1 g/L PVP (Figure 1-D-E-F). In addition, in one of our treatment light was reduced to 3000 lx and MS + 2 mg/L BAP + 2 mg/L KIN were conducted. Direct shoot regeneration started in one week and after regeneration, regenerated shoots were immediately removed from explants, and then subcultured in one week intervals.

Different regeneration ratios were obtained from both genotypes in two weeks on different media. The best regeneration processes were different from each other for used two genotypes. After germination, the most suitable regeneration medium were chosen with many treatments and for a routine regeneration system, cotyledonary nodes of Nazilli 84S were cultured on MS + 0.1 mg/L KIN + 1 g/L PVP, and Çukurova 1518's were cultured on MS + 0.1 mg/L KIN + 2 mg/L NAA + 1 g/L PVP (Figure 1-G).

In rooting phase, a two-step rooting protocol was used. We obtained rooting with Çukurova 1518 genotype together with the regeneration on MS + 0.1 mg/L KIN + 2 mg/L NAA + 1 g/L PVP, while we obtained only the regeneration with Nazilli 84S genotype on MS + 0.1 mg/L KIN + 1 g/L PVP. After this result, we decided to subculture both genotypes on the same medium (MS + 0.1 mg/L KIN + 2 mg/L NAA + 1 g/L PVP). In the second week of regeneration, both regenerated plantlets of two genotypes were subcultured on MS + 0.1 mg/L KIN + 2 mg/L NAA + 1 g/L PVP and after a week, thin and weak roots were obtained (Figure 1-H). After that, plantlets of both genotypes were transferred onto WPM (Woody plant medium) supplemented with 1 mg/L IBA (indole-3-butyric acid) (Figure 1-I Left). After one week, the basal sides of explants became very thick and then tick and strong roots obtained from the young plantlets (Figure 1-I Middle and Right). The regenerated plants were potted in a mixture of soil and perlite (1:1, v/v), and grown in a greenhouse conditions for adaptation to natural environments (Figure 1J).

Results and Discussion

Rapid *in vitro* direct shoot regeneration and efficient rooting were obtained in a very short time (5 weeks) from cotyledonary nodes of two different cotton varieties (Nazilli 84S and Çukurova 1518) (Figure 2-A). Both genotypes were germinated on hormone free MS media and the germination abilities were high for them (98 % Nazilli 84S - 95 % Çukurova 1518). Regenerated plants were all fertile, they did not show obvious changes in overall morphology, and short time in culture did not affect their normal developments. All the plants that regenerated from both genotypes tested exhibited normal phenotype and they were similar. Nazilli 84S showed 80 % regeneration success on MS + 0.1 mg/L KIN + 1 g/L PVP while Çukurova 1518 showed 75 % on MS + 0.1 mg/L KIN + 2 mg/L NAA + 1 g/L PVP as the best regenerations. In addition, they also showed lower regeneration responses on their the most regenerated media (Nazilli 84S - 65 % on MS + 0.1 mg/L KIN + 2 mg/L NAA + 1 g/L PVP and Çukurova 1518 - 60 % MS + 0.1 mg/L KIN + 1 g/L PVP).

In this study, we obtained the most successful regeneration with 7-day-old explants in both genotypes. In previous studies, the researchers generally cultured explants in 30 days after germination and they generally obtained responses between the 3rd and the 7th days or between the 28th and the 35th days (Gould *et al.,* 1991; Kumria *et al*.2003, Ozyigit *et al.,* 2007). In a similar study, Gupta *et al.* (2000) obtained the best regeneration response from 6-day-old explants using 5, 6, 7, 8, 9 and 10-day-old cotyledonary nodes. Luo & Gould (2000) cultured 7, 14, 21, 28, 34 and 45-day-old cotyledonary nodes containing hypocotyl pieces (like our study) and obtained the best regeneration response from 14 and 35-day-old explants. In another study, Kumria *et al.* (2003) cultured 5 and 7-day-old hypocotyl and cotyledonary nodes of *var*. Coker, they obtained callus and then somatic embryos. In a different study carried out by, Ozyigit *et al.* (2007), relation between explant age, total phenolics and regeneration success were studied and they reported that the amounts of total phenolics were less within the first 7 days and after 28th days of germination. They also obtained the highest regeneration 72.2 % with 7-day-old and 61.2 % with 35-day-old cotyledonary nodes.

In another step of this study, we reduced the light from 7500 to 3000 lx and cultured explants on MS supplemented with 2 mg/L BAP and 2 mg/L KIN like Gupta *et al.* (2000). As a result of this attempt, all of our explants were browned and darkened, so we did not obtain any regeneration.

Literature indicates that, in cotton tissue culture studies, indirect somatic embryogenesis and direct organogenesis are the main methods. In addition, cotyledonary nodes had been used and multiple shoots were mostly obtained from indirect somatic embryogenesis (Kumar *et al.,* 1998; Kumria *et al.,* 2003: Sun *et al.,* 2006). By the way, callus induction and indirect somatic embryogenesis take long times (5-6 months). This prolonged culture period could be one of the important disadvantages of cotton cultures, especially for gene transfer studies (Kumria *et al.,* 2003). Moreover, tissue browning and blackening could also be one of the major problems for *in vitro* culturing of cotton too (Ozyigit *et al.,* 2007). Using liquid media, frequent subculturing, some antioxidants such as citric acid and ascorbic acid, PVP and activated carbon, are some possible solutions for this problem. Those methods usually reduce phenolic oxidation and contribute to regeneration from explants (Toth *et al.,* 1994). In this study, 1 g/L PVP that added into both media and culturing explants in its early stages (7-day-old) mostly solved this problem.

After regeneration, both regenerated plantlets of two genotypes were subcultured on MS + 0.1 mg/L KIN + 2 mg/L NAA + 1 g/L PVP and then rooting was obtained from Çukurova 1518 60 % and from Nazilli 84S 45 % one week later. The obtained roots were thin and weak and there was a little browning on the basal sides of plantlets (Figure 2-B). The weak roots were removed and the plantlets were transferred to WPM + 1 mg/L IBA. After one week, the basal sides of explants became thicker and strong rooting obtained (Figure 2-C). One more week later, the rooted plants became ready to transfer to the soil. At that phase, basal sides of shoots became as callus-like structures and strong roots were elongated from both callus-like structure and shoots (Figure 2-D). The regenerated plants were then transferred to the soil and after development period, they plants were all fertile and they did not show obvious changes in overall morphology (Figure 2-E). Furthermore, some of the non-rooting plantlets, which subcultured on MS + 0.1 mg/L KIN + 2 mg/L NAA + 1 g/L PVP media also started rooting on WPM + 1 mg/L IBA too.

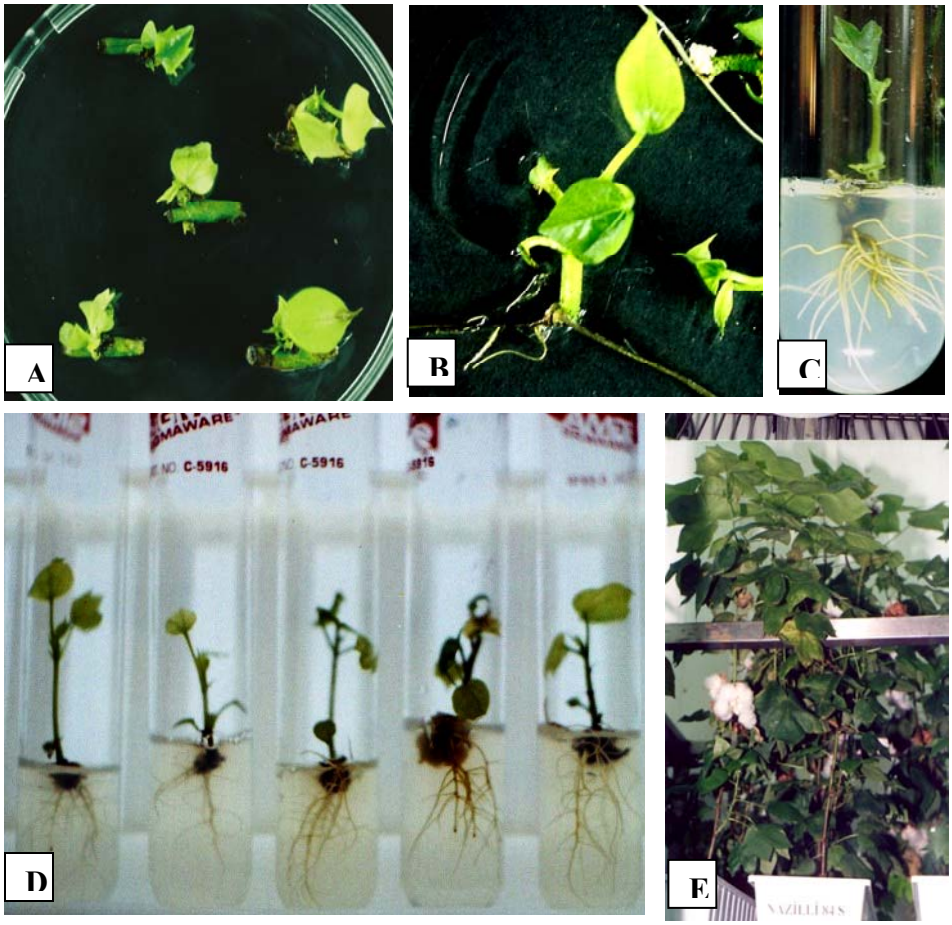

Figure 2: A) *In vitro* direct regeneration from cotyledonary nodes of cotton within the 2nd week. B) Obtained weak roots on MS + 0.1 mg/L KIN + 2 mg/L NAA + 1 g/L PVP and also browning on the basal sides of plantlets within 3rd week. C) Strongly rooted plantlet on WPM + 1 mg/L IBA at the end of the 4th week. D) Plants, which are ready to transfer to the soil at the end of the 5th week. E) Plants in pots in climate room.

In cotton tissue cultures, rooting problems are also one of the major problems and many scientists applied different methods to solve this problem (Ouma *et al.,* 2004; Ozyigit *et al.,* 2007). However, results of rooting success in many literatures are unclear and there are less data on rooting. In this study, the best rooting obtained with two stages as mentioned above. Gould *et al.* (1991) and Hemphill *et al.* (1998), tried to solve the rooting problem by putting the young plantlets directly to the soil. In another study, Gould & Cedeno (1998) transferred some shoots to the soil, and they also grafted non-rooted shoots onto new germinated cotton seedlings. Similar Gupta *et al.* (1997) used

NAA as a stimulator for rooting (like our study), they used nutrient agar supplemented with 2.7 µM/L NAA, and they obtained 60 % rooting with Khandwa-2 genotype. As it explained above, efficient rooting will be always a big problem for this species and it will be the criteria of successful tissue culture and gene transfer study.

Culturing cotyledonary nodes could be very popular method to examine regeneration potential of many other important species like cotton. Regarding the obtaining of shoots and roots, then adaptation, the young plants to greenhouse conditions and seed production are important features to have successful tissue culture based transgenic plants. However, this phenomenon has recently been shown to be influenced by both genotype and culture conditions. Both genotypes, which selected in this study and the methods used for *in vitro* regeneration and especially rooting, can now be applied to increase the efficiency of transformation protocols using cotyledonary nodes as explant source.

Direct shoot multiplication is preferred for generating true-to-type plants than callus regeneration. This study supports the rapid multiplication (in 5 weeks) of this commercially important plant by *in vitro* conditions. This report provides a simple protocol for the micropropagation of two different *G. hirsutum* genotypes (Nazilli 84S and Çukurova 1518). Both genotypes, which selected in this study and the methods used for *in vitro* regeneration and rooting, can be applied to increase the efficiency of transformation protocols using cotyledonary nodes as explant source.

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